Abstract—Nuclear RNA and DNA in muscle cell nuclei of laboratory-reared larvae of Walleye Pollock (*Gadus chalcogrammus*) were simultaneously measured through the use of flow cytometry for cell-cycle analysis during 2009–11. The addition of nuclear RNA as a covariate increased by 4% the classification accuracy of a discriminant analysis model that used cell-cycle, temperature, and standard length to measure larval condition, compared with a model without it. The greatest improvement, a 7% increase in accuracy, was observed for small larvae (<6.00 mm). Nuclear RNA content varied with rearing temperature, increasing as temperature decreased. There was a loss of DNA when larvae were frozen and thawed because the percentage of cells in the DNA synthesis cell-cycle phase decreased, but DNA content was stable during storage of frozen tissue.

Using measurements of muscle cell nuclear RNA with flow cytometry to improve assessment of larval condition of Walleye Pollock (*Gadus chalcogrammus*)

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Marine fish larvae are most vulnerable to starvation during the first few weeks after they begin to feed (O'Connell, 1980; Theilacker, 1986; Theilacker and Porter, 1995). Starvation may contribute directly to mortality, or it may do so indirectly by making larvae more vulnerable to predation (Bailey and Yen, 1983; Folkvord and Hunter, 1986). Many methods, such as the RNA–DNA ratio (Buckley et al., 1999), lipid composition (Lochmann et al., 1995), histological condition of tissues (Theilacker, 1978), morphological measurements (Theilacker, 1978), and flow cytometry (Theilacker and Shen, 1993a), have been developed to measure the physiological condition of fish larvae. Mortality rate has been shown to correlate with nutritional condition of fish larvae (Theilacker et al., 1996). Hence, accurate assessment of condition can improve understanding of environmental processes that affect early life survival and recruitment.

Previous studies have assessed the condition and growth of fish larvae with flow-cytometric cell-cycle analysis, which is a technique that measures the DNA content of individual cells in a population to determine the proportion of cells in different phases of the cell cycle (Theilacker and Shen, 1993b, 2001; Bromhead et al., 2000; González-Quirós et al., 2007; Porter and Bailey, 2011; Domingos et al., 2012). Cells that are in the process of dividing can have up to twice the amount of DNA as cells that are not dividing. The cell cycle consists of discrete phases: gap 1 (G1), DNA synthesis (S), gap 2 (G2), and mitosis (M) (Murray and Hunt, 1993). Cell growth occurs during the G1 phase before cell division begins, and cells may enter a G0 resting state from this phase in response to starvation or other unfavorable environmental conditions (Murray and Hunt, 1993). For cells to divide, they must first replicate their DNA (S phase) and then grow and produce the structures (G2 phase) necessary for mitosis. Cell division occurs during mitosis.

The proportion of cells in the S, and G2 and M (G2/M) phases are indicative of cells that may divide, and the use of flow-cytometric cell-cycle analysis to assess physiological condition is founded on the premise that cell proliferation is related to condition. Specific tissue types (e.g., Theilacker and Shen, 1993b; González-Quirós et al., 2007; Porter and Bailey, 2011) or individual whole larval homogenates (Domingos et al., 2012) have been used with this method,

and flow-cytometric cell-cycle analysis is as responsive to starvation as the RNA:DNA ratio in that it is able to detect starvation within a few days of no feeding (Porter and Bailey, 2011).

Flow cytometry has been used to measure the relative amount of cellular RNA and DNA in the brain cells of fish larvae (Theilacker and Shen, 1993b, 2001). Brain cells in the G0 and G1 phases were separated through the use of ribonuclease (RNAse): G0 were quiescent cells with low RNA content, and G1 were growing cells with high RNA content (Theilacker and Shen, 1993b). Theilacker and Shen (1993b) showed that the proportion of high-RNA-content G1 cells differed between fed and starved larvae, and it was suggested that those cells gave an indication of short-term changes in past feeding history. Nuclear RNA (nRNA) may react faster to metabolic changes than does cellular RNA, making it more sensitive to environmental change (Piwnicka et al., 1983) and potentially useful for assessment of the condition of fish larvae.

Most nRNA is contained in the nucleolus (Li et al., 2006), the site of ribosome biogenesis (Sirri et al., 2008), and ribosome production correlates with cellular growth (Caldarola et al., 2009). For many cell types grown in culture, nRNA content of G1 cells is highly variable, and a specific amount may be required for entry into the S phase (Darzynkiewicz et al., 1980; Piwnicka et al., 1983; Staiano-Coico et al., 1989). The threshold amount needed for G1 cells to progress into the S phase has been defined as the minimum nRNA content of S-phase nuclei determined from plots of nRNA and DNA fluorescence measured by flow cytometry (Darzynkiewicz et al., 1980).

In a previous study with flow cytometry (Porter and Bailey, 2011), cell-cycle information (fraction of nuclei in the S and G2/M phases), larval standard length (SL), and temperature were used as covariates in a laboratory-developed model for measurement of the condition of larvae of Walleye Pollock (*Gadus chalcogrammus*). In the study that we describe here, we found that an additional covariate based on nRNA improved the classification accuracy of a similar condition model by more clearly defining healthy (i.e., feeding, growing) and unhealthy (i.e., starving) larval conditions. We also examined the effect of rearing temperature on nRNA measurements and the effect of storage on frozen tissue used for measurements with flow cytometry.

Materials and methods

Larval rearing

Adult Walleye Pollock were collected by trawl in Shelikof Strait, Gulf of Alaska, during the spawning season in March 2009 and 2010 by the Alaska Fisheries Science Center (AFSC). For our experiments in 2009 and 2010, eggs from a single fish pairing (one female and one male) were fertilized and maintained aboard ship in the dark at 3°C before they were transported to the AFSC in Seattle, Washington. Eggs used for 2011 experiments came from a brood stock of adults kept at the AFSC laboratory at the Hatfield Marine Science Center in Newport, Oregon, and were also the result of a single fish pairing.

In 2009 and 2010, larvae were reared in 2 feeding treatments: an *always-fed* treatment, in which larvae were considered healthy, and an *unfed* (starved) treatment in which larvae were considered unhealthy. Only the always-fed treatment was used in 2011. Rearing methods are described in Porter and Bailey (2011). Two replicate tanks were used for each feeding treatment. The size of the tanks varied with each experiment: 120 L in 2009, 20 L in 2010, and 60 L in 2011. The diet of larvae in the always-fed treatment consisted of laboratory-cultured rotifers (*Brachionus plicatilis*) that were fed an algal diet (*Isochrysis galbana* and *Pavlova lutheri*) and a commercial rotifer supplement. Rotifers were maintained in the rearing tanks at a concentration of 10 mL^{-1}. Natural zooplankton, which included primarily copepod nauplii (*Acartia* spp.) and gastropod veligers, were collected from a local lagoon and screened through 202-µm mesh; they also were maintained in the always-fed rearing tanks, at a concentration of 3 mL^{-1}. A 16-h daylight cycle with a light level of 2.5 µmol photon m^{-2} s^{-1} from overhead, full-spectrum fluorescent lights was used, and larvae were sampled 4–6 h after the lights turned on at 0600 h. To avoid sampling larvae that were not actively feeding and possibly unhealthy, only larvae that had prey in their gut were sampled from the always-fed treatment. Rearing temperatures varied: 6.0°C in 2009, 2.9°C, 5.9°C, and 8.7°C in 2010, and 6.5°C in 2011.

2009 experiments: nRNA staining protocol and covariate

Three methods for preservation of larvae were tested to determine which of them was optimal for simultaneous staining of DNA and nRNA in muscle cell nuclei of larvae of Walleye Pollock: 1) storage at –80°C, 2) a methanol treatment, and 3) storage at –80°C followed by a methanol treatment for 15 min before tissue processing. To stain DNA for cell-cycle analysis with flow cytometry, 4′,6-diamidino-2-phenylindole (DAPI), a fluorescent DNA stain, was used at a concentration of 10 µg mL^{-1}, and nRNA was stained with Invitrogen Syto RNASelect[1] green fluorecent cell stain (S32703, Life Technologies Corp., Carlsbad, CA), hereafter referred to as Syto RNASelect stain.

