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Assessing the condition of walleye pollock *Theragra chalcogramma* (Pallas) larvae using muscle-based flow cytometric cell cycle analysis

Steven M. Porter *, Kevin M. Bailey

NOAA, National Marine Fisheries Service, Alaska Fisheries Science Center, 7600 Sand Point Way NE, Seattle, WA 98115, USA

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ABSTRACT

Flow cytometric cell cycle analysis was used to determine the fraction of muscle cells in the S and G2 phases of the cell cycle, which were used as covariates with temperature and standard length, in a laboratory-developed model to assess the physiological condition of wild walleye pollock, *Theragra chalcogramma*, larvae. The assay was calibrated to the range of temperatures larvae are likely to encounter in the eastern Bering Sea, and it was sensitive to changes in condition within 3 days of starvation. The S and G2 phases of the cell cycle gave an indication of larval walleye pollock condition. Healthy larvae had a larger fraction of cells in the S phase than G2 phase, and unhealthy larvae had a larger fraction of cells in the G2 phase than the S phase. Validation tests showed that the model classified 75% to 83% of the larvae correctly. The assessment of the condition of walleye pollock larvae collected from the southeastern Bering Sea in 2007 indicated that unhealthy larvae were located on the continental shelf (6%), and this may be due in part to the coldest temperatures occurring there and less abundant prey. In the continental slope/ocean basin waters, where prey levels were higher and temperatures warmest, no larvae in unhealthy condition were found.

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1. Introduction

Mortality is high and variable during the larval stage of marine fishes and can greatly influence year-class strength. In addition to predation and environmental effects, starvation may significantly contribute to mortality occurring during the larval stage (Houde, 1987). Using a histological index, walleye pollock, *Theragra chalcogramma*, larvae in the Gulf of Alaska were determined to be most vulnerable to starvation during the first 2 weeks after first feeding, and at some locations and seasons, significant numbers of starving larvae were found (Theilacker and Porter, 1995).

Walleye pollock are an important component of the eastern Bering Sea (EBS) ecosystem due to their vast numbers and biomass and are of great commercial importance (Macklin and Hunt, 2004). Their recruitment in the EBS is not well understood but it is largely determined during the first year (Mueter et al., 2006), so the larval stage may play an important role. The EBS is composed of a deep basin and a broad continental shelf region (Fig. 1). The shelf has three hydrographic domains bounded by fronts and defined by bathymetry: coastal (<50 m depth), middle shelf (50 to 100 m depth), and outer shelf (100 to 200 m depth). The oceanic region consists of the continental slope and ocean basin (>200 m depth). Environmental conditions that favor strong walleye pollock recruitment include above normal temperatures, and reduced sea-ice or an early ice retreat (Quinn and Niebauer, 1995; Mueter et al., 2006). The biophysical processes that influence larval survival differ between the oceanic and shelf areas (Napp et al., 2000). In the oceanic area, effective prey concentration (i.e., larvae are selective feeders preferring late-stage calanoid copepod nauplii over other species, Hillgruber et al., 1995), and advection and mesoscale variability (e.g., presence of eddies) are important (Napp et al., 2000). On the shelf important factors are sea-ice, water temperature, wind mixing, and timing of the spring bloom (Napp et al., 2000). Measurements of larval walleye pollock condition in the EBS are very limited and have indicated that there were either no or few larvae in unhealthy condition (Walline, 1985; Napp et al., 2000), but the histological condition indices utilized by one study did not account for the low temperatures occurring there (Napp et al., 2000).

Methods that have been developed for assessing the condition (i.e., healthy or starving) of fish larvae include biochemical assays such as RNA/DNA ratio (Buckley et al., 1999), and lipid composition (Lochmann et al., 1995), as well as histological condition of tissues (Theilacker, 1978) and morphological measurements (Theilacker, 1978; Lochmann and Ludwig, 2003). Because starvation affects various tissues of fish larvae at different rates, rather than homogenizing whole larvae to determine condition, specific tissues should be examined to reduce confounding effects. Tissues that have been previously used to assess the condition of fish larvae include the brain (Theilacker and Shen, 1993; Bromhead et al., 2000), midgut (Theilacker and Porter, 1995), and muscle (Catalán and Olivar, 2002; Catalán et al., 2007). Muscle is useful for assessing condition because it is one of the most sensitive tissues to feeding conditions,

* Corresponding author. Tel.: +1 206 526 4271; fax: +1 206 526 6723.
E-mail address: steve.porter@noaa.gov (S.M. Porter).

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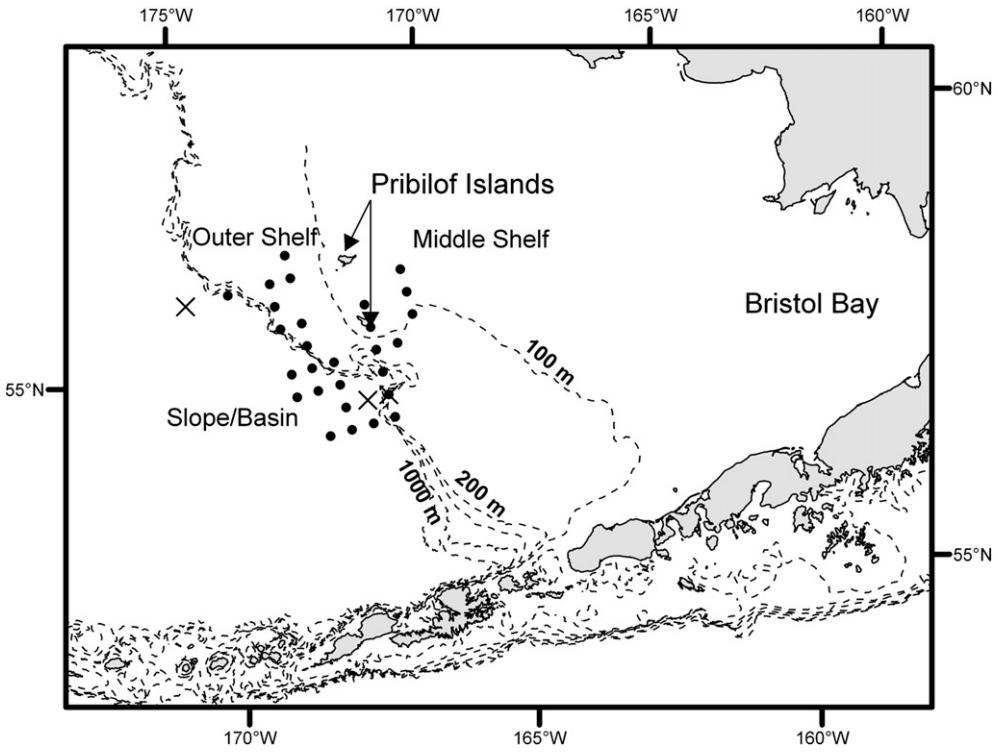

