

Cell cycle analysis of brain cells as a growth index in larval cod at different feeding conditions and temperatures

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SUMMARY: The percentage of cells dividing in a specific tissue of individual larvae can be estimated by analyzing DNA per cell by flow cytometry. An experimental test was carried out with cod (*Gadus morhua*) larvae, with brain as the target tissue, to validate this technique as an appropriate growth index for larval fish. Standard length (SL), myotome height, and %S-phase (% of cells in the S-phase of the cell-division cycle) variability were analyzed, with temperature (6 and 10°C), food level (high- and no-food) and larval developmental stage (first feeding, pre-metamorphosis and post-metamorphosis) as independent factors. Cod larvae grew faster (in SL) and presented a higher %S-phase under high-food conditions. Larval SL increased with temperature in rearing and experimental tanks. However, there was a significant interaction between temperature and food in the %S-phase. There were no significant differences in the %S-phase between 6 and 10°C at high-food levels. We suggest that this result is a consequence of temperature-dependency of the duration of the cell cycle. In the absence of food, larvae at 10°C had a lower %S-phase than larvae at 6°C, which may be related to increased metabolic costs with increasing temperature. Considering the effect of temperature, the mean % S-phase explained 74 % of the variability in the estimated standard growth rate.

Keywords: brain, cell cycle, cod larvae, flow cytometry, food, growth index, temperature.

RESUMEN: ANÁLISIS DEL CICLO CELULAR EN CÉLULAS DEL CEREBRO COMO ÍNDICE DE CRECIMIENTO EN LARVAS DE BACALAO A DIFERENTES CONDICIONES DE ALIMENTO Y TEMPERATURA. – El porcentaje de células en división en un determinado tejido de una larva de pez se puede estimar analizando la cantidad de ADN por célula mediante citometría de flujo. Se realizó un experimento con larvas de bacalao (*Gadus morhua*), analizando células de cerebro, para validar esta técnica como índice de crecimiento en larvas de peces. Se analizó la variabilidad de la longitud estándar (SL), la altura del tronco medida en el ano, y el %S (% de células en fase S del ciclo celular), con temperatura (6 y 10°C), nivel de alimento (alto y sin alimento) y estado de desarrollo larvario (comienzo de la alimentación, pre-metamorfosis y post-metamorfosis) como factores independientes. Las larvas de bacalao crecieron más rápido (en SL) y presentaron mayor %S bajo condiciones de nivel alto de alimento. La SL larvaria incrementó con la temperatura. Sin embargo, se observó una interacción significativa entre temperatura y alimento sobre %S. No hubo diferencias significativas en %S entre 6 y 10°C en condiciones de nivel alto de alimento. Sugerimos que este resultado es consecuencia de una termo-dependencia en la duración del ciclo celular. En ausencia de alimento, las larvas a 10°C presentaron %S más bajos que las larvas a 6°C, lo que puede estar relacionado con un incremento de los costes metabólicos a mayor temperatura. Considerando el efecto de la temperatura, el %S medio explicó el 74% de la variabilidad de la tasa de crecimiento específica estimada.

Palabras clave: alimento, cerebro, ciclo celular, citometría de flujo, índice de crecimiento, larva de bacalao y temperatura.

INTRODUCTION

The processes affecting survival of larval and early juvenile stages are the main factors that determine recruitment of pelagic fish. Growth has been proposed to be an important factor for regulating survival due to its influence on the duration of these stages and its interaction with characteristic high-mortality rates (Houde, 1987; Pepin, 1991).

A variety of growth and condition indices have been developed in order to understand how environmental conditions affect individual growth rates (see review from Ferron and Leggett, 1994). These include morphometric and histological indices, otolith microstructure analysis and others based on a variety of organic compounds that correlate to growth, such as nucleic acids and certain enzymes. Physiological larval status resulting from recent environmental conditions has been estimated by biochemical analysis of RNA and DNA content of larval homogenates (Clemmesen and Doan, 1996; Folkvord *et al.*, 1997; García *et al.*, 2007). The RNA/DNA index relies on the assumption that the amount of DNA per cell is relatively constant while the amount of RNA varies with physiological status. However, the RNA/DNA index may reflect potential protein synthesis more than growth itself (Ferron and Leggett, 1994).

Theilacker and Shen (1993a, b, 2001), taking into account that larval growth is mainly attained by cell division, proposed analyzing the percentage of cells that are dividing in a specific tissue as a more direct approach to estimating recent growth rate. The division cycle of most cells consists in four coordinated processes: cell growth, DNA replication, distribution of the duplicated chromosomes to daughter cells, and cell division (cytokinesis) (Cooper, 2000). Mitosis (nuclear division) and the timing of DNA synthesis divide the cell cycle in eukaryotes into four discrete phases. The M-phase of the cycle corresponds to mitosis, which is usually followed by cytokinesis. This phase is followed by the G_1 phase (gap 1), which corresponds to the interval (gap) between mitosis and the initiation of DNA replication. During G_1 , the cell is metabolically active and grows continuously, but does not replicate its DNA. G_1 is followed by the S-phase (synthesis), during which DNA replication takes place. The completion of DNA synthesis is followed by the G_2 phase (gap 2), during which cell growth continues and proteins are synthesized in preparation for mitosis. Cells in G_1 may enter a qui-

escent stage of the cell cycle called G_0 , in which they remain metabolically active but no longer proliferate unless induced to return to G_1 by appropriate external signals (Cooper, 2000). In the technique used by Theilacker and Shen (1993a, b; 2001), DNA is stained in single cells or nuclei with a fluorescent dye. The intensity of fluorescence per cell is measured by flow cytometry (FC) and, since it corresponds to its amount of DNA, the proportion of cells at each intensity represents the proportion of cells at different cell cycle phases (Fig. 1): (1) cells in the G_0 and G_1 phases (G_0 - G_1) are represented by a peak at low fluorescence; (2) cells in the G_2 and M phases (G_2 -M) correspond with a population that peaks at double fluorescence intensity compared to G_0 - G_1 cells; (3) cells that are going through DNA replication (S-phase) fluoresce at intensities ranging between G_0 - G_1 and G_2 -M peaks. However, this apparently straightforward estimation depends on some critical aspects. FC requires a suspension of individual cells. Cell dissociation from a tissue may produce a certain amount of debris, whereas some cells may still remain aggregated. Fluorescence of aggregates and debris interfere with the target cell populations (see Fig. 1) and therefore with adequate estimation of the percentage of cells at each cell cycle phase (Shankey *et al.*, 1993).

