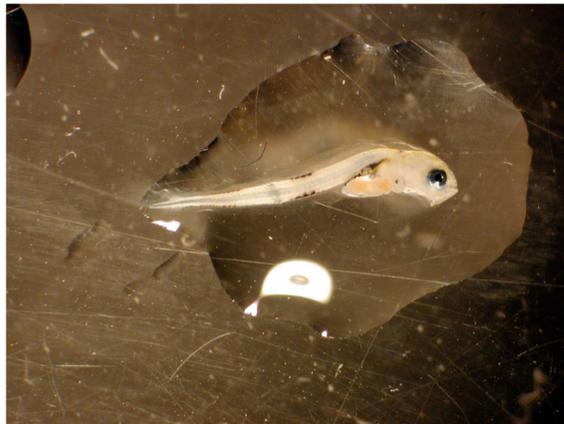


# Assessing the Condition of Walleye Pollock (*Theragra chalcogramma*) Larvae Using Muscle-Based Flow Cytometric Cell Cycle Analysis

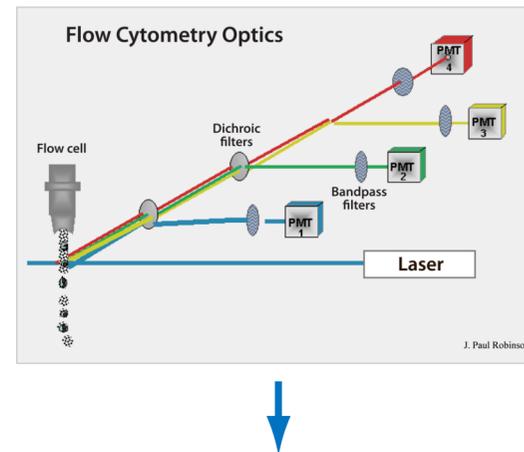
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**INTRODUCTION** We developed an assay that measures the condition of walleye pollock, *Theragra chalcogramma*, larvae using flow cytometric cell cycle analysis of muscle cell nuclei. The assay was calibrated using always-fed (healthy) and unfed (unhealthy) laboratory-reared larvae reared over a range of temperatures that they are likely to experience in the eastern Bering Sea. Advantages of this assay are that sample preservation (frozen storage at -80°C) and tissue preparation are relatively simple, and individual larvae can be quickly assessed (about 10 min per larva to process the tissue and analyze it). 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.

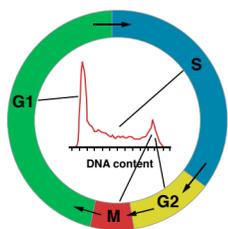


**1. SAMPLE PREPARATION** A frozen larva is thawed on ice, placed into a drop of DAPI stain (diamidino-2-phenylindole, a DNA stain that is commonly used for flow cytometric cell cycle analysis) on a glass depression slide, and the head and gut are dissected away from the trunk musculature. The muscle tissue is sliced into pieces with scalpels and the solution containing them is syringed a few times to release nuclei from the muscle cells. The solution is filtered through a 48- $\mu$ m filter to separate the stained nuclei from large cellular debris. Prepared samples are kept on ice until their fluorescence is measured using a BD Influx flow cytometer.

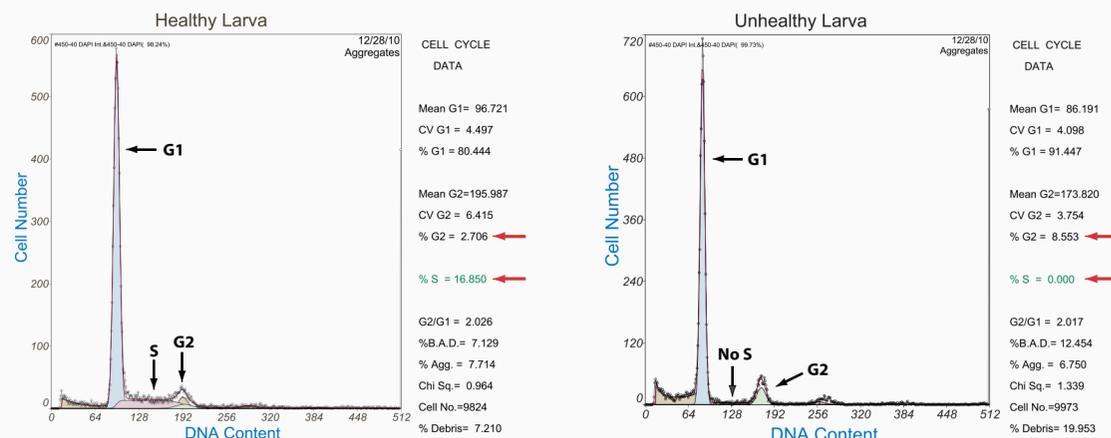


**2. FLOW CYTOMETRY** is a technique by which individual cells are stained with target-specific dyes, passed through laser beams, and their fluorescence and light-scattering properties are measured. Cells that are in the process of dividing can have up to twice the amount of DNA as those not dividing, therefore more DAPI stain binds to them and their fluorescence is higher.