Fig. 1. Southeast Bering Sea walleye pollock, *Theragra chalcogramma*, collection sites for larval condition analysis in May 2007 (circles). "X"s indicate where microzooplankton were sampled.

serving as a reserve of protein that is used for metabolism during times of starvation (Houlihan et al., 1988). When comparing protein synthesis and loss in starved juvenile Atlantic cod, *Gadus morhua*, white muscle contributed 66% of the protein loss of the whole body (Houlihan et al., 1988). The effect of starvation on muscle tissue has been histologically identified as fiber separation, and this has been used as a way to assess condition (Catalán and Olivar, 2002).

Flow cytometry has also been used to assess condition of fish larvae (Theilacker and Shen, 1993; Bromhead et al., 2000). Flow cytometry is a technique by which individual cells are stained with target-specific dyes, passed through laser beams, and their fluorescence and light-scattering properties are measured. DAPI (diamidino-2-phenylindole) is a stain that binds to DNA and is commonly used for flow cytometric cell cycle analysis. Cells that are in the process of dividing can have up to twice the amount of DNA as those not dividing, therefore more stain binds to them and their fluorescence is higher. Flow cytometry data are processed with software that calculates the fraction of cells in G1, S, and G2 phases of the cell cycle. The cell cycle is divided into two basic parts, interphase and mitosis. Interphase consists of three phases: gap 1 (G1), DNA synthesis (S), and gap 2 (G2). Cell growth occurs during the G1 phase before cell division begins. For cells to divide they must first replicate their DNA (S phase), and then grow and produce the structures (G2 phase) necessary for mitosis. There are checkpoints located between G1and S, and G2 and mitosis that are sensitive to starvation and will arrest the cell cycle if nutrient supply is low (Murray and Hunt, 1993). Using flow cytometric cell cycle analysis it has been possible to calculate the fraction of brain or muscle cells in each phase of the cell cycle for individual fish larvae to assess condition and growth (Theilacker and Shen, 1993, 2001; Bromhead et al., 2000; Catalán et al., 2007; González-Quirós et al., 2007), and this technique is based on the premise that cell proliferation is related to growth and condition. The fraction of cells in the S and G2 phases are indicative of cells that may divide and therefore can be associated with growth and condition. For walleye pollock, sea bass (*Dicentrarchus labrax*), and Atlantic cod larvae, cell cycle analysis has been used to distinguish between feeding treatments (Theilacker and Shen, 2001; Catalán et al., 2007; González-Quirós et al., 2007).

Previous flow cytometry studies on larval fishes have not developed statistical models incorporating environmental covariates such as temperature to classify their condition, nor has the method been widely applied to field-collected fish larvae. In this study we developed an assay for classifying the condition of walleye pollock larvae using flow cytometric cell cycle analysis of muscle cell nuclei. The classification model included temperature and fish length as covariates and was calibrated using walleye pollock larvae of known condition reared over a range of temperatures that they are likely to experience in the EBS. The effect of discontinuous feeding on larval walleye pollock condition was examined at one temperature to investigate how responsive the assay is to changes in the feeding environment, and the condition of field-collected larvae from the southeastern Bering Sea in 2007 is reported.

2. Methods

2.1. Laboratory rearing

Adult walleye pollock were collected by trawl in Shelikof Strait, Gulf of Alaska, during the spawning season in March 2006, 2007, 2008, and 2009. Eggs from single fish pairings (one female and one male) were fertilized and were maintained aboard ship in the dark at $3° \pm 0.3$ °C before being transported to the Alaska Fisheries Science Center, Seattle, WA. All experiments used one-micrometer filtered seawater (33 PSU) and a 16-hour daylight cycle with 2.5 μmol photon m^{-2} s^{-1} light level from overhead, full spectrum fluorescent lights. An ocular micrometer was used to measure the standard length (SL) of each larva to the nearest 0.08 mm before placing it into a microcentrifuge tube and storing it at −80 °C.

2.2. Flow cytometry

The protocol of Theilacker and Shen (2001) was modified to prepare larval walleye pollock muscle tissue for flow cytometric analysis. To briefly summarize, a frozen larva was thawed on ice,

Table 1
Summary of analyses used to examine cell cycle and condition of laboratory-reared walleye pollock, *Theragra chalcogramma*, larvae.

Analysis	Year	Feeding treatments	Rearing temperature	N[e]
Larval condition model	2006–2009	Always-fed and unfed	Low, intermediate-low, intermediate-high, and high[d]	342
Effect of discontinuous feeding	2007	Always-fed, F3S[a], S3F[b], and unfed	Intermediate-high	125
Temperature effect on the cell cycle	2006–2009	Always-fed and unfed	Low, intermediate-low, intermediate-high, and high	316
Comparing independent groups of larvae	2006–2008	Prefeeding[c], always-fed, and unfed	Intermediate-low, intermediate-high, and high	269

[a] Fed for 3 days and then starved.
[b] Starved for 3 days and then fed.
[c] Larvae sampled prior to first feeding.
[d] Low = 1.4 °C ± 0.02°; Intermediate-low = 3.2 °C ± 0.03°; Intermediate-high = 5.9 °C ± 0.02°; High = 9.0 °C ± 0.05°.
[e] Number of larvae analyzed.

placed into approximately 100 μL of DAPI stain on a glass depression slide, the head and gut were dissected away from the trunk musculature leaving mainly muscle tissue, and then the muscle tissue was sliced into four to five pieces using two scalpels. The solution containing the tissue pieces was transferred into a microcentrifuge tube containing 230 μL of DAPI stain and triturated six times using 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 their fluorescence was measured using a BD Influx[1] flow cytometer (BD Biosciences, San Jose, California, USA), typically within 4 to 5 h of preparation. For each larva a minimum of 5000 nuclei were analyzed using MutliCycle software (Phoenix Flow Systems, San Diego, California, USA) to calculate the fraction of muscle cells in the G1, S, and G2 phases of the cell cycle. Samples that had <5000 nuclei and/or a G1 coefficient of variation (CV) greater than 9.00 were not used in any further analyses. Large G1 CVs can affect the accuracy of the S phase calculations (Shankey et al., 1993).