Models suggest that small changes in larval growth rates cause large differences in recruitment size (Houde, 1987; Pepin and Myers, 1991). Temperature and the production and abundance of larval prey have been proposed as the major factors

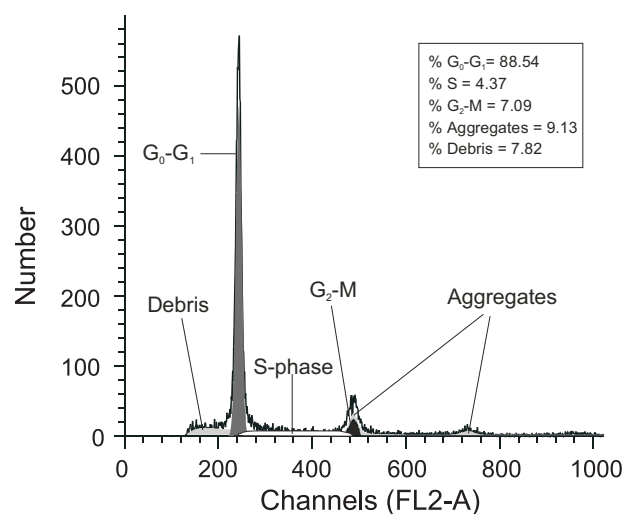


FIG. 1. – A typical display of the computer program used for cell cycle analysis. It shows a histogram of the number of particles at each fluorescence intensity, and modelled fits for each cell cycle phase (G_0 - G_1 , S and G_2 -M), debris and aggregates.

that regulate larval growth (e.g. Houde, 1989; Leggett and Deblois, 1994). In addition, larval growth variability is also related to the ontogenetic state (Otterlei *et al.*, 1999). Theilacker and Shen (1993a, b, 2001) showed significant differences in the percentage of S + G₂-M in brain cells and muscle nuclei between fed and unfed *Theragra chalcogramma* larvae, although their analysis was restricted to first-feeding larvae under a homogeneous temperature. Bromhead *et al.* (2000) also observed differences in the (G₂-M)/(G₀-G₁) ratio in brain cells between fed and unfed *Galaxias olidus* larvae at two different temperatures. However, the interaction between both factors was not tested. In addition, they did not observe significant differences in (G₂-M)/(G₀-G₁) between larvae growing at 12 and 16°C. Lower values from larvae at 20°C were associated with high mortality and attributed to disease and (or) temperature shock. They did observe a significant relationship with larval size, which they attributed to growth variability related to developmental stage. Catalan *et al.* (2007) observed significant differences in S + G₂-M of muscle nuclei between fed and fasted, post-flexion *Dicentrarchus labrax* larvae after 2 days.

Our objective was to test the adequacy of cell cycle analysis to study the effect of factors that may have a larger influence on growth variability of fish larvae, including their possible interactions. We used a factorial experimental design which considered temperature, food availability and larval developmental stage. We selected larval *Gadus morhua* for our experimental test because of the large amount of background information on metabolic rates and condition indices for this species, in field and experi-

mental studies (e.g. Otterlei *et al.*, 1999; Finn *et al.*, 2002). We used larval brain as the target tissue for estimating the percentage of cell division, even though neural tissue growth presents a slower temporal response under unfavourable growth conditions (Theilacker and Shen, 2001). Brain cells were still preferred, as preliminary FC analysis showed that we were able to obtain more appropriate cell suspensions from larval brain than from larval muscle, based on quality parameters obtained from the FC analysis (see Table 1 for details about quality parameters).

MATERIAL AND METHODS

Fish and rearing conditions

Cod eggs were obtained on 20 March 2001 from a naturally spawning broodstock of about 100 repeat spawning Norwegian coastal cod. Temperature during incubation was 6.7±0.1°C (SD), and 50% hatching (day 0) occurred on April 4. Larvae were transferred on day 2 post hatching to four 1-m rectangular tanks with a rearing volume of 500 L. Approximately 3000 larvae were transferred to four tanks, two of which were kept in a temperature controlled room at 6°C and the other two were kept at 10°C. The seawater was kept stagnant initially and later up to 10 % was exchanged per day in connection with addition of food. The larvae were fed cultured algae, *Isochrysis* and *Rhodomonas* (2 L tank⁻¹ day⁻¹), and natural zooplankton. The larvae were fed in excess, and prey levels were adjusted daily to attain a nominal density of 2000 prey L⁻¹. The main prey items were initially rotifers and copepod nauplii, and subsequently copepodites and adult copepods. Aeration was used in the tanks to maintain oxygen levels sufficiently high and disperse the food for the cod larvae (Otterlei *et al.*, 1999).

Experimental design

We used a 2 × 2 × 3 × 2 factorial design to examine the effects of temperature (T; 6 and 10°C), food (High-Food and 0-Food), developmental stage (DS; first feeding, pre metamorphosis and post metamorphosis) and tank (n = 2), which was nested in T and food.

Larvae were transferred from the rearing tanks to eight experimental tanks, four in each controlled temperature room, at three distinctive developmen-

TABLE 1. – Unacceptable and medium quality values of parameters, used to establish criteria for discarding cell cycle data. % BAD, % of background aggregates and debris is defined as the ratio of model estimated aggregates and debris to total cellular (or nuclei) events in the histogram, over the region from the lowest G₀-G₁ mean to the highest G₂-M mean. CCE, cell cycle events. NECh, number of events per channel between lowest G₀-G₁ and highest G₂-M of the histogram. CV, the variation coefficient of the normal diploid (G₀-G₁) cell population. RCS, reduced Chi-square value. RCS is a measure of how well the model describes the observed data.

Quality parameter	Unacceptable quality	Medium quality
% of aggregates	> 20	>10
% of debris	> 15	>10
% of BAD	> 25	> 20
CCE	< 6000	< 10000
NECh	< 90	< 100
CV of G ₀ -G ₁	> 7	> 5
RCS	> 5	> 3

tal stages: established first feeding larvae (FF; about 5 mm standard length (SL) and with no visible yolk remains), a high-growth phase at a late larval stage (Pre-M; 9-10 mm SL; (Otterlei *et al.*, 1999)), and the stage after initiation of metamorphosis (Post-M; about 15 mm SL). See Figure 2 for the timing of transfer to the experimental tanks at each temperature in relation to larval size. These tanks were 60-cm rectangular tanks with approximately 150 L rearing volume. At each temperature, two of the tanks received algae and zooplankton at the same densities as the rearing tanks (High-Food), while the other two tanks did not receive any plankton (0-Food). Larvae were initially transferred equally from all four rearing tanks to the corresponding experimental tanks at the same temperature, but due to lower availability of larvae in one of the rearing replicates at 10°C, larvae were sampled from one rearing tank during the latter two developmental stages (transfers). A total of 60 larvae were transferred to each of the experimental tanks during the FF developmental stage, 45-50 in Pre-M, and 45 in Post-M. Ten larvae were sampled from each of the experimental tanks on day 1 and day 5 after transfer between 13:00 and 15:00 hours (local time). The remaining larvae in the experimental tanks were sampled and counted to estimate survival during the experimental period. We considered that 5 days of starvation vs. high feeding conditions would cause a clear effect on larval growth without causing a large larval mortality in starvation treatments that may put into question any significant differences between treatments.