Preservation of tissue by freezing works well for DAPI-DNA staining (Theilacker and Shen, 2001), and methanol-preserved tissue has been stained successfully with Syto RNASelect stain (Life Technolo-

[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

gies Corp.[2]; Molecular Probes, Inc.[3]). Syto RNASelect stain is known to also bind to DNA and emit an extremely weak fluorescent signal; therefore, we examined through the use of a staining study the effect that varying concentrations of Syto RNASelect stain had on the DAPI-DNA interaction to confirm that the stain concentration we used did not interfere with DAPI staining. Three concentrations of Syto RNASelect stain were mixed in DAPI and tested: 500 nM (concentration recommended by Invitrogen), 1000 nM (maximum concentration recommended by Invitrogen), and 2000 nM. The optimal preservative and stain concentration was defined as that which provided the highest nRNA fluorescence that did not affect DNA fluorescence as measured by flow cytometry. Each preservative was tested with the 3 concentrations of RNA stain previously described and a DAPI-only negative control that was used to determine background RNA fluorescence.

For preservative and stain testing, larvae were removed from a rearing tank, anesthetized in a 1% solution of tricaine methanesulfonate (MS222), measured for their SL with an ocular micrometer, and placed in a 1.5-mL microcentrifuge tube that was either filled with methanol or inserted into a gel-filled microcentrifuge tube holder that was frozen at –80°C. Tubes were kept in the holder until they were transferred to a –80°C freezer. The nuclei of 29 always-fed larvae were pooled to have enough material to simultaneously test 3 stain concentrations and the control group for each type of preservative. Six replicate aliquots were taken for each stain concentration and control. A specific volume of Syto RNASelect stain was then added to each aliquot to equal the desired final concentration. Larvae were processed and analyzed as described in the section *Materials and methods, Flow cytometry*. To determine optimal RNA stain concentration, analysis of variance (ANOVA), Tukey's tests, and Dunnett's tests were used to compare nRNA and DNA fluorescence associated with each RNA stain concentration and the control. SYSTAT, vers. 13 (Systat Software, Inc., Chicago), was used for all statistical testing.

After the optimal preservation method and stain concentration were determined, nRNA staining was confirmed through comparison of untreated muscle cell nuclei to those nuclei treated with RNAse. Nuclei were treated in a mixture of DAPI and RNAse A (50 μg mL^{-1} RNAse A, Sigma R6513, DNAse free) at room temperature for 25 min before adding the Syto RNASelect stain. The nuclei of 12 always-fed larvae were pooled to have enough nuclei to simultaneously test 3 treatments: a positive control (DAPI+RNA stain), a negative control (DAPI only), and a treatment (RNAse A+DAPI+RNA stain). Four replicate aliquots were taken for each treatment, and ANOVA and Tukey's tests were used to compare RNAse A-treated nuclei to both the positive and negative controls.

After the staining protocol, an nRNA covariate to test in a larval condition model was determined with larvae from the always-fed and unfed treatments. Ten individuals were taken from each replicate tank of both treatments at first feeding (defined as the day when 50% of the larvae had prey in their gut) and then at 4, 8, 11, and 14 days after first feeding. Five potential nRNA covariates were investigated: geometric mean fluorescence of nuclei in the G0 and G1 (G0/G1), S, and G2/M phases of the cell cycle pooled, mean fluorescence of each cell-cycle phase (G0/G1, S, and G2/M) separately, and the ratio of the number of S-phase nuclei to the number of G1-phase nuclei with high RNA content (hereafter, this ratio will be referred to as RSG1; see *Materials and methods, Flow cytometry* section). RSG1 is a measure of potential cell division based on progression of nuclei from the G1 to S phase. An unhealthy larva would not be expected to grow or it would grow more slowly than a healthy individual. Therefore, an unhealthy larva would have fewer dividing cells and potentially less S-phase nuclei, and its ratio of S-phase nuclei to G1-phase nuclei with high RNA content would be smaller than the ratio for healthy individuals. Nuclear RNA fluorescence between treatments was compared with the 2-sample *t*-test. The Mann-Whitney *U* test was used to compare RSG1 between treatments.

2010 experiment: nRNA, temperature, and condition

Five larvae were sampled from each always-fed tank on 3 separate days after feeding began. A degree-day model (degree-day=temperature*fish age in days) was used so that larvae were sampled on the day of similar developmental stage after first feeding, not on the same calendar day after first feeding (Table 1). Eight larvae from the unfed treatment were sampled from each tank daily, beginning at first feeding and ending at yolk exhaustion. All larvae were frozen at –80°C. Geometric mean nRNA fluorescence for nuclei in the G0/G1, S, and G2/M phases of the cell cycle pooled, and RSG1 for each larva was determined (see *Materials and methods, Flow cytometry* section). ANOVA and Tukey's tests were used to examine differences in fluorescence and RSG1 between feeding treatments and among temperatures.

2011 experiment: effect of freezing and storage on nRNA and DNA

After 4 days of feeding, 20 control larvae (not frozen) were processed directly from the rearing tanks (10 from each replicate tank) and analyzed by flow cytome-

[2] Life Technologies Corp. 2013. Life Technologies: Syto RNASelect green fluorescent cell stain—5 mM solution in DMSO. Life Technologies Corp., Carlsbad CA. [Product information webpage; Available from http://products.invitrogen.com/ivgn/product/S32703, accessed August 2013.]

[3] Molecular Probes, Inc. 2004. Syto RNASelect green fluorescent cell stain (S32703). Molecular Probes, Inc., Eugene, OR. [Product information document; Available from http://tools.invitrogen.com/content/sfs/manuals/mp32703.pdf.]

Table 1

Days after first feeding when laboratory-reared larvae of Walleye Pollock (*Gadus chalcogrammus*) were sampled to determine the effect of temperature on nuclear RNA. Rearing tanks were maintained at 3 different temperatures. Because temperature can affect the growth rate of fishes, a "degree-day" model (degree-day=temperature*fish age in days) was used to ensure that that fish were taken at similar developmental stages.

Rearing temperature (°C)	Sampling days after first feeding (determined by degree-day model)	Sampling days (calendar days) after first feeding
2.9	17, 35, 61	6, 12, 21
5.9	18, 35, 65	3, 6, 11
8.7	17, 33, 58	2, 4, 7

try (see *Materials and methods, Flow cytometry* section). On the same day, 60 larvae were frozen at –80°C in individual tubes (30 from each tank) for flow-cytometric cell-cycle analysis at later dates. At 4 weeks after they were frozen and at additional intervals of 3, 6, and 10 months, 15 of these frozen larvae were randomly selected and analyzed with flow cytometry. Geometric mean nRNA fluorescence for nuclei in the G0/G1, S, and G2/M phases of the cell cycle pooled and RSG1 was determined for each larva (see *Materials and methods, Flow cytometry* section). The fraction of nuclei in the G0/G1, S, and G2/M phases of the cell cycle also was determined for each larva (see *Materials and methods, Flow cytometry* section). ANOVA, Dunnett's tests, and Tukey's tests were used to compare nRNA fluorescence, DNA fluorescence, and fractions of nuclei in the G0/G1, S, and G2/M phases between the control and frozen samples and among the frozen samples over time.

Flow cytometry

The tissue preparation protocol described in Porter and Bailey (2011), modified from Theilacker and Shen (2001), was followed. A frozen larva was placed on ice to thaw just before it was processed. A methanol-preserved larva was processed directly from the preservative. The larva was then placed on a glass depression slide into an approximately 100-µL mixture of DAPI and Syto RNASelect stains. The head and gut were dissected away from the trunk musculature, and the muscle tissue was sliced into 4 or 5 pieces with 2 scalpels.