2.3. Larval condition model

Laboratory-reared walleye pollock larvae of known feeding condition were used to develop a model (LCM) for assessing condition in the field (Table 1). Two feeding treatments (always-fed and unfed) were conducted at four temperatures (low = 1.4° ± 0.02 °C, intermediate-low = 3.2° ± 0.03 °C, intermediate-high = 5.9° ± 0.02 °C, and high = 9.0° ± 0.05 °C; overall mean ± standard error). In 2006, experiments using both feeding treatments were conducted at the intermediate-low, intermediate-high, and high temperatures. Experiments conducted in 2007 and 2008 were used to increase the number of replicates for those treatments and temperatures, and for independent cross-validation testing of the LCM. Larvae were reared using both feeding treatments at the low temperature in 2009 to add colder temperature data to the model, and to also provide samples for cross-validation testing.

The diet of larvae in the always-fed treatment consisted of laboratory-cultured rotifers (*Brachionus plicatilis* fed an algal diet of *Isochrysis galbana*, *Pavlova lutheri*, and a commercial rotifer supplement), and natural zooplankton (screened through 202 μm mesh, which included primarily *Acartia* spp. nauplii and gastropod veligers) collected from a local lagoon and maintained in the rearing tanks at concentrations of 10/mL and 3/mL, respectively. For each temperature, larvae were sampled at first feeding (defined as the day when 50% of the larvae had prey in their gut) and then at three other intervals based on their SL, so that larvae would be of similar size among the four temperatures. To avoid sampling larvae that were not actively feeding and possibly unhealthy, only larvae that had prey in their gut were sampled. At the intermediate-low, intermediate-high, and high temperatures there were two replicate 120-L tanks containing approximately 500 larvae each, and 5 larvae were taken from each replicate tank at each sampling. For the low temperature treatment, 4 replicate 20-L tanks were stocked with approximately 500 larvae each, and 7 larvae from each tank were taken each sampling. Only zooplankton prey was used with this treatment because the rotifers became inactive at this temperature and settled out of the water quickly. For the unfed treatment at all temperatures there were two replicate 120-L tanks containing approximately 500 larvae each, and 5 to 14 larvae were taken from each replicate tank each sampling. The larvae were sampled at first feeding, then again when approximately 10% of their first feeding yolk volume remained, at approximately 1% of their yolk remaining, and finally at yolk absorption.

2.4. Effect of discontinuous feeding

Four feeding treatments (always-fed, fed-for-3-days-then-starved (F3S), starved-for-3-days-then-fed (S3F), and unfed) were used to examine the effect of discontinuous feeding on the S and G2 phases of the cell cycle at the intermediate-high temperature in 2007 (Table 1). Each treatment consisted of two replicate 120-L tanks containing approximately 500 larvae each, and 5 larvae were sampled from each tank at first feeding and then at 3, 6, 11, and 14 days after first feeding. For all fed treatments, only larvae that had been actively feeding (i.e., had prey in their gut) were sampled. The fraction of muscle cells in the S and G2 phases for larvae from both the always-fed and unfed treatments were also included in the data set used to formulate the LCM. To better classify large unhealthy larvae (i.e., larvae that fed for a period of time and then starved), individuals that had starved for 8 and 11 days from the F3S treatment (n = 14, SL = 6.49 ± 0.07) were included in the model data set. Also included were larvae that were recovering after a period of starvation, these had fed for 8 and 11 days from the S3F treatment (n = 12, mean SL = 6.21 ± 0.10).

2.5. Temperature effect on the cell cycle

Always-fed and unfed larvae first feeding and older from the low, intermediate-low, intermediate-high, and high temperatures reared in 2006 through 2009 were used to examine temperature effect on the S and G2 phases of the cell cycle (Table 1).

2.6. Comparing independent groups of larvae

The fraction of cells in the S and G2 phases are associated with growth and condition, these values were added together (called fraction of muscle cells dividing, FMCD) and used to examine variability among larvae of different parentage. Groups of larvae at different developmental stages produced by single female and single male crosses and reared under different temperatures and feeding treatments were compared (Table 1). In 2007, two independent groups of larvae were reared at 5.8 °C ± 0.1° in 4-L glass tanks. There were two replicate tanks of 50 larvae for each group, and 7 prefeeding larvae from each tank were sampled at 1 and 3 days after hatching to eliminate confounding effects due to feeding. Groups of larvae reared in 2006, 2007, and 2008 from the always-fed and unfed treatments

[1] Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

at the intermediate-low, intermediate-high, and high temperatures first feeding and older were also compared to examine group effect.

2.7. Condition of walleye pollock larvae in the Bering Sea

Walleye pollock larvae were sampled from the continental shelf (middle shelf and outer shelf), and the continental slope/ocean basin areas of the southeastern Bering Sea near the Pribilof Islands and Pribilof Canyon for condition analysis from 8 to 14 May 2007 during an ichthyoplankton survey (Fig. 1). Samples were collected using a 60 cm bongo frame fitted with 505-μm mesh nets towed obliquely to a depth of 300 m or 10 m off bottom, whichever was shallower. Walleye pollock larvae were immediately removed from the net codend, their standard length was measured to the nearest 0.1 mm using an ocular micrometer, and they were individually placed into microcentrifuge tubes and frozen at −80 °C. The standard lengths of the larvae were corrected for shrinkage during collection using equations presented in Theilacker and Porter (1995) because the duration of the bongo tows varied with bottom depth. Water temperature was measured during each tow using a Sea-Bird SBE 19 SeaCat attached to the towing wire. Mean temperature between the surface and 50 m depth was used in the LCM because most walleye pollock larvae in the EBS are located in this depth interval (Napp et al., 2000). The objective of the survey was to describe ichthyoplankton abundance and distribution so there were only three microzooplankton samples collected to estimate prey abundance. One sample was collected on the continental shelf, and two were from the continental slope/ocean basin area (Fig. 1). Samples were collected using Niskin bottles during CTD casts. Prey abundance (number per liter) included all copepod nauplii and eggs between 0 and 50 m depth. The mean SL, mean temperature, mean fraction of cells in the S phase, and mean fraction of cells in the G2 phase was calculated for each station using larvae meeting the previously described criteria for an acceptable flow cytometry sample (i.e., nuclei count >5000, and G1 CV ≤9.00), and these values were then entered into the LCM.