The same day of the transfer to the experimental tanks and the day corresponding to day 5 in the experimental tanks, 10-20 larvae were sampled from the rearing tanks to estimate larval size at the beginning and end of the feeding experiments. Additional samples were taken from the rearing tanks on approximately a weekly basis between the transfers.

Sample processing

All sampled larvae were length measured (SL) live under a calibrated dissecting microscope, and a digital photo was taken of each larva for later morphometric analysis. After photography, the larvae were rinsed in distilled water, put in an eppendorf tube with a cryoprotectant solution (Table 2), and frozen in liquid nitrogen. SL and myotome height measured at the anus (MYO, Ellertsen *et al.*, 1980) were subsequently measured from the photo images

by Image Pro analysis software. Larvae from High-Food treatments that did not have any apparent gut contents were excluded from further analysis ($n = 4$).

Hereafter, only up to 5 larvae sampled on day 5 from each experimental tank were further processed and used for FC analysis (35 in FF, 39 in Pre-M and 40 in Post-M). Larvae were thawed, immediately rinsed in citrate buffer (Table 2) and placed on a microscope slide in a drop of citrate buffer. Under the stereomicroscope, the brain was dissected out and transferred with a micropipette into an eppendorf tube containing 100 μ L of citrate buffer. The tissue was broken up into a suspension of individual cells by pipetting the solution in and out very gently 10 to 15 times with a 50 to 200 μ L micropipette set at approximately 100 μ L, trying to avoid creating bubbles. This procedure is one of the most critical aspects of the method as excessively strong pipetting causes a high percentage of debris and aggregates that reduces the quality of the results (see below). Immediately after, 100 μ L of 0.08 N HCL were added to the solution and the sample was kept on ice until DNA staining procedures. Up to 20 larvae were processed at a time within 1.5 h.

Subsequently, samples were centrifuged at 4 G during 5 minutes and the supernatant taken out. Two hundred μ L of solution A (Table 2) was added to each sample and the eppendorf tube was gently shaken. The sample was left for 10 minutes at room temperature. Then, 200 μ L of solution B (Table 2) was added and left at room temperature for another 10 minutes. Finally, 250 μ L of solution C (Table 2) was added and samples were immediately placed on ice in the dark until FC analysis (not before 10 minutes).

Flowcytometry analysis

Samples were analyzed with a FACScalibur (Beckton & Dickinson) FC with an argon-ion laser that produced 15 mW at 488 nm. The percentage of cells in each cell division phase (G_0 - G_1 , S and G_2 -M) was estimated from data obtained from the FL2-A channel by modelling carried out with ModFit version 3.0 (Verity Software) (Fig. 1). The model was set with autoaggregates, autodebris, diploid cell cycle without aneuploid population and the S-phase population as a rectangle.

The accuracy of the percentage of cells in each phase estimate is dependant on the number of cells analyzed, the proportion of debris and aggregates, the degree of overlap between G_0 - G_1 and S popula-

TABLE 2. – Solutions used in preserving fish larvae and DNA staining.

Solution	Components	Quantity
<i>Cryoprotectant</i> (500 ml)	Bovine serum Dimethylsulfoxide Eagles MEM (Biochrom)	100 ml 100 ml 400 ml
<i>Citrate buffer</i> (1000 ml) (pH adjusted to 7.6)	Sucrose Trisodium citrate.2H ₂ O Dimethylsulfoxide Distilled water	85.50 g (250 mM) 11.76 g (40 mM) 50 ml added to a volume of 1000 ml
<i>Stock solution for</i> <i>solutions A, B and C</i> (1000 ml)	Trisodium citrate.2H ₂ O Nonidet-P40 Spermine tetrahydrochloride Distilled water	1000 mg (3.4 mM) 1000 µl (0.1% v/v) 522 mg (1.5 mM) added to a volume of 1000 ml
<i>Solution A</i> (pH is adjusted to 7.6)	Stock solution Trypsin	1000 ml 30 mg
<i>Solution B</i> (pH is adjusted to 7.6)	Stock solution Trypsin inhibitor Ribonuclease A	1000 ml 500 mg 100 mg
<i>Solution C</i> (pH is adjusted to 7.6)	Stock solution Propidium Iodide Spermine tetrahydrochloride	1000 ml 416 mg 1160 mg

tions and to what extent the model adjusts to the observed distribution of events at each fluorescence value (Bauer *et al.* 1993; Shankey *et al.* 1993; Verity Software, Inc. unpublished data). In order to ensure standardization and appropriate quality across experimental treatments, sample processing was stopped when the cell counter reached 15000 cells and the criteria for discarding samples from further data analysis (Table 1) were established from a series of parameters obtained from the model, namely: (1) Number of cell cycle events (CCE). Theoretical considerations and empirical observations suggest that a minimum of 10000 (not including debris and aggregates) is necessary for reproducible determination of the S-phase, although this value is controversial (Bauer *et al.* 1993; Shankey *et al.* 1993). (2) Number of events per channel (NECh), defined as the number of events per channel between the lowest G_0 - G_1 and highest G_2 -M of the histogram. Simulation studies indicate that for accurate S-phase estimates there should be an average of approximately 100 NECh (Verity Software, Inc. unpublished data). (3) Percentage of aggregates. (4) Percentage of debris. (5) Background aggregates and debris (% BAD), defined as the ratio of the model estimated aggregates and debris to total cellular (or nuclei) events in the histogram over the region from the lowest G_0 - G_1 mean to the highest G_2 -M mean. Low levels of cell aggregates and debris, and their adequate assessment, are critical for

estimating the percentage of cells at each cell cycle phase (Shankey *et al.*, 1993). (6) Variation coefficient of the normal diploid (G_0 - G_1) cell population (CV). CV affects the accuracy of S-phase calculations. It should be <8% (Bauer *et al.* 1993; Shankey *et al.* 1993). (7) Reduced Chi-Square value (RCS) is a measure of how well the model describes the observed data. Values less than 3.0 represent good fits. Values of 3.0 to 5.0 are considered to be fair, and values greater than 5.0 are poor, and may indicate that the model is not a good match for the histogram (Verity Software, Inc. unpublished data). Samples were rejected if they were over or below a critical value considered unacceptable according to these parameters, or when more than two exceeded medium quality values (Table 1).