Only the pieces of muscle tissue were transferred into a microcentrifuge tube that contained a 230-µL mixture of DAPI and Syto RNASelect stains and the mixture was triturated 6 times with a 1-mL syringe with a 25-gauge needle to release the nuclei from the muscle cells. The solution was filtered through a 48-µm filter into another microcentrifuge tube to separate the stained nuclei from large cellular debris. Prepared samples were kept on ice until they were analyzed with a BD Biosciences Influx flow cytometer (BD Biosciences, San Jose, CA) typically within 4–5 h of preparation. DAPI was excited with a 350-nm UV laser, and Syto RNASelect stain was excited with a 488-nm laser. The DAPI/DNA detector filter was 450/40, and the detector filter for Syto RNASelect stain/RNA was 525/30. Flourochrome compensation for overlapping emissions spectra of RNA and DNA stains is unnecessary when exciting DAPI and Syto RNASelect stain with the BD Biosciences Influx flow cytometer. The beams of the 350-nm and 488-nm lasers intercept the stream at spatially separate points. Each beam excites only the stain for DNA (350 nm) or RNA (488 nm). The emission light is focused on separate mirror pinholes and is detected in separate modular detection blocks. Chicken and trout erythrocyte nuclei (Biosure, Inc., Grass Valley, CA) stained with the same mixture of DAPI and Syto RNASelect stains used for the muscle cell nuclei were used as controls.

At the beginning of each flow cytometry session, each control type was run and necessary adjustments were made to the flow cytometer to keep control fluorescence values similar to previous sessions. DNA and RNA fluorescence values for larvae within the same experiment but measured during different flow cytometry sessions were made comparable by standardizing to a common control value. Samples that had <5000 nuclei analyzed or a coefficient of variation >9.00 for the cell-cycle phase of G0/G1 were not used in further analyses, and the use of this criteria resulted in rejection of about a third of all samples.

For each larva, the fraction of nuclei in the G0/G1, S, and G2/M phases was calculated with MultiCycle AV software, vers. 4.0 (Phoenix Flow Systems, San Diego, CA). FCS Express flow cytometry analysis software, vers. 3.0 (De Novo Software, Los Angeles, CA) was used to calculate RSG1 and geometric mean fluorescence values for nRNA and DNA. DAPI area (DNA content, linear scale) in relation to DAPI height (DNA content, linear scale) was plotted for each larva, and a gate (a boundary used to enclose specific data points) was made to exclude debris, doublets, triplets, and large aggregates from nuclei in the G0/G1, S, and G2/M phases (Fig. 1A). Nuclei in those phases were located within the gate (Fig. 1B).

To calculate geometric mean values of nRNA and DNA fluorescence for each larva, a scatter plot of nRNA fluorescence (log scale) values in relation to DNA fluorescence (linear scale) values was made from the data enclosed within the gate for nuclei in the G0/G1, S, and G2/M phases. Each phase was gated separately for mean fluorescence values (Fig. 2), and a single gate that enclosed nuclei of all phases was used for the pooled mean fluorescence value. RSG1 was calculated with the same nRNA and DNA fluorescence scatter plot.

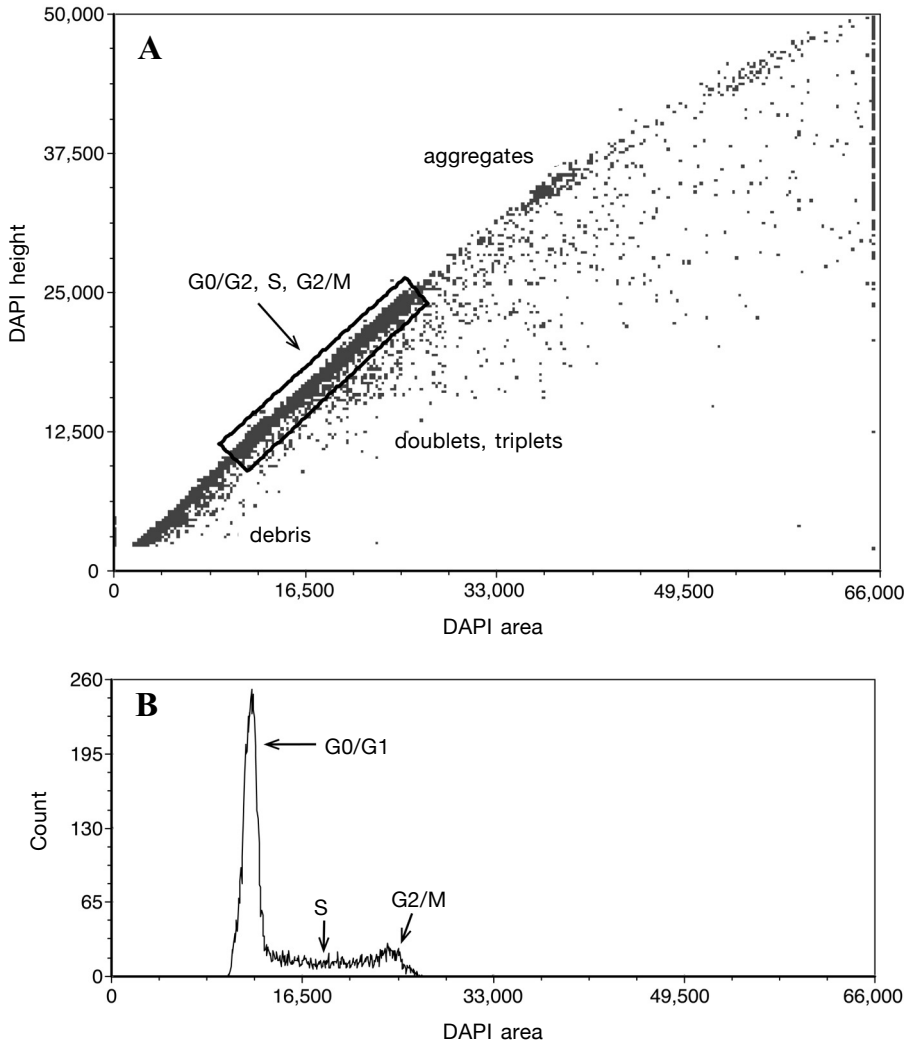

Figure 1

(**A**) Scatter plot of 4′,6-diamidino-2-phenylindole (DAPI) area (DNA content, linear scale) and DAPI height (DNA content, linear scale) for a laboratory-reared larva of Walleye Pollock (*Gadus chalcogrammus*) sampled in 2009, showing the gating strategy used to exclude debris, doublets, triplets, and large aggregates from muscle cell nuclei in the G0 and G1 (G0/G1), S, and G2 and M (G2/M) phases of the cell cycle. Those nuclei are enclosed within the gate, which is represented by a rectangle. (**B**) Histogram showing the distribution pattern of the nuclei within the gate. Phases of the cell cycle: G1=gap 1 or cell growth before cell division, G0=resting state from G1, S=DNA synthesis, G2=gap 2 or cell growth before mitosis, and M=mitosis.

A gate that divided G1-phase nuclei into fractions of low and high nRNA content on the basis of nRNA fluorescence of S-phase nuclei was made by following Staiano-Coico et al. (1989). The smallest value of S-phase nRNA fluorescence was the minimum nRNA content needed for G1 nuclei to enter the S phase, and it was assumed that any nuclei with greater fluorescence also could enter the S phase (Staiano-Coico et al., 1989). A gate that enclosed G1 nuclei was made, beginning at the smallest nRNA fluorescence value of the S-phase nuclei and extending to enclose the G1 nuclei with the highest nRNA fluorescence values (designated G1B; Fig. 3, A and B). The G1B group was defined as the number of nuclei with the potential to progress from the G1 to S phase. The number of S-phase nuclei was determined through the use of a gate that enclosed those nuclei (Fig. 3, A and B), and RSG1 was calculated by dividing that number by the number of G1B nuclei.