2.8. Data analysis

Systat 11 (Systat Software, Inc., Richmond, California, USA) was used for all statistical tests, and the Tukey multiple comparison test used when ANOVA or ANCOVA were significant.

2.8.1. Replicate tanks

Replicate tanks from each year and experiment were compared using ANOVA to determine if they could be pooled. Treatment and replicate tank were used as factors, and FMCD was used as the dependent variable. Replicate tanks that were not significantly different were pooled in other analyses.

2.8.2. Larval condition model

Discriminant analysis with fraction of muscle cells in the S phase, fraction of muscle cells in the G2 phase, SL, and temperature as covariates was used to formulate the LCM to classify a larva as healthy (always-fed treatment, actively feeding larvae) or unhealthy (unfed treatment, starving larvae). The fraction of cells in the S and G2 phases were arcsin $\sqrt{fraction-of-cells}$ transformed to normalize their distribution. Larvae from the always-fed treatment that had a value of zero for the fraction of cells in the S phase were assumed to be in poor condition and were removed from the model data set (see Section 3.3, Effect of discontinuous feeding). For each larva the model calculates a probability of belonging to the healthy and unhealthy groups, and the highest probability is used as the classification for that larva.

2.8.3. Effect of discontinuous feeding

Changes over time (days after first feeding) to the fraction of muscle cells in the S and G2 phases of the cell cycle for the always-fed and unfed feeding treatments were examined with linear regression. Eleven and fourteen days after first feeding were excluded from the analysis of the unfed treatment due to low sample numbers (n = 1). Differences in the fraction of cells in the G2 phase among feeding treatments were compared using ANOVA with treatment as a factor. The Kruskal–Wallis test with treatment as a factor was used to compare the fraction of cells in the S phase among treatments because the data were not normally distributed. The Dunn multiple comparison test was used when the Kruskal–Wallis test was significant.

2.8.4. Temperature effects on the cell cycle

ANCOVA with temperature as a factor and SL as a covariate was used to examine the effect of temperature on the fraction of cells in the S phase. The always-fed and unfed treatments were pooled by temperature, and data were transformed using arcsin $\sqrt{fraction-of-cells}$ to normalize their distribution. ANOVA with temperature as a factor was used for temperature effects on the G2 phase. ANCOVA was not appropriate for analyzing this data set because, except for the highest temperature, the slope of the regression between the fraction of cells in the G2 phase and SL for each temperature was not significantly different from zero.

2.8.5. Comparing independent groups of larvae

ANOVA with group, days after hatching, and replicate as factors was used to test for differences in the FMCD between prefeeding groups. ANOVA using treatment and year as factors, and FMCD pooled across temperatures as the dependent variable was used to compare groups of always-fed and unfed larvae first feeding and older at the intermediate-low, intermediate-high, and high temperatures reared in 2006, 2007, and 2008.

3. Results

3.1. Replicate tanks

For all laboratory samples (n = 636), G1 CVs ranged between 3.07 and 8.98 (5.79 ± 0.05). There was no significant difference in the FMCD between replicate tanks for all treatments and temperatures in 2008 and 2009 (Table 2). There was a significant difference in the FMCD between replicate tanks for the 2007 prefeeding larvae groups, but no differences between replicate tanks for all other experiments conducted during that year (Table 2). For experiments where replicate tanks were not significantly different, tanks were pooled for all other analyses. There was a significant difference in the FMCD between replicate tanks for the 2006 experiments (Table 2). The mean FMCD for all the temperatures and treatments combined for the first replicate set analyzed (0.1437 ± 0.0048) was significantly greater than for the second replicate set (0.1202 ± 0.0049; ANOVA, $F_{1,175} = 11.67$, $p = 0.0008$). A factor that may have contributed to the difference between replicates is that the protocol used to process the samples for flow cytometry was being refined during the time that

Table 2
ANOVA results comparing replicate rearing tanks.

Year/experiment	F	p
2006/fed and unfed treatments at the intermediate-low, intermediate-high, and high temperatures[a]	$F_{6,165} = 3.13$	0.006
2007/prefeeding group at 5.8 °C	$F_{2,42} = 5.19$	0.01
2007/discontinuous feeding experiment at the intermediate-high temperature	$F_{4,130} = 0.681$	0.61
2007/unfed treatment at the intermediate-low temperature	$F_{1,22} = 2.07$	0.16
2007/unfed treatment at the high temperature	$F_{1,17} = 1.13$	0.30
2008/fed treatment at the intermediate-low, intermediate-high, and high temperatures	$F_{3,50} = 0.42$	0.74
2009/fed and unfed treatments the low temperature[b]	$F_{3,113} = 1.71$	0.17

[a] Intermediate-low = 3.2 °C ± 0.03°; intermediate-high = 5.9 °C ± 0.02°; high = 9.0 °C ± 0.05°.
[b] Low = 1.4 °C ± 0.02°.

the first replicate set was being analyzed. There was a large amount of cellular debris in the early samples analyzed, so changes were made to the protocol to reduce this. The protocol for processing flow cytometry samples had been finalized when the second replicate set was analyzed, therefore only the second replicate set of 2006 data was used in the model.

3.2. Larval condition model

A total of 342 larvae were utilized to formulate the LCM using discriminant analysis (151 always-fed, "healthy" larvae, that included 12 recovering from starvation, see Section 2.4, Effect of discontinuous feeding; 191 unfed, "unhealthy" larvae, that included 14 large, starved individuals, see Section 2.4, Effect of discontinuous feeding; Table 3A and B), and a quadratic model was chosen because of unequal variance among the covariates ($\chi^2 = 42.69$, df = 10, $p < 0.0001$). The model significantly discriminated between the always-fed and unfed groups (Wilks' $\lambda = 0.56$, approx. $F_{4,337} = 66.82$, $p < 0.001$), and overall 83% were correctly classified using the jackknifed classification procedure (Table 4A). This procedure indicated that the LCM performed somewhat better at classifying unhealthy larvae than healthy ones (Table 4A). The main problem with classifying "healthy" laboratory-reared larvae is the fact that not all individuals in the always-fed treatment tanks fed at a high enough rate to remain healthy so their condition may be somewhere between healthy and unhealthy. Laboratory-reared walleye pollock larvae of known condition but not used to formulate the model were classified as another way to test model accuracy (independent cross-validation). The test data set consisted of 89 larvae: 50 healthy larvae from the low, intermediate-low, intermediate-high, and high temperatures; and 39 unhealthy larvae from the low, intermediate-low, and intermediate-high temperatures. The overall percentage correctly classified was less than the jackknifed procedure (75%), and in this case the percentage of healthy and unhealthy larvae correctly classified was nearly equal (76% and 74%, respectively, Table 4B).