Statistical analysis

Two ANOVAs were carried out on Ln SL and % S-phase as dependent factors, with T (6 and 10°C), DS (FF, Pre-M and Post-M), Food (0-Food and High-Food) and tank (two tanks nested in each combination of T and food) as independent factors. Exposure to suboptimal growth conditions such as the absence of food may cause a reduction in larval weight that may not be reflected in SL. SL*MYO is a better proxy for larval weight than SL (van der Meeren, 1991). Therefore, differences in the morphometric relationship between SL and MYO may

be used as a better index of the effect that experimental conditions may exert on larval weight. An ANCOVA analysis was carried out to determine this morphometric variability in the different experimental treatments. Variables were logarithmically transformed to fit variance homogeneity conditions and confirmed to be non-significant using a Cochran C-test. LnMYO was the dependent variable and LnSL the covariate. The independent factors were the same as in the other ANOVAs. In each analysis, data were balanced for the different treatments using the mean of the corresponding dependent variable and the mean of the covariate. F-values and their corresponding *p*-values that were obtained from the ANOVAs and ANCOVA of balanced data sets were recalculated following Underwood (1997). Taking into account that Tank was nested in Food and Temperature, a SNK *post-hoc* test was carried out following Underwood (1997). Average daily mortality after transfer was estimated by assuming a constant mortality rate and correcting for sampled and remaining larvae in the respective tanks.

Estimate of specific growth rate

In order to analyze the relationship between %S-phase and larval growth we estimated the mean larval specific growth rate (SGR) of the larvae from day 1 to day 5 in each experimental treatment (T × Food × DS × Tank). Larval dry weight (DW; mg) in each larva was estimated based on data from SL (mm) and MYO (mm) (Finn *et al.*, 2002), yielding the equation:

$$\ln DW = -3.21 + 1.37 \ln (SL \cdot MYO),$$

$$n = 245, R^2 = 0.997, SE \text{ est.} = 0.01.$$

The specific growth rate (SGR) between day 1 and day 5 was:

$SGR = (\ln DW_5 - \ln DW_1)/4$, where DW_5 and DW_1 are average estimated dry weights five and one day after transfer respectively.

An ANCOVA analysis was carried out to test for significant effects of temperature on the relationship between SGR and %S-phase. We considered SGR as the dependent variable, temperature (6 and 10°C) as an independent factor and %S-phase as the covariate. We tested for significant differences in slopes and intercepts between the linear regression fits for 6 and 10°C. We fitted a linear regression model of SGR against %S according to the ANCOVA results.

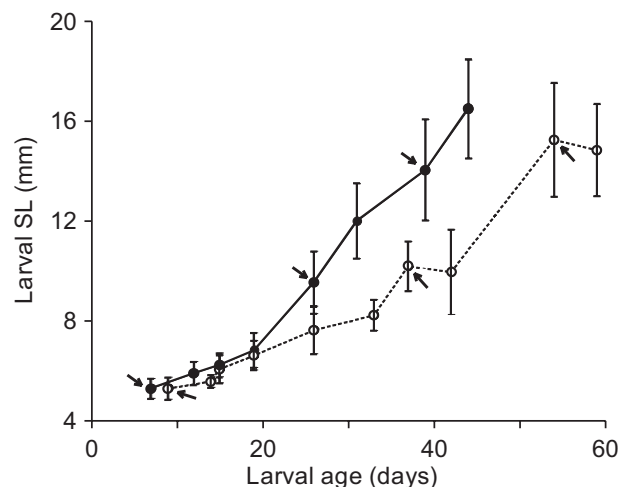


FIG. 2. – Mean and standard deviation of standard length (SL; mm) in rearing tanks at 6°C (open circles and dashed line) and 10°C (filled circles and continuous line) from the beginning to the end of the experiments. The arrows mark the beginning of the experiments at each Temperature – Developmental Stage.

RESULTS

Larvae grew faster (in terms of SL) at 10°C than at 6°C. Larvae took 44 days from hatching to grow up to 16.5 mm (S.D. 1.98) SL at 10°C and 59 days to grow up to 14.8 mm (S.D. 1.84) SL at 6°C (Fig. 2). Daily mortality rate after transfer to the experimental tanks was similar in the fed and starved groups and averaged 3.3 and 3.2 % day⁻¹ respectively. The mortality rate averaged 8.6% day⁻¹ in the FF period and 0.5% day⁻¹ in the Pre-M and Post-M stages (Table 3). High mortality of FF larvae was possibly due to transfer stress.

T and Food showed a significant effect on Ln SL of larvae from the experimental treatments (ANOVA, *p* < 0.05, Table 4). As expected, larger larvae were observed at experimental day 5 under

TABLE 3. – Average daily mortality rates (day⁻¹) in experiments after transfer at 3 different developmental stages: (1) established first feeding larvae, about 5 mm standard length (SL) and with no visible yolk remains (FF); (2) high-growth phase at late larval stage, 9–10 mm SL (Pre-M); and after initiation of metamorphosis, about 15 mm SL (Post-M).

Stage	Temp	Daily mortality rate	
		Fed	Starved
FF	6°C	0.062	0.089
FF	10°C	0.124	0.068
Pre-M	6°C	0.009	0.003
Pre-M	10°C	0.000	0.014
Post-M	6°C	0.000	0.006
Post-M	10°C	0.000	0.009
Average		0.033	0.032

TABLE 4. – Results of the ANOVA analysis on larval LnSL at experimental day 5 in each experimental treatment. T, temperature; DS, developmental stage; df, degrees of freedom; MS, mean squares; F, F value; *p*, level of significance. Asterisks denote level of significance: * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001.