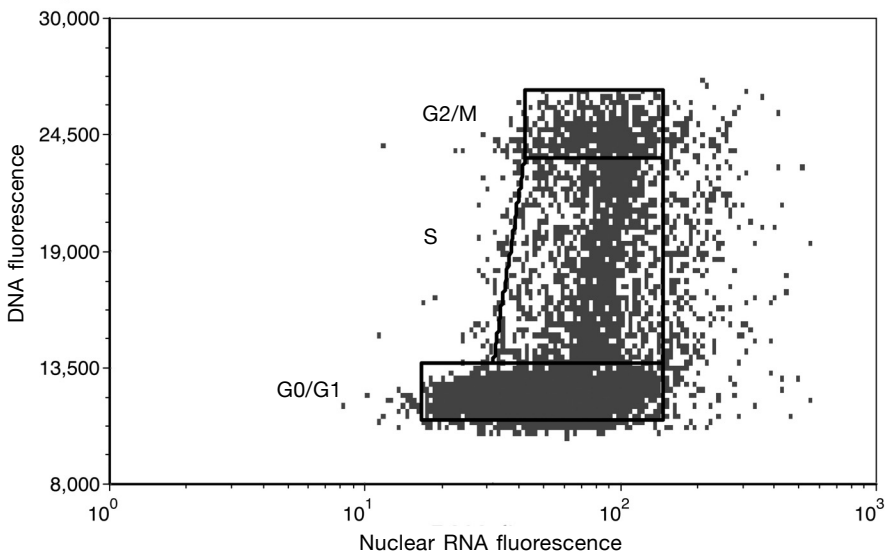

Figure 2

Scatter plot of nuclear RNA (log scale) and DNA fluorescence (linear scale) of muscle cell nuclei in the G0 and G1 (G0/G1), S, and G2 and M (G2/M) phases of the cell cycle for a laboratory-reared larva of Walleye Pollock (*Gadus chalcogrammus*) sampled in 2009. The rectangles outlined in black indicate the gates used to calculate geometric mean values of nuclear RNA and DNA fluorescence. Fluorescence values are arbitrary units. Phases of the cell cycle: G1=gap 1 or cell growth before cell division, G0=resting state from G1, S=DNA synthesis, G2=gap 2 or cell growth before mitosis, and M=mitosis.

Model testing

Data from all 3 years were pooled for model testing. One-third of the data were randomly removed from that data set and used for independent cross-validation testing. The remaining data were used to formulate discriminant analysis models similar to the model described in Porter and Bailey (2011) to classify larvae as healthy (feeding and growing) or unhealthy (starving). Models had SL, temperature, fraction of cells in the S phase, and fraction of cells in the G2/M phases as covariates, and were tested with and without the nRNA covariate included. The arcsin $\sqrt{x}$ transformation was used to normalize the fraction of cells in the S phase, fraction of cells in the G2/M phases, and RSG1. Models were compared on the basis of the accuracy of their classification of the cross-validation data set and Akaike's information criterion values (Burnham and Anderson, 2002).

Results

Nuclear RNA staining protocol

Flow-cytometric cell-cycle analysis showed that the tissue from frozen samples had a small amount of debris and distinct peaks in G0/G1 and G2/M phases. Fixing larvae in methanol for either a short period of time or long-term storage did not work as well; samples contained a large amount of cellular debris and aggregates, and the nuclei did not disassociate from the tissue easily. Samples from larvae preserved long term in methanol contained too much debris to be usable for further analysis. Debris was not as great in the samples that were frozen and then received a short-term methanol treatment as it was in the samples stored long term in methanol. The latter samples were usable, but they were not as clean as the samples that were frozen and not treated with methanol. Comparison of the RNA fluorescence between frozen tissues and tissues that were frozen and then treated with methanol indicated that short-term methanol preservation did not improve RNA staining; therefore, frozen tissue (stored at –80°C) worked best for preservation of muscle tissue from larvae of Walleye Pollock for nRNA and DNA staining and was used for all further tests and experiments.

Syto RNASelect stain concentration affected both nRNA and DNA fluorescence. The fluorescence of nRNA for all stain concentrations was significantly higher than the values observed for the DAPI–only control (ANOVA, $F_{(3,19)}$=165.94, P<0.001; Dunnett's test, P<0.001 for each concentration; Fig. 4A). The nRNA fluorescence values of the DAPI+1000-nM Syto

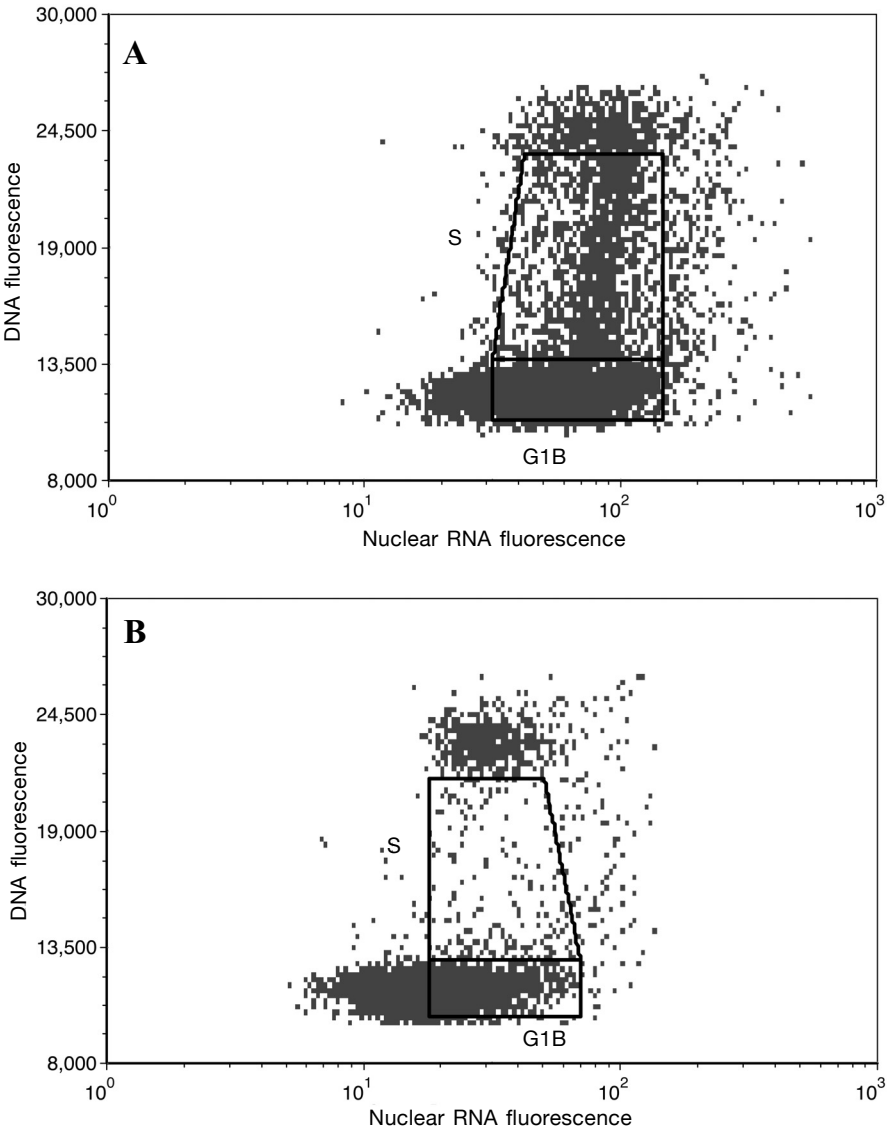

Figure 3

Scatter plots of cell-cycle analysis by flow cytometry of muscle cell nuclei of (**A**) an always-fed larva (fed 4 days) and (**B**) an unfed larva (starved 14 days) of Walleye Pollock (*Gadus chalcogrammus*) reared in 2009. The rectangles outlined in black indicate the gates used to determine the number of G1-phase nuclei with elevated nuclear RNA content needed to enter the S phase (G1B), and the total number of nuclei in the S phase of the cell cycle (S). The G1 phase of the cell cycle is when cell growth occurs before cell division, and the S phase is when DNA replicates. The always-fed larva had a distinct group of S-phase nuclei, and, for the unfed larva, S-phase nuclei were fewer and dispersed. Fluorescence values are arbitrary units.

RNASelect stain concentration and DAPI+2000-nM Syto RNASelect stain concentration were significantly higher than the values for the DAPI+500-nM Syto RNASelect stain concentration (ANOVA, $F_{(3,19)}$=165.94, P <0.001; Tukey's test, P<0.01 for both stain concentrations; Fig. 4A), but there was no significant difference in fluorescence between them (ANOVA, $F_{(3,19)}$=165.94, P<0.001; Tukey's test, P=0.44; Fig. 4A).