3.3. Effect of discontinuous feeding

The fraction of muscle cells of the unfed larvae in the G2 phase increased over time (linear regression, slope = 0.012, p = 0.0001; Fig. 2A), and the fraction muscle of cells in the S phase decreased from 0.0334 ± 0.0053 at first feeding to 0.0000 after 6 days of starvation (linear regression, slope = −0.006, $p < 0.0001$; Fig. 2B). Based on this observation, fed larvae from this and other experiments that had a value of 0.0000 for the fraction of cells in the S phase were assumed to be in poor condition and not used in any further analyses. The cell cycle of the always-fed larvae reacted in the opposite way as the unfed larvae. The fraction of muscle cells in the G2 phase decreased over time (linear regression, slope = −0.003, p = 0.002; Fig. 2A), and the fraction of muscle cells in the S phase increased (linear regression, slope = 0.008, p = 0.001; Fig. 2B).

Significant changes in the fraction of muscle cells in the S and G2 phases of the cell cycle could be detected in 3 to 11 days after feeding conditions changed. The S phase responded faster to starvation than the G2 phase. After 3 days of starvation the fraction of muscle cells in the S phase of unfed larvae was significantly less than that of the always-fed group (Kruskal–Wallis test, p = 0.0002; Dunn multiple comparison test, $p < 0.001$; Fig. 2B), and the G2 phase of starving larvae was significantly larger than the always-fed larvae after 8 days of starvation (larvae 11 days after first feeding from the F3S treatment; ANOVA, $F_{2,22} = 8.48$, p = 0.002; Tukey test, p = 0.003; Fig. 2A). The fraction of muscle cells in the S phase for the F3S treatment was intermediate between the always-fed and unfed treatments after 3 days of starvation (6 days after first feeding; Kruskal–Wallis test, p = 0.0009; Dunn multiple comparison test,

Table 4
Larval condition model classification accuracy for laboratory-reared walleye pollock, *Theragra chalcogramma*, larvae of known condition. Number of correct and incorrect classifications for each treatment, and the percentage correctly classified using the jackknifed procedure (A) and independent cross-validation procedure (B).

Treatment	Classification		Percent correct
	Healthy	Unhealthy	
A. Jackknifed procedure			
Always-fed	116	35	77
Unfed	24	167	87
		Overall correct	83
B. Independent cross-validation			
Always-fed	38	12	76
Unfed	10	29	74
		Overall correct	75

Table 3
Mean standard length (SL, mm) of walleye pollock, *Theragra chalcogramma*, larvae for the always-fed (A) and unfed (B) treatments for each temperature and year analyzed by flow cytometry and used in the larval condition model.

A. Always-fed treatment

Sample	2009 Low[a] SL ± SE[b] (n)	2006 Intermediate-low[c] SL ± SE (n)	2008 Intermediate-low SL ± SE (n)	2006 Intermediate-high[d] SL ± SE (n)	2007 Intermediate-high SL ± SE (n)	2006 High[e] SL ± SE (n)	2008 High SL ± SE (n)
FF[f]	5.83 ± 0.05 (13)	5.71 ± 0.04 (5)	5.79 ± 0.04 (5)	5.41 ± 0.03 (5)	5.52 (1)	5.31 ± 0.04 (5)	No sample
2	6.12 ± 0.02 (16)	5.92 ± 0.00 (3)	6.08 ± 0.08 (5)	6.03 ± 0.03 (3)	5.95 ± 0.05 (9)	5.81 ± 0.03 (5)	5.92 ± 0.05 (4)
3	6.30 ± 0.06 (14)	6.16 ± 0.10 (5)	No sample	6.11 ± 0.11 (3)	6.33 ± 0.08 (7)	6.10 ± 0.06 (5)	No sample
4	6.36 ± 0.04 (2)	6.64 ± 0.18 (5)	6.51 ± 0.11 (3)	6.80 ± 0.08 (4)	6.40 ± 0.12 (5)	6.59 ± 0.05 (3)	6.56 ± 0.10 (4)

B. Unfed treatment

Sample	2009 Low SL ± SE (n)	2006 Intermediate-low SL ± SE (n)	2007 Intermediate-low SL ± SE (n)	2006 Intermediate-high SL ± SE (n)	2007 Intermediate-high SL ± SE (n)	2006 High SL ± SE (n)	2007 High SL ± SE (n)
FF	5.77 ± 0.03 (20)	5.70 ± 0.02 (4)	5.82 ± 0.07 (7)	5.36 ± 0.03 (4)	5.55 ± 0.01 (9)	5.30 ± 0.03 (5)	5.08 ± 0.10 (6)
10% FF yolk volume	5.84 ± 0.05 (19)	5.70 ± 0.10 (5)	5.85 ± 0.06 (6)	5.68 ± 0.07 (5)	5.62 ± 0.07 (9)	5.62 ± 0.02 (4)	5.41 ± 0.08 (6)
1% FF yolk volume	5.85 ± 0.05 (17)	5.68 ± 0.05 (5)	5.92 ± 0.02 (7)	5.62 ± 0.08 (4)	5.63 ± 0.05 (5)	5.52 ± 0.08 (3)	5.28 ± 0.32 (2)
Yolk exhaustion	5.67 ± 0.05 (10)	No sample	5.54 ± 0.11 (4)	5.68 ± 0.00 (2)	5.52 (1)	5.52 ± 0.05 (3)	5.36 ± 0.11 (5)

[a] Low = 1.4 °C ± 0.02°.
[b] Standard error.
[c] Intermediate-low = 3.2 °C ± 0.03°.
[d] Intermediate-high = 5.9 °C ± 0.02°.
[e] High = 9.0 °C ± 0.05°.
[f] First feeding.