Factors and interactions	df effect	MS effect	df error	MS error	F	<i>p</i>
T	1	0.180	4	0.009	19.084	0.020*
DS	2	25.074	8	0.019	1296.5	0.000***
Food	1	0.650	4	0.009	68.99	0.001**
Tank	4	0.009	210	0.011	0.876	0.479
T x DS	2	0.057	8	0.019	2.970	0.108
T x Food	1	0.041	4	0.009	4.379	0.105
DS x Food	2	0.047	8	0.019	2.466	0.146
DS x Tank	8	0.019	210	0.011	1.798	0.079
T x DS x Food	2	0.060	8	0.019	3.097	0.101

TABLE 5. – Results of the 3 ANCOVAs for each DS at experimental day 5. LnMYO, dependent variable; LnSL, covariate. T, temperature; DS, developmental stage; df, degrees of freedom; MS, mean squares; F, F value; *p*, level of significance. Asterisks denote level of significance: * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001.

DS	Factors and interactions	df effect	MS effect	df error	MS error	F	<i>p</i>
FF	T	1	0.033	4	0.027	1.252	0.326
	Food	1	0.228	4	0.006	8.527	0.043*
	Tank	1	0.027	61	0.027	4.571	0.003**
	T x Food	4	0.035	4	0.027	1.311	0.316
Pre-M	T	1	0.006	4	0.003	1.813	0.249
	Food	1	0.277	4	0.003	81.36	0.001**
	Tank	4	0.003	65	0.004	0.812	0.522
	T x Food	1	0.011	4	0.003	3.303	0.143
Post-M	T	1	0.000	4	0.0025	0.001	0.974
	Food	1	0.441	4	0.0025	173.9	0.000***
	Tank	4	0.003	70	0.0027	0.940	0.446
	T x Food	1	0.017	4	0.0025	6.790	0.060

High-Food or 10°C treatments. These differences were less apparent in the FF stage than in Pre-M and Post-M stages. There were significant differences between the linear regression slopes of LnMYO against LnSL for the 24 different combinations of treatments ($n = 223$; $F = 2.14$; $p = 0.003$). The slopes of the Post-M stage cases, mainly at 10°C, were consistently lower than the slopes of the FF and Pre-M. We therefore carried out 3 separate ANCOVAs, one for each developmental stage. The slopes within FF, Pre-M and Post-M were not significantly different (FF: $n = 70$; $F = 1.52$, $p = 0.177$. Pre-M : $n = 74$; $F = 1.044$; $p = 0.410$. Post-M: $n = 79$; $F = 0.955$; $p = 0.472$). The effect of the covariant (LnSL) was significant in each DS (FF: $n = 70$; $F = 13.34$; $p < 0.01$. Pre-M: $n = 74$; $F = 9.27$; $p < 0.01$. Post-M: $n = 79$; $F = 475.4$; $p < 0.01$). ANCOVAs also showed significant effect of Food in each DS (Table 5). Adjusted means were calculated in order to remove all differences that could account for LnSL (the covariate). Lower adjusted means, reflecting a slenderer morphology for a certain SL (and therefore lower larval weight), were observed in larvae at 0-Food com-

pared with High-Food in each of the different cases (Fig. 3). Larvae at 0-Food and 10°C presented lower adjusted means than larvae at 0-Food and 6°C in each larval stage (Fig. 3), although the interaction between T and Food was not significant at any DS.

According to criteria in Table 1, 45.7, 5.1, and 7.5% (16, 2, 3 in numbers) of FF, Pre-M and Post-M samples respectively, were discarded from further data analysis. Most FF, rejected cases (*c.* 70%; $n = 11$) presented <6000 CCE. The low number of replicates in FF treatments (Fig. 4) prevented us from including this whole data set in the %S-phase ANOVA. There was a significant effect of the interaction T x Food on %S-phase (only Pre-M and Post-M cases). There were significant differences in all T x Food pairs, except between 6°C – High-Food and 10°C – High-Food (SNK test, $p < 0.05$; Fig. 5). Treatments with 0-Food had significantly lower %S-phase cells than High-Food groups, and among 0-Food, 10°C treatments presented significantly lower %S-phase than 6°C treatments (Fig. 5). This overall pattern was similar to the pattern observed for the adjusted means

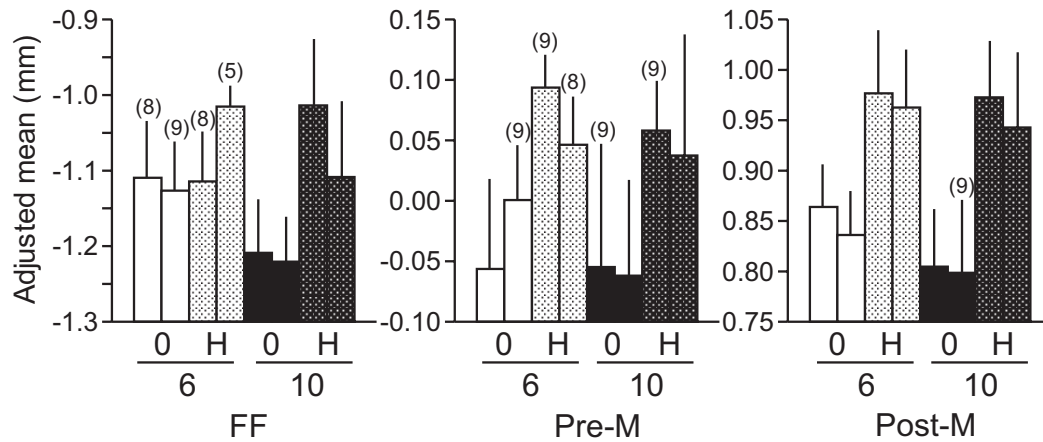


FIG. 3. – Adjusted means (bars) and their standard deviation (vertical lines) of Ln MYO (Ln SL as covariate) in each experimental Temperature x Developmental Stage x Food interaction at experimental day 5. 6°C, white background bars; 10°C, black background bars; 0-Food (0), solid bars; High-Food (H), dotted bars. Each pair case corresponds to two different tanks. FF, established first feeding larvae, about 5 mm standard length (SL) and with no visible yolk remains; Pre-M, late larval stage, c. 9-10 mm SL; Post-M, after initiation of metamorphosis, c. 15 mm SL. $n = 10$ in all treatments except when indicated in parenthesis on top of the corresponding bar. Adjusted means and their standard deviation were calculated independently at each developmental stage.