There was no significant difference in DNA fluorescence between the control and the 500-nM and 1000-nM Syto RNASelect stain concentrations (ANOVA, $F_{(3,19)}$=10.90, P<0.001; Dunnett's test, P=0.57 and

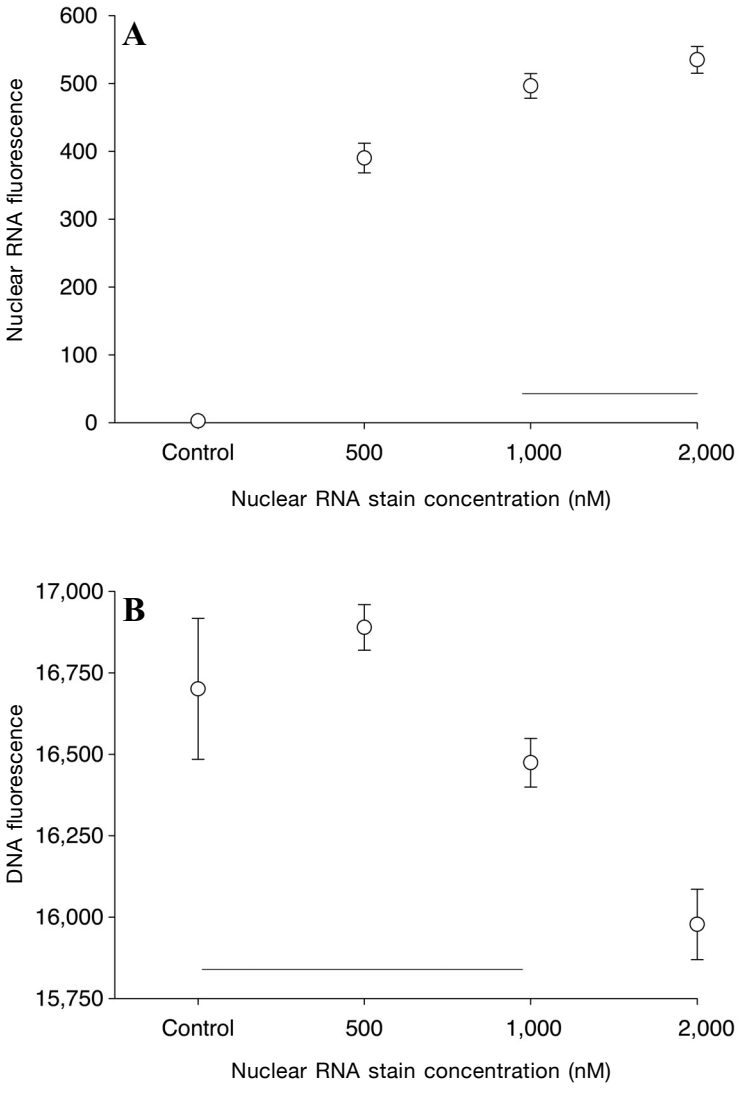

Figure 4

Mean fluorescence of (**A**) nuclear RNA and (**B**) DNA as a function of nuclear RNA stain concentration observed in muscle cell nuclei from larvae of Walleye Pollock (*Gadus chalcogrammus*) reared in 2009. The horizontal bar indicates fluorescence values that are not significantly different (P>0.05). Error bars indicate ±1 standard error of the geometric mean. Fluorescence values are arbitrary units.

0.44 respectively; Fig. 4B). The 2000-nM concentration caused a significant reduction of DNA fluorescence compared with the DNA fluorescence of the control (ANOVA, $F_{(3,19)}$=10.90, P<0.001; Dunnett's test, P=0.002; Fig. 4B), indicating that a Syto RNASelect stain concentration >1000 nM negatively affected DNA staining. Additionally, no significant difference was observed in DNA fluorescence between the 500-nM and 1000-nM concentrations (ANOVA, $F_{(3,19)}$=10.90, P<0.001; Tukey's test, P=0.10; Fig. 4B). Therefore, the 1000-nM concentration was optimal for nRNA staining because it produced the highest nRNA fluorescence and had no effect on DNA staining.

The nRNA fluorescence of the RNAse-treated nuclei was significantly less than the values seen for the positive control (DAPI+1000-nM Syto RNASelect stain), indicating that nRNA was being stained (ANOVA, $F_{(2,9)}$=386.26, P<0.0001; Tukey's test, P<0.0001; Fig. 5A); however, the treated nuclei had a higher fluorescence than the negative control (DAPI only; ANOVA, $F_{(2,9)}$=386.26, P<0.0001; Tukey's test, P<0.0001; Fig. 5A), indicating fluorescence signal from stained DNA or the incomplete removal of nRNA from the samples. There was no significant difference in DNA fluorescence among the 3 treatments (ANOVA, $F_{(2,9)}$=0.009, P=0.99; Fig. 5B); therefore, it is most likely that the treatment with RNAse A did not completely remove all of the nRNA. Additionally, this experiment independently confirmed that the 1000-nM concentration of the Syto RNASelect stain does not affect DNA staining because there was no significant difference in DNA fluorescence between the group stained with DAPI only and the group stained with DAPI+1000-nM Syto RNASelect stain.

Nuclear RNA covariate

For each treatment, larval SL was not significantly different between replicate tanks (always-fed treatment, 2-sample t-test, t_{61}=0.196, P=0.85; unfed treatment, 2-sample t-test, t_{48}=0.45, P=0.65), indicating that larvae in those tanks responded similarly to the same treatment. Therefore, replicate measurements of nRNA fluorescence from each tank for each treatment were pooled. The growth rate of larvae in the always-fed treatment from hatching to 19 days after hatching was 0.11 mm d^{-1}, a rate that is typical for larvae of Walleye Pollock reared in a 6°C laboratory (Porter and Theilacker, 1999). To formulate the nRNA covariate, we used 113 larvae, 63 always-fed (healthy) and 50 unfed (unhealthy). There was no significant difference in nRNA fluorescence between feeding treatments when all phases of the cell cycle were pooled or when phases were examined separately (Table 2), indicating that nRNA fluorescence was not a useful indicator of condition. Unlike nRNA fluorescence, RSG1 was responsive to feeding conditions; therefore, it was chosen as the nRNA covariate for model testing.

Plots of nRNA and DNA fluorescence showed that always-fed larvae had a distinct group of aggregated S-phase nuclei that joined the G0/G1 and G2/M phases, and S-phase nuclei were fewer and dispersed for unfed larvae (Fig. 3, A and B). Overall RSG1 was significantly larger for always-fed larvae than for unfed larvae (0.23 [standard error of the mean 0.01] for always-fed and 0.16 [SE 0.01] for unfed larvae; Mann-Whitney U test, U=2299.5, N_1=63, N_2=50, P<0.001), clearly distinguishing larvae between the 2 feeding treatments. For always-fed larvae, there was an initial increase in RSG1 and then a gradual decline to its initial value after 2 weeks of feeding (Fig. 6A). For unfed individuals, RSG1 declined throughout the time period to less than half its initial value after 2 weeks of feeding (Fig. 6A). Nuclear RNA fluorescence did not show a distinct difference between feeding treatments until after 2 weeks of feeding (Fig. 6B).