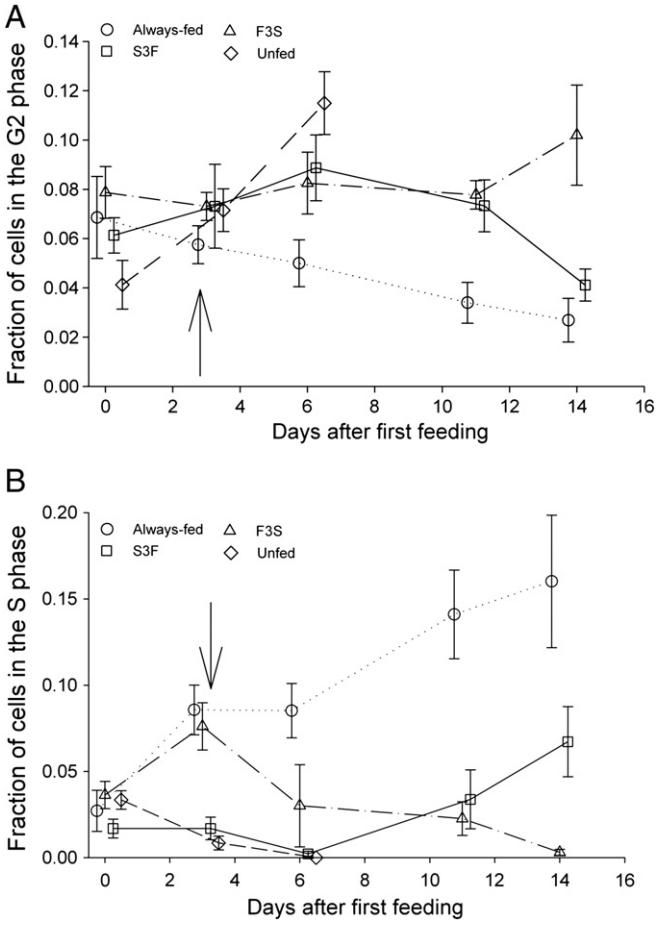

Fig. 2. The fraction of muscle cells (mean ± standard error) in the G2 (A) and S (B) phases of the cell cycle for walleye pollock, *Theragra chalcogramma*, larvae reared in the laboratory under four feeding treatments (always-fed, fed-3-days-then-starved (F3S), starved-3-days-then-fed (S3F), and unfed). Sample numbers for each treatment on each day ranged from 3 to 9, with the majority being 7 or greater. The unfed treatment at 11 and 14 days after first feeding was excluded due to low sample numbers (n = 1). Arrows indicate the day that the diet was changed.

$p > 0.05$; Fig. 2B). After 8 days of starvation both the S and G2 phases of the F3S treatment were significantly different than the corresponding phases in the always-fed treatment (11 days after first feeding; for the S phase, Kruskal–Wallis test, $p = 0.002$; Dunn multiple comparison test, $p = 0.005$; Fig. 2B; for the G2 phase, ANOVA, $F_{2,22} = 8.48$, $p = 0.002$, Tukey test, $p = 0.003$; Fig. 2A) showing that the larvae were beginning to starve. The S and G2 phases of larvae in the F3S treatment after 11 days of starvation (14 days after first feeding) were not significantly different from the unfed treatment on day 6, indicating that the larvae were starving (for the S phase, Kruskal–Wallis test, $p = 0.08$, Fig. 2B; for G2 phase, ANOVA, $F_{1,9} = 0.26$, $p = 0.62$, Fig. 2A).Thus for the F3S treatment, the effects of starvation began after 3 days of starvation and had full effect after 11 days. It took 11 days of feeding for larvae from the S3F treatment to recover from starvation. There was an increasing trend in the fraction of muscle cells in the S phase beginning after 3 days of feeding (6 days after first feeding), showing that larvae were recovering (Fig. 2B), but it took 11 days of feeding (14 days after first feeding) for the fraction of muscle cells in the S phase to significantly increase after the period of starvation (Kruskal–Wallis test, $p = 0.01$; Dunn multiple comparison test, $p = 0.004$; Fig. 2B). There was no significant difference in the fraction of muscle cells in the G2 phase between the always-fed and S3F treatments after 11 days of feeding (14 days after first feeding), indicating that larvae had recovered from initial starvation (ANOVA, $F_{2,17} = 9.71$, $p = 0.002$; Tukey test, $p = 0.69$; Fig. 2A).

The condition of larvae from the discontinuous feeding treatments not used to formulate the LCM was classified with the model. For F3S larvae, 83% on the last day of feeding were healthy (5/6), and after starving for 3 days only 25% were classified as healthy (1/4), showing that the model could detect unhealthy larvae within 3 days of the onset of starvation. Larvae from the S3F treatment at first feeding, 3, and 6 days after first feeding were classified as unhealthy, supporting the previously described results that it takes 11 days of feeding for recovery to occur.

3.4. Temperature effect on the cell cycle

The fraction of larval walleye pollock muscle cells in the S phase significantly increased with temperature (ANCOVA, $F_{3,311} = 8.68$, $p < 0.0001$; Table 5). The low, intermediate-low, and intermediate-high temperatures were not significantly different from each other (Tukey test, $p > 0.05$; Table 5), but all were significantly less than the high temperature (Tukey test, $p < 0.05$; Table 5). Temperature did not significantly affect the fraction of cells in the G2 phase over the temperature range examined (ANOVA, $F_{3,312} = 0.93$, $p = 0.43$; Table 5).

3.5. Comparing independent groups of larvae

The FMCD was not significantly different between independent groups of prefeeding walleye pollock larvae (ANOVA, $F_{1,42} = 0.03$, $p = 0.87$; 0.0771 ± 0.0067, and 0.0771 ± 0.0043 respectively). It also did not significantly vary between independent groups of larvae reared under similar feeding conditions, but groups of always-fed and unfed larvae were consistently different from each other (ANOVA, $F_{3,207} = 41.42$, $p < 0.001$; similar feeding conditions Tukey Test, $p > 0.30$; different feeding conditions Tukey Test, $p < 0.001$; Table 6). Thus, distinct cell cycle differences exist between larvae reared under different feeding conditions independent of their parentage.