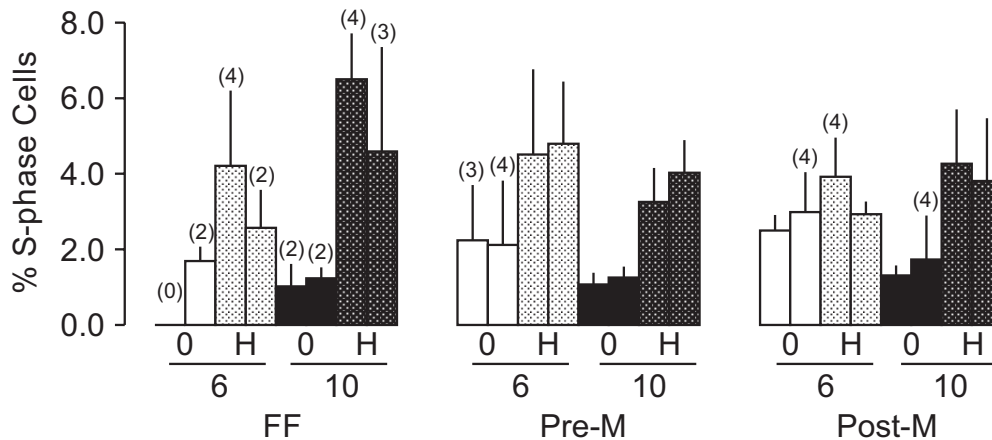


FIG. 4. – As in 3 but for %S cells and $n = 5$ in all treatments except when indicated in parenthesis on top of the corresponding bar.

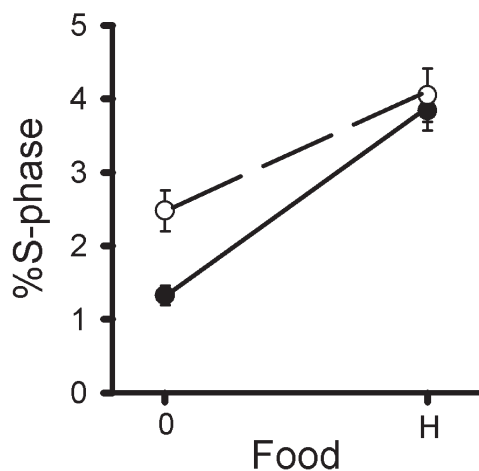


FIG. 5. – Temperature x Food interaction plot. Mean (circles) and standard error (vertical lines) of % S-phase for Pre-M and Post-M stages (as defined in Fig. 3 caption). 6°C, open circles and dashed line, and 10°C, filled circles and continuous line. 0, 0-Food; H, High-Food.

in the $\ln\text{MYO} - \ln\text{SL}$ ANCOVAs: (1) there were lower adjusted means in treatments with 0-Food, (2) there were no apparent differences between 6°C – High-Food and 10°C – High-Food, and (3), although the effect of T was not significant, the T x Food interaction and adjusted means in 6°C – 0-Food in general, presented lower mean values than 10°C – 0-Food for each larval stage (Fig. 3). The number of replicates was too low to include FF in the ANOVA. Treatments with 0-Food also presented a lower %S-phase than High-Food, and among them the 10°C treatments were the lowest. Differences in the %S-phase between developmental stages did not reveal any consistent pattern (Fig. 4) and no significant interactions or main effects were found for this factor (Table 6). The ANOVA analysis for % $G_2\text{-M}$ did not show any significant effect of food, temperature or their

TABLE 6. – Results of the ANOVA with % S-phase on day 5. as dependent variable experimental (Only Pre-M and Post-M stages included). T, temperature; DS, developmental stage; df, degrees of freedom; MS, mean squares; F, F value; *p*, level of significance. Asterisks denote level of significance: * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001.

Factors and interactions	df effect	MS effect	df error	MS error	F	p
T	1	8.709	4	0.343	25.42	0.007**
DS	1	0.010	4	1.108	0.009	0.928
Food	1	82.99	4	0.343	242.3	0.000***
Tank	4	0.343	59	1.489	0.230	0.920
T x DS	1	2.508	4	1.108	2.264	0.207
T x Food	1	4.221	4	0.343	12.32	0.025*
DS x Food	1	3.854	4	1.108	3.469	0.136
DS x Tank	8	1.108	59	1.489	0.744	0.566
T x DS x Food	1	1.166	4	1.108	3.760	0.125

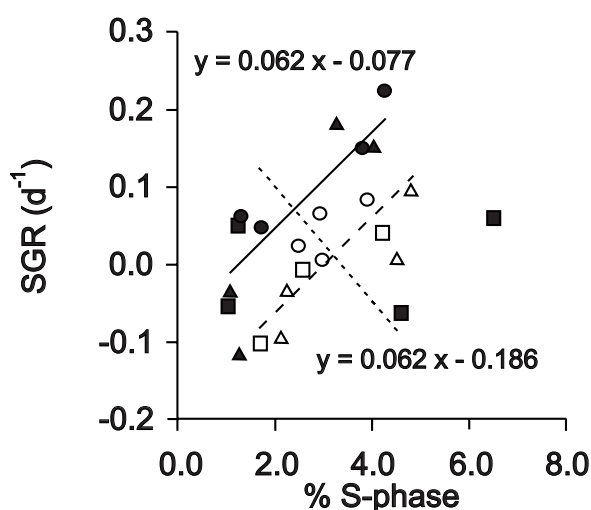


FIG. 6. – Relationship between mean standard growth rate (SGR, d^{-1}) against mean %S-phase in each experimental treatment. Open symbols correspond to 6°C and filled symbols to 10°C. Squares correspond to FF larvae; triangles to Pre-M larvae and circles Post-M larvae (as defined in Fig. 3 caption). Continuous and dashed lines and their corresponding equations represent linear regression fits for 10 and 6°C treatments respectively (r^2 values are given in the text). The dotted line is used as a visual reference to differentiate 0-Food (below and to the left) from High-Food (above and to the right).

interaction (data not shown), and for % G_0 – G_1 only food was significant ($p = 0.023$).

The SGRs from each experimental treatment were positively related to their corresponding average %S-phase in general (Fig. 6). However, the estimated SGRs of FF larvae at 10°C – High-Food tanks were low compared to their corresponding %S-phase values, and SGR was negative in one case (with %S ~ 4). However, the SGR of FF larvae from one of the 10°C – 0-Food tanks was positive. This inconsistency between SGR and %S for FF larvae at 10°C was not observed between MYO-SL adjusted means (Fig. 3) and %S (Fig. 4). We decided to exclude FF larvae at 10°C from the ANCOVA and the subsequent linear regression model since this group had also experienced unexpected high mortality (Table 3), possibly

due to transfer stress. There were no significant differences between the slopes of linear regression fits of SGR against %S-phase for 6 and 10°C ($n = 19$; $F = 1.68$; $p > 0.20$). However, there were significant differences between the intercepts ($n = 19$; $F = 19.7$; $p < 0.001$). A linear regression model, considering different intercepts and forcing equal slopes for each temperature, explained 73.8 % of the variability ($n = 19$; $r^2 = 73.8$; $F = 22.6$; $p < 0.001$) and showed that for the same value of %S-phase, SGR was 0.11 higher at 10°C than at 6°C (Fig. 6). According to the regression equations (Fig. 6), the limit between positive and negative SGR was at 1.24 and 3.00 %S-phase for 10 and 6°C respectively.