**3. DATA PROCESSING** Flow cytometry cell counts are processed with software that calculates the fraction of cells in G1, S, and G2 phases of the cell cycle. The fraction of cells in the S and G2 phases are indicative of cells that may divide and therefore are associated with growth and condition. In our study, the S and G2 phases of the cell cycle gave an indication of 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. The larger fraction of cells in the G2 phase of unhealthy larvae is most likely due to a checkpoint between this phase and mitosis that slows or halts cell division if energy reserves are low.



**CELL CYCLE** The cell cycle is divided into two basic parts, interphase and mitosis (cell division). Interphase consists of three phases: gap 1 (G1), DNA synthesis (S), and gap 2 (G2). Cell growth occurs during the G1 phase. For cells to divide they must first replicate their DNA (S phase), and then grow and produce the structures necessary for mitosis (G2 phase). There are checkpoints located between G1 and S, and G2 and mitosis that are sensitive to starvation and will arrest the cell cycle if nutrient supply is low.



**HISTOGRAMS** of a healthy and unhealthy larva showing the G1, S, and G2 phases of the cell cycle after the flow cytometry data has been processed with MultiCycle software. The x-axis of each histogram is DNA fluorescence and the y-axis is the number of nuclei with a specific fluorescence value. Note that for the unhealthy larva there are no S phase nuclei present.

## MODEL DEVELOPMENT AND TESTING

**FORMULATION** A quadratic discriminant analysis model was formulated to classify a larva as healthy (feeding) or unhealthy (starving) using the fraction of cells in the S and G2 phases of the cell cycle, temperature, and standard length. For each larva the model calculates the probability of belonging to the healthy and unhealthy groups, and the highest probability is used as the classification for that larva.

**TESTING** Laboratory-reared larvae of known condition were used to check classification accuracy using both the jackknifed and independent cross-validation procedures. The assay is sensitive, detecting changes in condition within 3 days of starvation, and its classification accuracy is high; 75% to 83% of the larvae were correctly classified depending on the testing procedure used.

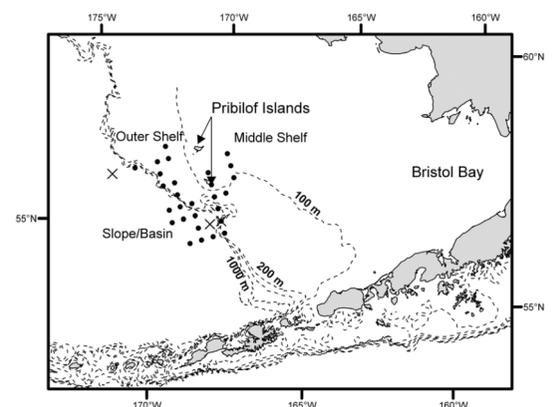
Jackknifed procedure			
Classification			
Treatment	Healthy	Unhealthy	Percent correct
Always-fed	116	35	77
Unfed	24	167	87
		overall correct	83

Independent cross-validation			
Classification			
Treatment	Healthy	Unhealthy	Percent correct
Always-fed	38	12	76
Unfed	10	29	74
		overall correct	75

## ASSESSING THE CONDITION OF FIELD-COLLECTED LARVAE

We assessed field-collected walleye pollock larvae from the southeastern Bering Sea and found unhealthy larvae located on the continental shelf (6%). This may be due in part to the coldest temperatures occurring there and low prey availability. In the continental slope/ocean basin waters, where prey levels were higher and temperatures warmest, no larvae in unhealthy condition were found.

Condition of walleye pollock larvae in the southeastern Bering Sea in May 2007						
Area	Number of stations	Water temp (°C)	Number analyzed	Size range (mm)	Prey concentration	% Unhealthy
Continental shelf	17	2.0 ± 0.2	47	5.9 - 9.7	7.5	6
Continental slope/ocean basin	11	3.1 ± 0.1	28	6.8 - 9.4	14.6 ± 1.1	0



Southeastern Bering Sea walleye pollock collection sites for larval condition analysis in May 2007 (circles). "X"s indicate where microzooplankton were sampled.

## CONCLUSION

Tissue preparation and analysis for our assay is relatively simple and quick. The assay was calibrated to the range of temperatures larvae are likely to encounter in the eastern Bering Sea (1.4° to 9° C), and it was sensitive to changes in condition within 3 days of starvation. The assessment of the condition of walleye pollock larvae collected from the southeastern Bering Sea in 2007 indicated that a small percentage of larvae located on the continental shelf were unhealthy.