3.6. Condition of walleye pollock larvae in the southeastern Bering Sea

Walleye pollock larvae were collected from 5 stations on the middle shelf, 12 stations on the outer shelf, and 11 stations from the continental slope/ocean basin area (Fig. 1). One to six larvae per station were analyzed. All shelf stations were included as a single group to increase spatial coverage of this area, and 6% of the larvae were classified as unhealthy (Table 7). All larvae from the continental slope/ocean basin area were healthy (Table 7). The larvae collected from the shelf were smaller than those collected from the continental slope/ocean basin area (Two-sample t-test, $p = 0.02$; Table 7). Mean water column and sea surface temperatures were colder on the shelf than in the continental slope/ocean basin area (Two-sample t-test, $p = 0.0006$, and $p = 0.0009$ respectively; Table 7). The lowest mean water column temperatures (−0.4°, 0.8°, 0.9 °C) occurred at the 3 northernmost stations on the middle shelf, possibly due to melting sea-ice. Sea-ice surrounded Saint Paul Island (northernmost Pribilof Island) on May 7, although the main ice edge was located farther

Table 5
The fraction of muscle cells in the S and G2 phases of the cell cycle (always-fed and unfed treatments pooled for each temperature; mean ± standard error) for walleye pollock, *Theragra chalcogramma*, larvae reared in the laboratory at different temperatures. For each phase the symbol indicates treatments that were not significantly different.

Temperature	n	S phase fraction[a]	G2 phase fraction
Low (1.4 °C ± 0.02°)	111	$0.0266 \pm 0.0001^{\blacklozenge}$	$0.0528 \pm 0.0034^{\blacktriangle}$
Intermediate-low (3.2 °C ± 0.03°)	69	$0.0330 \pm 0.0002^{\blacklozenge}$	$0.0497 \pm 0.0036^{\blacktriangle}$
Intermediate-high (5.9 °C ± 0.02°)	76	$0.0411 \pm 0.0002^{\blacklozenge}$	$0.0589 \pm 0.0036^{\blacktriangle}$
High (9.0 °C ± 0.05°)	60	0.0648 ± 0.0002	$0.0534 \pm 0.0049^{\blacktriangle}$

[a] Values are adjusted for size based on ANCOVA results.

Table 6
Comparing the fraction of muscle cells dividing (FMCD, fraction of cells in the S and G2 phases of the cell cycle combined and pooled across temperatures, mean ± standard error) among different feeding treatment groups of laboratory-reared walleye pollock, *Theragra chalcogramma*, larvae from independent single-pair fertilizations each year. Symbols indicate treatments that were not significantly different.

Year	Treatment	n	FMCD
2006	Unfed	44	0.0930 ± 0.0053◆
2006	Fed	51	0.1436 ± 0.0065▲
2007	Unfed	68	0.0806 ± 0.0035◆
2008	Fed	56	0.1431 ± 0.0063▲

north (Ice Analysis, National/Naval Ice Center, http://www.natice.noaa.gov). One week later, sea-ice around the island was gone and the main ice edge had retreated northward indicating that melting had occurred (Ice Analysis, National/Naval Ice Center, http://www.natice.noaa.gov). Prey densities varied from 7.5 prey per liter (outer shelf station) to 14.6 (mean for the two slope/ocean basin stations; Table 7).

4. Discussion

The assay described in this study represents a unique development in measuring larval fish condition that combines flow cytometric cell cycle analysis as an indicator of muscle cell proliferation, with morphometric and environmental covariates. The main premise of our assay is that muscle growth reflects larval feeding condition and that most muscle growth in larvae occurs by cell division. Muscle growth in fish can occur by hyperplasia (growth by the addition of new muscle cells), hypertrophy (enlargement of existing fibers), or by a combination of the two. For walleye pollock larvae the type of muscle growth has not been determined, but most fish larvae have hyperplasic component (Bone et al., 1995). For a closely related gadid species, Atlantic cod, the number of muscle fibers in fish less than 20 cm in length increased at a faster rate than fiber diameter, indicating hyperplasia was an important factor in muscle growth during this stage (Greer-Walker, 1970). Two advantages of the new assay are that sample preservation (freezing) and tissue preparation are relatively simple, and larval condition can be quickly assessed (about 10 min per larva to process the tissue and analyze it in a flow cytometer). An additional advantage over biochemical assays that use whole homogenized larvae, whose organs may have differential responses, is that this assay uses mainly muscle cells, which have been shown to be highly sensitive to nutritional stress (Houlihan et al., 1988). Furthermore, our assay can measure individual variation without pooling samples as some protocols require. The assay is sensitive, detecting changes in condition within 3 days of starvation, and its classification accuracy was high; 75% to 83% of laboratory-reared walleye pollock larvae were correctly classified depending on the testing procedure used. These values fall within the range of values of other discriminant analysis models used to assess the condition of early developmental stages of fish (70% to 98.6%) that require much more time consuming morphometric (Theilacker, 1978; Lochmann and Ludwig, 2003) or biochemical measurements (Lochmann et al., 1995; Cunha et al., 2003). Other studies using flow cytometry could detect starvation in fish larvae within 1 to 3 days, but did not consider temperature effects and did not apply the results of their laboratory work to assessing the condition of larvae in the field (Theilacker et al., 1996; Catalán et al., 2007). A disadvantage of using flow cytometry is that the instrument is relatively expensive to purchase and maintain, and a complete calibration of the assay for each fish species examined has to be accomplished.

The fraction of muscle cells in the S and G2 phases varied with feeding conditions, and the S phase responded faster to starvation than the G2 phase. Healthy larvae had a larger fraction of cells in the S phase than G2, and unhealthy larvae had a larger fraction of cells in the G2 phase than S. Fed Atlantic cod larvae were also found to have a greater percentage of cells in the S phase than starved ones (González-Quirós et al., 2007). The larger fraction of cells in the G2 phase of unhealthy larvae is probably due to a checkpoint between this phase and mitosis that slows or halts cell division if energy reserves are low (Murray and Hunt, 1993). The fraction of cells in the G2 phase increased, and the fraction of cells in the S phase correspondingly decreased to zero during 6 days of starvation, possibly indicating a negative feedback mechanism was present. We hypothesize that a purpose for the increased fraction of cells in the G2 phase during starvation could be for compensatory growth. Since a relatively large fraction of cells are arrested near mitosis, growth could quickly occur when conditions were favorable. Compensatory growth has been shown to occur in Atlantic cod larvae when feeding resumed after a period of starvation (Zhao et al., 2001). The fraction of cells in various stages of the cell cycle were unique to feeding conditions and significantly differed between feeding and starving walleye pollock larvae regardless of parentage, affirming that the LCM can be applied to field-collected larvae. Genetic variation and maternal effects on egg quality do not appear to have a significant effect on condition classification by the LCM.