DISCUSSION

The growth rates of cod larvae within the rearing tanks in this study are comparable with results from other studies from the same facility. The estimated time from hatching until initiation of metamorphosis (12 mm), was approx. 31 and 46 days at 10 and 6°C respectively. This is about four days longer than in the study by Otterlei *et al.*, (1999) at the same temperatures, but about 4 days shorter than observed in full ration groups in a subsequent study (Høie *et al.*, 2003). The growth rates from the lab studies have previously been shown to be of similar magnitude as those observed from mesocosm after temperature correction (Folkvord, 2005), and we therefore conclude that the High-Food groups had a normal growth pattern up to the time of experimentation.

Our general objective was to test the adequacy of cell cycle analysis to study the effect that food availability and temperature have on larval growth. We set an experimental design with contrasting factor conditions in order to generate differentiated larval

growth rates. It should be noted that mortality rates of FF larvae were higher and more variable than at Pre-M and Post-M stages. It seems that not all fed-FF larvae were in good condition and therefore not growing at the expected rate. This interpretation is consistent with higher variability in SL, MYO and %S-phase in FF larvae compared with later stages, which presented lower and less variable mortality rates. Some larvae which would have recently metabolized energy from yolk may have further amplified the spread in larval condition at the FF stage.

The small brain size in FF larvae made dissecting them difficult and tissue loss often occurred. This fact may have been the reason for the high percentage of samples rejected due to a low number of cells (< 6000 CCE) and questions the applicability of this technique in small-sized larvae. However, the number of cells seemed to be enough for the analysis in most FF larvae. Refinement of the dissecting and manipulating techniques may improve the success rate. It should be noted that muscle must be the target tissue in the analysis of larval growth variability in field studies, which is the main purpose of condition indices. Larval growth is mainly attained by an increase in muscle mass and other tissues, such as brain, may not scale isometrically with growth at different developmental stages. Furthermore, muscle is more sensitive to unfavourable conditions than brain. However, we were unable to obtain samples of an acceptable quality for cell cycle analysis from muscle samples in preliminary trials with *Sparus aurata* and Catalan *et al.* (2007) obtained only 30 % of samples with CV < 6, which they used as their quality threshold. Neural tissue is softer and it was easier to obtain cell suspensions with a low percentage of debris and aggregates. However, stronger mechanical force is needed to obtain cell suspensions from muscle. Therefore, a higher percentage of debris may be generated, resulting in low quality samples for cell cycle analysis. Successfully applying this technique to muscle largely depends on developing an appropriate technique for obtaining cell suspensions with low amounts of debris and aggregates.

Under the experimental conditions and methods in our study, and according to observed larval growth patterns, the %S-phase was a better proxy for cell division rate than %G₂-M or %G₀-G₁ (%G₀-G₁ = 100 - (%S + %G₂-M)). Previous studies have used the total percentage of S + G₂-M (Theilacker and Shen, 1993a, b; 2001; Catalan *et al.* 2007) or the

ratio (G₂-M)/(G₀-G₁) as indices of cell division rate. Whereas S + G₂-M may be a better proxy for the total number of cells dividing and it apparently increases the power of statistical analysis, Shankey *et al.* (1993) recommended that only %S should be used as the index of cell division rate. They argued that G₂-M measurements have greater variability than S-phase due to the arrest of cells in G₂-phase and aggregates overlying G₂-M. Our results agree with the argument that %S-phase is a better estimate of cell proliferation than % S + G₂-M. However, Bromhead *et al.* (2000) reported that including S-phase data in their cell division index (CDI; G₂-M/G₀-G₁) had little or no effect on the statistical significance of their results. The software used to model cell cycle phases (G₀-G₁, S and G₂-M) calculates the percentage of cells in each phase. Therefore, G₀-G₁ = 100 - (S + G₂-M), so it is not surprising that %S had little effect on their CDI as it is already indirectly included in it. For a fixed percentage of G₂-M, if %S increases, G₀-G₁ (the denominator) decreases, and the CDI will have a higher value. In essence, the CDI is positively related and behaves similarly to the total percentage of S + G₂-M used in other studies (Theilacker and Shen, 1993a, b; 2001; Catalan *et al.* 2007). Still, the absence of G₂-M significance in our study may be related to larger numbers of aggregates caused by methodological procedures and difficulties in their appropriate estimation, more than theoretical considerations such as arrest in G₂. Even low aggregates may exert important variability when calculating G₂-M (Shankey *et al.*, 1993). Light scatter measured by a flowcytometer is related to particle size. A procedure frequently used to differentiate G₂-M from aggregates is to separate them by their pattern in light scatter, based on the assumption that aggregates may be larger than cells in the G₂-M phase. However, we did not observe two clearly differentiated populations and there was no consistent pattern in the samples. It should be taken into account that within larval brains there may be differences in cell sizes that may cause some degree of overlap between G₂-M and aggregates. Furthermore, the pattern of this overlap may change as proportions of cell sizes vary during larval development. Until better techniques are developed to appropriately differentiate aggregates and G₂-M, at least % S vs. % S + G₂-M must be evaluated to assess their relative predictive power with each specific methodology used (Bauer *et al.*, 1993), including target tissue.

Nevertheless, %S-phase in brain cells in our study was significantly related to food and temperature, the interaction between both factors in the Pre-M and Post-M stages and consistent with the observed pattern in FF larvae. Larvae presented a higher increase in the %S-phase under High-Food conditions, which is in agreement with patterns of S + G₂-M observed in previous studies (Theilacker and Shen, 1993a, b, 2001; Bromhead *et al.*, 2000; Catalan *et al.*, 2007). High %S-phase at High-Food was coincident with high SL and adjusted means from the morphological relationship between MYO and SL (a proxy for individual larval weight). Our results suggest that cell cycle analysis may be appropriate as a growth index under contrasting feeding conditions.