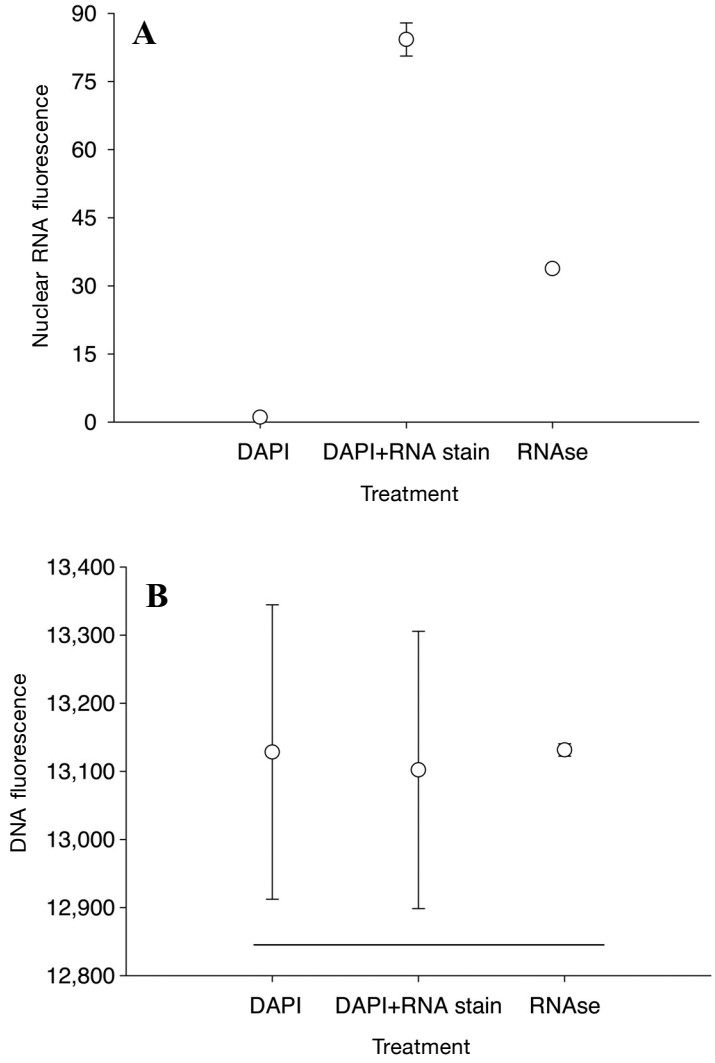

Figure 5

Mean fluorescence of (**A**) nuclear RNA and (**B**) DNA as a function of 3 treatments used to confirm nuclear RNA staining—4′,6-diamidino-2-phenylindole (DAPI) stain only (negative control), DAPI+nuclear RNA stain (positive control), and ribonuclease (RNAse) A—of muscle cell nuclei from larvae of Walleye Pollock (*Gadus chalcogrammus*) reared in 2009. The horizontal bar indicates fluorescence values that are not significantly different (P>0.05). Error bars indicate ±1 standard error of the geometric mean. Fluorescence values are arbitrary units.

Nuclear RNA, temperature, and condition

Growth in the always-fed treatment at the warmest temperature (8.7°C) was poor; therefore, larvae from this treatment were not used for further analyses. A growth rate of about 0.15 mm d^{-1} would be expected (senior author, unpubl. data), but larvae grew 0.08 mm d^{-1}. Both mean percentage of nuclei in the S-phase (8.90) and mean RSG1 (0.17) were small for a typical healthy, feeding larva, supporting the observation that those larvae were not growing well. Growth rates at the temperatures of 5.9°C (0.10 mm d^{-1}) and 2.9°C (0.06 mm d^{-1}) were typical for larvae reared at those temperatures.

There was no significant difference in overall nRNA fluorescence between the always-fed and unfed treatments (ANOVA, $F_{(1,61)}$=1.25, P=0.27; Table 3). Rearing temperature significantly affected overall nRNA fluorescence. Larvae reared at 2.9°C had a higher fluorescence than larvae reared at 5.9°C (ANOVA, $F_{(1,61)}$=4.47, P=0.04; Table 3), indicating more RNA in muscle nuclei from larvae reared at the colder temperature. RSG1 was significantly higher for the always-fed treatment than for the unfed treatment at both temperatures (ANOVA, $F_{(1,87)}$=59.65, P<0.01, Tukey's test, P<0.01; Table 3), similar to the result for the experiment conducted in 2009.

The effect of temperature on RSG1 was dependent on feeding treatment. RSG1 was smaller for always-fed larvae reared at 5.9°C compared with RSG1 of larvae reared at 2.9°C (ANOVA, $F_{(1,87)}$=18.56, P<0.001, Tukey's test, P<0.01; Table 3), and this result may indicate shorter duration for the cell cycle at warmer temperatures. RSG1 was not significantly different between unfed larvae reared at 8.7°C and at 2.9°C (ANOVA, $F_{(2,55)}$=3.61, P=0.03, Tukey's test, P=0.97; Table 3), indicating that temperature affected RSG1 only of

Table 2

Nuclear RNA fluorescence of muscle cell nuclei in various phases of the cell cycle from laboratory-reared larvae of Walleye Pollock (*Gadus chalcogrammus*). Larvae were sampled from the always-fed and unfed treatments used in experiments in 2009. Fluorescence values are arbitrary units and were adjusted on the basis of controls to make samples comparable among sessions of flow cytometry. Standard errors of geometric means are reported in parentheses. Cell-cycle phases: G1=gap 1 or cell growth before cell division, G0=resting state from G1 phase, S=DNA synthesis, G2=gap 2 or cell growth before mitosis, and M=mitosis. G0/G1=fluorescence of G0 and G1 phases combined, G2/M=fluorescence of G2 and M phases combined.

Treatment	RNA fluorescence	Mean	*n*	Statistical test	*P*
Always-fed	G0/G1, S, G2/M phases pooled	54.9 (1.9)	63	2-sample *t*-test, t_{111}=1.03	0.31
Unfed	G0/G1, S, G2/M phases pooled	52.1 (2.0)	50	–	–
Always-fed	G0/G1 phases	49.2 (1.6)	63	2-sample *t*-test, t_{111}=1.01	0.31
Unfed	G0/G1 phases	46.9 (1.6)	50	–	–
Always-fed	S phase	87.2 (2.7)	63	2-sample *t*-test, t_{111}=0.57	0.57
Unfed	S phase	90.5 (3.3)	50	–	–
Always-fed	G2/M phases	87.2 (2.7)	63	2-sample *t*-test, t_{111}=0.24	0.81
Unfed	G2/M phases	86.3 (3.1)	50	–	–

growing larvae. The data for unfed larvae were re-analyzed with the 8.7°C treatment removed to determine if the exclusion of the data from the 8.7°C always-fed treatment biased the conclusions from this study. The results were unchanged; there was no significant difference in RSG1 between unfed larvae at 2.9°C and 5.9°C (ANOVA, $F_{(1,87)}$=59.65, P<0.001, Tukey's test, P=0.05).

Model testing

Data from experiments in 2009, in 2010 (temperatures: 2.9°C and 5.9°C), and in 2011 were pooled for model testing (n=237). The 8.7°C experiment in 2010 was excluded because larvae grew poorly in the always-fed treatment. A control model used temperature, SL, arcsin $\sqrt{x}$ -transformed fraction of cells in the S phase, and arcsin $\sqrt{x}$ -transformed fraction of cells in the G2/M phases; and a test model added arcsin $\sqrt{x}$ -transformed RSG1 to the covariates used in the control model (n=158). For independent cross-validation testing, 49 always-fed larvae ranging from 5.36 to 8.64 mm SL and 30 unfed larvae from 5.20 to 5.92 mm SL were used (n=79). Both models significantly discriminated between the always-fed and unfed treatment groups (control model, Wilks's lambda=0.46, $F_{(4,153)}$=44.19, P<0.001; test model, Wilks's lambda=0.45, $F_{(5,152)}$=37.13, P<0.001).

The test model improved overall classification accuracy by 4%, and accuracy for both always-fed and unfed larvae increased (Table 4). The improvement in the always-fed treatment larvae was due to the correct classification of additional small, feeding larvae (<6.00 mm SL). The classification accuracy in the test model for those larvae improved 14%, increasing from 53% (8/15) to 67% (10/15) when RSG1 was used. Classification accuracy of unfed treatment larvae improved 3%, and the test model correctly classified all the unfed larvae tested (Table 4). For larvae <6.00 mm SL from both the always-fed and unfed treatments, classification accuracy improved 7% (an increase from 37/45 to 40/45). There was no difference in classification accuracy between models for larvae ≥6.00 mm SL, and both models correctly classified all of those larvae. Akaike's information criterion value for the test model was less than that value for the control model (–1130.53 and –1001.38, respectively), indicating that the addition of RSG1 improved model fit.