For a variety of organisms cell cycle duration is influenced by temperature (yeast, *Saccharomyces cerevisiae*, Jagadish and Carter, 1978; fish, *Harpagifer bispinis*, Brodeur et al., 2003), but the conclusions conflict with regards to the effect of temperature on the proportion of cells in specific phases of the cell cycle (Moore et al., 1997; Takeo et al., 2003; González-Quirós et al., 2007). For larval walleye pollock muscle cells, the effect appears phase-dependent, as the fraction of cells in the S phase increased with temperature but the G2 phase was unaffected, and this result is similar to that for cultured cells. Reducing the incubation temperature of Chinese hamster ovary cells from 37° to 30° or 33 °C decreased the percentage of cells in the S phase from about 30% to approximately 3% and remained at this level for 8 days, while control cells kept at 37 °C ranged between 15 and

Table 7
Area, seawater temperature, number and size of walleye pollock, *Theragra chalcogramma*, larvae examined for condition, prey density, and percentage of larvae determined to be unhealthy in the southeast Bering Sea in May 2007.

Area[a]	No. stations[b]	Temp[c] (°C)	No. larvae[d]	Size Range[e] (mm)	Mean Size[f] (mm)	Prey[g]	Unhealthy[h]
Continental shelf	17	2.0 ± 0.2	47	5.9–9.7	7.2 ± 0.3	7.5	6
Continental slope/ocean basin	11	3.1 ± 0.1	28	6.8–9.4	8.2 ± 0.2	14.6 ± 1.1	0

[a] Shelf is 50 to 200 m depth, and continental slope/ocean basin is >200 m.
[b] Number of stations where walleye pollock larvae were collected.
[c] Mean seawater temperature between the surface and 50 m depth for all sampled stations within the area ± standard error.
[d] Total number of larvae analyzed for condition.
[e] Mean standard length range for all sampled stations within the area. All length measurements were adjusted for shrinkage during collection.
[f] Mean standard length ± standard error for all sampled stations within the area.
[g] Number per liter, includes all copepod nauplii and eggs. One station was sampled on the shelf, and two stations were sampled on the continental slope/ocean basin (mean density ± standard error).
[h] Percentage of sampled stations within the area classified as having unhealthy larvae present.

20% in the S phase during the same time period (Moore et al., 1997). The percentage of cells in the G2 phase remained unchanged when temperature was reduced from 37° to 30 °C (Moore et al., 1997). Likewise for Atlantic cod larvae, temperature did not affect the percentage of cells in the G2 phase (González-Quirós et al., 2007). The change in the proportion of cells in the S phase with temperature may be due in part to the effect of temperature on growth. Healthy larvae grow faster at warmer temperatures, so an increase in the proportion of cells replicating their DNA is not unexpected. The lack of a temperature effect on the fraction of cells in the G2 phase may be explained by greater variability in the G2 phase than the S phase. Factors that could contribute to increased G2 phase variability include the potential for cell aggregates to affect G2 phase calculations (Shankey et al., 1993), and the G2/mitosis checkpoint confounding temperature effects.

The relatively low percentage of unhealthy walleye pollock larvae reported here for the EBS may indicate that starvation of early stage larvae was not an important source of larval mortality, or alternatively, unhealthy larvae were removed from the system by another source (e.g., predation) prior to sampling. All unhealthy larvae were from the shelf area, where the incidence of starvation was 6%. Other studies have reported no unhealthy walleye pollock larvae in the EBS (Walline, 1985; Napp et al., 2000), but a relatively large proportion of unhealthy larvae have been reported in Alaskan waters elsewhere (up to 20% in Shelikof Strait, Gulf of Alaska; Theilacker et al., 1996). The smallest larvae were collected on the shelf, possibly reflecting slower growth or the presence of a more recently spawned cohort of larvae. Walleye pollock are known to spawn in the Bering Sea at different times throughout the year (Hinckley, 1987). Small walleye pollock larvae are more vulnerable to starvation than larger ones (Theilacker et al., 1996), and are most vulnerable to starvation during the first 2 weeks after first feeding (at approximately 6 °C; Theilacker and Porter, 1995).

Better conditions for larval walleye pollock survival may have existed in the continental slope/ocean basin area due to higher temperatures and abundances of prey. The mean shelf water column temperature (2.0 °C) was significantly colder than the temperature of the continental slope/ocean basin area (3.1 °C). The survival of early juvenile stage walleye pollock in the EBS was higher during warm years with an early ice retreat, indicating that warm conditions may improve their survival (Mueter et al., 2006). For walleye pollock larvae adapted to living in areas other than the Bering Sea, laboratory studies have shown that cold temperature (3 °C) can negatively affect their feeding (Gulf of Alaska; Paul, 1983), and that they avoid cold water and become inactive when entrained in it (Puget Sound, Washington, USA; Olla and Davis, 1990). Interannual prey densities in the southeastern Bering Sea are variable, but prey may be generally more abundant in the continental slope/ocean basin area than on the shelf (Clarke, 1984). Temperature can also affect prey availability, in cold conditions the abundance of small copepods (prey for walleye pollock larvae) on the shelf was lower than when conditions were warm (Coyle et al., 2008). In 2007, the mean May sea surface temperature in the Bering Sea was below average, making it a “cold” year (1.2 °C, data from the Bering Sea ecosystem and climate website, http://www.beringclimate.noaa.gov), which may have reduced prey abundance on the shelf contributing to poor feeding conditions for walleye pollock larvae. Our microzooplankton samples were collected opportunistically and only three samples were taken, but they did indicate that the abundance of prey in the continental slope/ocean basin area was higher than on the shelf.

Numerous methods have been developed to assess the condition of fish larvae, some of which are time consuming and require extensive training. Tissue preparation and analysis for our protocol was relatively simple and quick and could be conducted at sea. A higher incidence of starvation among walleye pollock larvae was found in our study compared to past studies of the Bering Sea (Walline, 1985; Napp et al., 2000), suggesting that the flow cytometeric assay described here may be more sensitive to detecting starvation. Since starvation may be a major cause of mortality of fish larvae in the sea, development of sensitive assays to determine larval condition improves our understanding of processes affecting their survival and recruitment.

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