There was no significant difference in %S-phase for fully-fed larvae at different temperatures (6 and 10°C), which was in agreement with the absence of significant differences in G₂-M/G₀-G₁ observed in larvae of *Galaxias olidus* reared at 12 and 16°C (Bromhead *et al.*, 2000). In our case, this result was unexpected as Otterlei *et al.* (1999) showed that growth rate of cod larvae increased at temperatures from 4 to 14°C, which is in agreement with the pattern observed in our rearing tanks (Fig. 2). The rate of increase in SGR with %S-phase was similar at each temperature (no significant differences between slopes), but SGR was higher at a certain %S-phase value at 10°C (significant differences between intercepts). The cause of this temperature dependency on the relationship between growth and %S-phase is as still unclear. We suggest that temperature affects the rate at which cells progress through the cell cycle, as shown in previous studies on different organisms (West *et al.*, 1981; Crossen, 1985; Francis and Barlow, 1988). Cell division rate for any one cell may be faster in larvae at 10°C than at 6°C (Aksnes *et al.*, 2006). The brain would grow faster at 10°C as the duration of the cell cycle is shorter at higher temperatures, although the proportion of cells in the brain cycling at any one time may not increase. Hence, temperature should be considered as a second independent variable, like in the RNA/DNA index (Buckley, 1984). However, the RNA/DNA index is inversely related to temperature at a given growth rate. Increased RNA at low temperatures has been proposed to be a compensatory response by cells to ensure that basic metabolic processes are maintained despite the slowing of biochemical reaction rates due to low temperatures [see

review by Bergeron (1997)]. Our results do not suggest a temperature-compensatory response in the percentage of cells dividing.

In addition, larvae from 6°C at 0-Food presented a significantly higher %S-phase than larvae from 10°C at 0-Food. Metabolic costs increase with temperature in larval cod (Finn *et al.*, 2002). Therefore, cell division would be less affected by suboptimal food availability at low temperatures. Although we should not expect a reduction in SL under low food availability, we can expect this to occur in terms of larval weight as a consequence of metabolic costs. This is consistent with the pattern in the morphometric relationship between MYO and SL; larvae at 6°C – 0-Food presented higher MYO in relation to SL compared with larvae at 10°C – 0-Food (although not significant). The interaction between food and temperature, mediated by temperature dependency of metabolic costs, may play a significant role in larval growth rate variability in the field, which increases with temperature (Houde, 1989; Buckley *et al.*, 2004). However, the magnitude of this effect in terms of % S-phase should be interpreted with caution. As suggested by the absence of differences in %S at High-Food conditions at different temperatures, the rate of cell proliferation in a tissue may depend both on the percentage of cells ‘cycling’ and on the rate at which individual cells progress through the cell cycle, which could be temperature dependent. Nevertheless, the capability of cell cycle analysis to detect growth differences at low temperatures is a potential advantage over RNA indices, as the compensatory increase in RNA at suboptimal temperatures reduces their sensitivity (Bergeron, 1997). A simultaneous analysis of RNA and cell cycle based indices may confirm this hypothesis.

The absence of a significant effect of developmental stage on the %S-phase or its interaction with other factors was also an unexpected result. We expected a larger percentage of cells dividing at larval stages characterized by higher growth rates (e.g. pre-metamorphosis stages (Otterlei *et al.*, 1999)). However, Pre-M larvae did not have higher SGR than Post-M larvae. Between-individual variability in SL and MYO within each experimental tank may account for part of the variability in SGR estimates. In addition, the SL at which cod larvae attained maximum growth rates may have varied between 6 and 10°C during experimental treatments. Growth allometry between different tissues may be an alter-

native explanation. In other fish, brain growth proceeds at a lower rate than body weight. The relationship between body mass, SL and MYO shows 3 distinct phases during cod ontogeny (Finn *et al.*, 2000), and the larval sizes that determined developmental stage in our study fitted each of these phases. Body mass increases at a higher rate in relation to SL between 7 and 12 mm, which is concurrent with a higher rate of increase in MYO with respect to SL (Finn *et al.*, 2002). Higher growth rate in pre-metamorphosis stages (Otterlei *et al.*, 1999) seems to be related to morphological changes, and may not scale isometrically with brain growth. Muscle tissue is more suitable to test for differences in growth variability related to ontogeny. However, muscle growth in larval cod can be either in the form of growth in fibre numbers (hyperplasia) or fibre size (hypertrophy) (Galloway *et al.*, 1999), and in a follow-up study it would be recommended to measure the total DNA content (as a measure of cell numbers) to see if this increase corresponds to the increase in dry weight (as a measure of total cell mass). The apparent discrepancy may also be due to growth phases in cell numbers as opposed to increases in cell size. Furthermore, larval development also includes tissue remodelling, in which apoptosis (programmed cell death) plays an important role (Yamashita, 2003). At certain developmental stages, apoptosis could represent a significant fraction compared to cell division in a specific tissue. Taking into account high characteristic growth rates in larval stages, we assumed that growth was mainly attained by cell division, at least in neural tissues. However, growth rate is in fact the consequence of cell division (positive growth) and apoptosis (negative growth), and their relative proportion changes during larval development and possibly under unfavourable conditions (e.g. fasting). Apoptosis can be estimated by flow cytometry and it is necessary to determine its relative importance and evaluate whether it is appropriate to include it in a cell cycle based index.

The linear regression between SGR and the %S-phase in our study explained a higher amount of the variance (74 %) than the regression between growth rate (AGR, mm d⁻¹) and %(S + G2) obtained by Theilacker and Shen (2001) in muscle tissue of *Theragra chalcogramma* larvae under different feeding conditions (54 %). This is somewhat lower than that obtained using RNA:DNA ratios in a study on herring larvae (Folkvord *et al.*, 1996), in which

multiple regression models explained up to 90% of the variability in average growth. A likely explanation for this difference is that most of the RNA and DNA in RNA:DNA analyses originate from relatively responsive muscle tissue as opposed to less nutritionally dependent brain cells.

In summary, the results presented herein support the argument by Theilacker and Shen (1996) that analyzing the cell cycle by flow cytometry may be an adequate growth index in fish larval studies. As in previous studies (Theilacker and Shen, 1992a, b, 2001; Bromhead *et al.*, 2000), our study suggests that cell cycle analysis by flow cytometry is appropriate for analyzing growth variability related to food availability. Furthermore, under food deprivation, the observed %S-phase variability was consistent with the effect that temperature has on metabolic costs. The absence of significant differences in the %S-phase under different temperatures may be the consequence of a temperature dependence of the duration of the cell cycle in a single cell. This effect should be tested under a wider range of temperature conditions.

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