Effect of freezing and storage on nRNA and DNA

There was a loss of DNA when larvae were frozen and thawed, but that process did not affect nRNA measurements. The DNA fluorescence of fresh tissue was significantly greater than DNA fluorescence of frozen

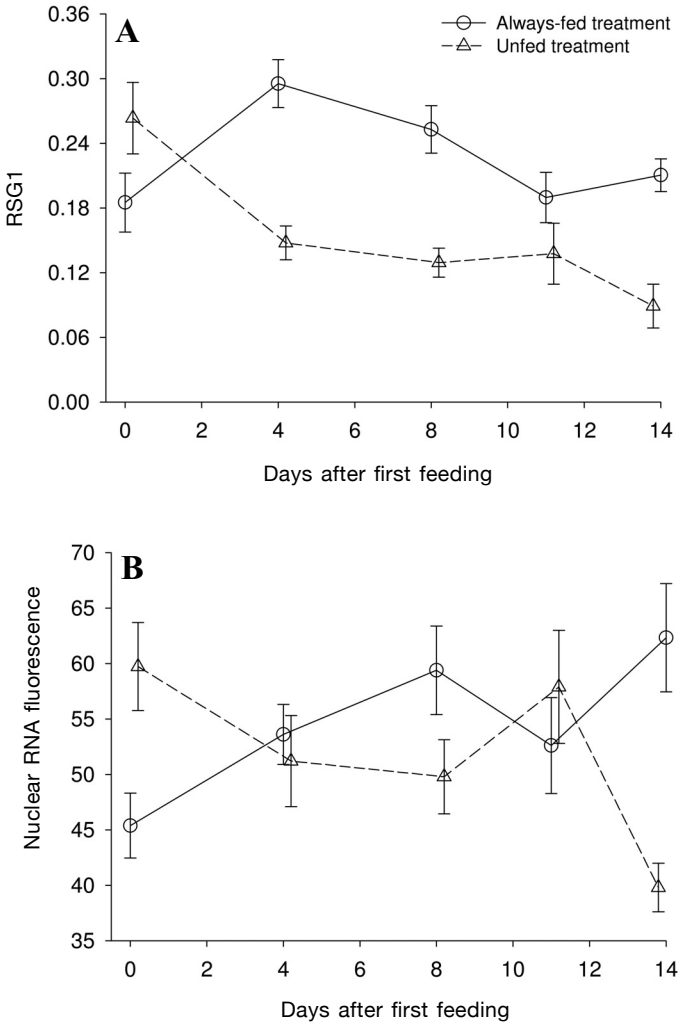

Figure 6

For always-fed and unfed treatments of larvae of Walleye Pollock (*Gadus chalcogrammus*) reared in 2009, (**A**) mean ratios of the number of S-phase nuclei to the number of G1-phase nuclei with high nuclear RNA content (RSG1) and (**B**) geometric mean fluorescence of nuclear RNA of all cell-cycle phases pooled. The G1 phase of the cell cycle is when cell growth occurs before cell division, and the S phase is when DNA replicates. Error bars indicate ±1 standard error of the mean. Fluorescence values are arbitrary units.

tissue (ANOVA, $F_{4,48}$=36.67, P<0.001, Dunnett's tests, P<0.05 for all frozen groups; Table 5), indicating that DNA was lost when tissue was frozen and thawed. The percentage of nuclei in the G2/M phases was not significantly different between the fresh and frozen tissue treatments (ANOVA, $F_{4,48}$= 2.00, P=0.11; Table 5), but freezing affected the percentage of nuclei in the G0/G1 and S phases. There was a significant increase in the percentage of nuclei in the G0/G1 phases between fresh and frozen tissue (ANOVA, $F_{4,48}$=19.90, P<0.001, Dunnett's tests, P<0.01; Table 5), and freezing had the opposite effect on S-phase nuclei, namely a decrease in the percentage of nuclei in that phase (ANOVA, $F_{4,48}$=19.14, P<0.001, Dunnett's tests, P<0.01; Table 5). The increase in the percentage of nuclei in the G0/G1 phases in the freezing treatment may be due to a loss of DNA from S-phase nuclei that caused them to be identified as nuclei in the G0/G1 phases on the basis of their fluorescence signal. There was no significant change in the percentage of G0/G1- or S-phase nuclei among the 4 frozen groups of larvae tested (Tukey's tests, P >0.35; Table 5), indicating that DNA was stable during storage of frozen tissue. This result indicates that DNA was lost by S-phase nuclei either during the freezing process or subsequent thawing. There was no significant difference in nRNA fluorescence between fresh and frozen tissues (ANOVA, $F_{4,48}$=2.38, P=0.06; Table 5), indicating no loss of nRNA. RSG1 showed results similar to those as DNA in that RSG1 of frozen tissue was significantly less than RSG1 of fresh tissue, and it was stable during the 10 months of storage of frozen tissue (ANOVA, $F_{4,48}$= 16.70, P<0.001, Dunnett's tests, P<0.01; Tukey's tests >0.90; Table 5).

Discussion

We developed a protocol for staining nRNA in muscle cell nuclei of larvae of Walleye Pollock for use in flow cytometry, and we showed that the inclusion of an nRNA covariate in a model resulted in more accurate measurement of physiological condition than did cell-cycle analysis alone. Accurate assessment of condition of fish larvae is essential because small changes in mortality rate over a long period of time can strongly influence future recruitment (Houde, 1987). RSG1, based on nRNA fluorescence, proved to be an indicator of potential growth that was responsive to feeding conditions, and it contributed meaningful improvement to the discriminant analysis model for assessment of larval condition, as shown by an increase in classification accuracy and a decrease in Akaike's information criterion value. Data from 3 years were used for model testing; therefore, results are not unique to a single group of larvae. The classification accuracy of small larvae (<6.00 mm SL) was most improved (7%). This result is important because that size class includes larvae that have just started to feed, and the S- and G2/M-phase fractions can be highly variable in first-feeding larvae and may not always distinctly indicate condition. Additionally,

Table 3

The ratio of the number of S-phase nuclei to the number of G1-phase nuclei with high nuclear RNA content (RSG1) and values of nuclear RNA (nRNA) fluorescence for larvae of Walleye Pollock (*Gadus chalcogrammus*) sampled from always-fed and unfed treatments reared at different temperatures in 2010. Fluorescence values are arbitrary units and were adjusted on the basis of controls to make samples comparable among sessions of flow cytometry. Standard errors of geometric means of nRNA fluorescence (all cell-cycle phases pooled) and of RSG1 means are reported in parentheses. Cell-cycle phases: G1=gap 1 or cell growth before cell division, G0=resting state from G1 phase, S=DNA synthesis, G2=gap 2 or cell growth before mitosis, and M=mitosis.

Rearing temperature (°C)	Treatment	*n*	RSG1	nRNA fluorescence
2.9	Unfed	28	0.14 (0.01)	32.3 (1.5)
2.9	Always-fed	26	0.26 (0.02)	32.5 (2.0)[1]
5.9	Unfed	13	0.09 (0.01)	26.7 (0.8)
5.9	Always-fed	24	0.19 (0.01)	30.5 (1.9)[2]
8.7	Unfed	17	0.13 (0.02)	26.5 (1.1)

[1] n=14.
[2] n=10.

the sizes of healthy and unhealthy larvae overlap in that size class.

Nuclear RNA varied with rearing temperature, increasing as temperature decreased, a result similar to the findings of other studies on the effect of temperature on RNA content of larval fishes (Canino, 1994; Malzahn et al., 2003). This result also indicates that our nRNA staining protocol worked as intended.

Temperature affected RSG1, and, therefore, it needs to be accounted for when our method is used to measure the condition of larvae sampled from the field. RSG1 for healthy larvae was smaller at warmer temperatures (5.9°C in our study), and this observation may indicate that nuclei were cycling faster through the cell cycle than nuclei at colder temperatures. Other studies have shown that increasing temperature decreases cell-cycle duration in other species, such as yeast (*Saccharomyces cerevisiae*) (Jagadish and Carter, 1978) and Magellan Plunderfish (*Harpagifer bispinis*) (Brodeur et al., 2003). There was no difference in the percentage of S-phase brain cells of fed larvae of Atlantic Cod (*Gadus morhua*) reared at 6°C and 10°C—a finding that was explained by an increased rate of progression by cells through the cell cycle at the higher temperature (González-Quirós et al., 2007). A similar result was also found for S-phase nuclei from muscle cells of larvae of Walleye Pollock reared at 3.2°C and 5.9°C, temperatures comparable to those used in our study (Porter and Bailey, 2011).

RSG1 of unhealthy larvae was not affected by temperature, probably as a result of the slow or ceased growth of these larvae. The brain cells of starved Atlantic Cod larvae reared at 10°C had a smaller percentage of S-phase cells than the brain cells of larvae starved at 6°C (González-Quirós et al., 2007), a difference that may be due to the length of time that the larvae were starved. Fish larvae in general starve faster at higher temperatures, and, for larvae of Walleye Pollock in a previous study, the percentage of S-phase nuclei decreased the longer larvae were starved (Porter and Bailey, 2011). Atlantic Cod larvae at both temperatures were starved for 5 days; therefore, the percentage of S-phase cells of the larvae starved at the higher temperature (10°C) would be expected to be less than the percentage for the larvae starved at the lower temperature.

The loss of DNA during freezing and thawing has been documented for human blood, where about 25% of the DNA was lost (Ross et al., 1990). Differences in nuclear membrane permeability among cell-cycle phases may account for the loss of DNA when larvae of Walleye Pollock were frozen and thawed, and they may explain why there was a decrease in the percentage of muscle cell nuclei in the S phase. S-phase nuclei may be more permeable than G2-phase nuclei (Coverly et al., 1993; Leno and Munshi, 1994), resulting in diffusion of DNA out of the nucleus, and they may rupture because they may be more fragile than nuclei at other phases—an outcome that also would contribute to loss of nuclei. Crytoprotectant has been used to stabilize DNA in brain cells of Walleye Pollock larvae during freezing (Theilacker and Shen, 1993a), and it could possibly be used to prevent the loss of DNA from muscle cell nuclei as well, but the use of cryptoprotectant needs further investigation, particularly for between-laboratory comparisons where standardized protocols are used (Caldarone et al., 2006). In our study, the loss of DNA was unchanged for up to 10 months when the

Table 4

Results of larval condition for laboratory-reared larvae of Walleye Pollock (*Gadus chalcogrammus*) sampled in 2009, 2010, and 2011. Independent cross-validation testing of models without (control) and with (test) the ratio of the number of S-phase nuclei to the number of G1-phase nuclei with high nuclear RNA content (RSG1) included as a covariate. The arcsin $\sqrt{x}$ transformation was used to normalize the fraction of cells in the S phase of the cell cycle, fraction of cells in the G2/M phases of the cell cycle, and RSG1. G2/M=fraction of nuclei in the G2 and M phases combined. Cell-cycle phases: S=DNA synthesis, G2=gap 2 or cell growth before mitosis, and M=mitosis.

Control model
Covariates: standard length, temperature, arcsin $\sqrt{\text{S-phase fraction}}$, arcsin $\sqrt{\text{G2/M-phase fraction}}$

	Classification		
Treatment	Healthy	Unhealthy	Percentage correct
Always-fed	42	7	86
Unfed	1	29	97
		Overall correct	90

Test model
Covariates: standard length, temperature, arcsin $\sqrt{\text{S-phase fraction}}$, arcsin $\sqrt{\text{G2/M-phase fraction}}$, arcsin $\sqrt{\text{RSG1}}$

	Classification		
Treatment	Healthy	Unhealthy	Percentage correct
Always-fed	44	5	90
Unfed	0	30	100
		Overall correct	94

Table 5

Percentages of nuclei in the G0/G1, S, and G2/M phases of the cell cycle, the ratio of the number of S-phase nuclei to the number of G1-phase nuclei with high nuclear RNA content (RSG1), nuclear RNA fluorescence, and DNA fluorescence for muscle cell nuclei from fresh and frozen larvae of Walleye Pollock (*Gadus chalcogrammus*) sampled in 2011. Fluorescence values are arbitrary units and were adjusted on the basis of controls to make samples comparable among sessions of flow cytometry. Standard errors of geometric means of nRNA and DNA fluorescence (all cell-cycle phases pooled), of means for RSG1 and percentages of nuclei in cell-cycle phases are reported in parentheses. G0/G1=percentage of nuclei in G0 and G1 phases combined, G2/M= percentage of nuclei in G2 and M phases combined. Cell-cycle phases: G1=gap 1 or cell growth before cell division, G0=resting state from G1 phase, S=DNA synthesis, G2=gap 2 or cell growth before mitosis, and M=mitosis.

Treatment	*n*	Percentage of nuclei in G0/G1	Percentage of nuclei in S phase	Percentage of nuclei in G2/M phases	RSG1	nRNA fluorescence	DNA fluorescence
Fresh tissue	18	76.7 (1.2)	20.64 (0.97)	2.63 (0.42)	0.37 (0.03)	22.22 (0.43)	14,691.03 (194.93)
Frozen 4 weeks	11	85.3 (0.8)	11.78 (1.27)	2.96 (0.73)	0.18 (0.01)	23.72 (0.68)	12,082.43 (137.11)
Frozen 3 months	10	87.4 (1.0)	8.22 (1.22)	4.36 (0.59)	0.18 (0.02)	21.30 (0.59)	12,866.44 (187.72)
Frozen 6 months	6	87.8 (1.4)	8.50 (1.85)	3.71 (1.05)	0.20 (0.02)	23.69 (0.68)	14,280.30 (144.20)
Frozen 10 months	8	88.2 (1.4)	10.01 (2.00)	1.79 (0.84)	0.19 (0.03)	22.83 (0.82)	14,104.69 (221.88)

tissue was frozen at –80°C, indicating that DNA was stable during that time and under that condition.

Neither freezing and thawing nor storage of frozen tissue affected RNA in muscle cell nuclei of larvae of Walleye Pollock in our study. For frozen human tissue, no significant RNA degradation (as measured by gene expression and electropherograms) was detected after 16 h on ice (Micke et al., 2006), and another study showed that significant RNA degradation did not begin until 30 min after thawing at room temperature (Botling et al., 2009). Although those studies did not measure the amount of RNA present, they do support our assertion that the protocol used in our study was adequate to preserve RNA because larvae were frozen quickly, thawed tissue was kept cool on ice, and the time from tissue thawing to analysis with flow cytometry was typically not longer than 5 h. Our results differ from the findings of Theilacker and Shen (1993b) in that their study indicated that a cryoprotectant and acid were needed before freezing to stabilize RNA in brain cells of larvae of Walleye Pollock. This difference in results may be due to the difference in type of tissue used (muscle cell nuclei versus whole brain cells) (Olivar et al., 2009) or in the method used for preparation of tissue for flow cytometry. In Theilacker and Shen (1993b), tissue dissection occurred before freezing and whole cells were analyzed; however, in our study, tissue preparation occurred after freezing, and only nuclei were used.

Our results indicate that only frozen tissue should be analyzed when condition is measured with the method described here. Results would be inaccurate if fresh tissue were used because of its higher fraction of S-phase nuclei. Cell-cycle measurements (fraction of S- and G2/M-phase nuclei pooled) of independent groups of larvae of Walleye Pollock reared under similar conditions were not significantly different (Porter and Bailey, 2011), indicating that the effect of freezing and thawing was consistent. Freezing and thawing of tissue was also part of the method used in another study where standardized protocols were used for spectrofluorometric analysis of RNA and DNA for assessing larval fish physiological condition (Caldarone et al., 2006).

Conclusions

An nRNA covariate improved larval condition measurements. For minimal cost (the cost of the Syto RNASelect stain), model accuracy increased, and the greatest improvement was for small larvae. The assay that we developed in our study quickly determines condition, and, therefore, many larvae can be analyzed in a short period of time. In addition, larvae can be kept frozen for at least 10 months without affecting condition measurements. There is no diel pattern in RNA content or measurements of the cell-cycle phases for larvae of Walleye Pollock (Bailey et al., 1995; Theilacker and Shen, 2001); therefore, our method can be applied in the field, where sampling can occur anytime during a 24-h period. Because we found that temperature affected RSG1, future studies should include measurements at additional temperatures to formulate a model for field-sampled larvae.

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