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Detection and evaluation of temperature effects on cell proliferation in somatic tissues of the eastern oyster, Crassostrea virginica, by flow cytometry

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DETECTION AND EVALUATION OF TEMPERATURE EFFECTS ON CELL PROLIFERATION IN SOMATIC TISSUES OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*, BY FLOW CYTOMETRY

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To

Mom and Dad

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FOREWORD

The eastern oyster, *Crassostrea virginica*, is an ecologically and economically important bivalve mollusk in the state of Louisiana. There has been a decline in oyster populations during the past century due to overharvesting, habitat destruction, and disease. Although overharvesting and habitat destruction have been major problems, along the Atlantic coast and Gulf of Mexico, diseases have plagued oyster populations since the 1950's (Burreson and Calvo 1996; Ford 1996; Kennedy 1996). These devastating diseases have lead to an increase in scientific research to restore eastern oyster populations.

The development of an oyster cell line would aid research into understanding of the physiology of oysters and the effects of viruses and pathogens. The overall goal of these studies was to evaluate the effect of differential growth temperatures on *in vivo* cell proliferation of oyster somatic tissues. The results of this study can be used for advancing the development of an oyster cell line.

This research was a collaborative effort between the Aquaculture Research Station (ARS), of the Louisiana State University (LSU) Agricultural Center, and the LSU Department of Biological and Agricultural Engineering (BAE). Portions of this project were presented at conferences and meetings (Table 1). This thesis was organized into seven chapters. Chapter one provides background on cell culture, oyster biology, and current methods for detection of cell proliferation. At the beginning of this project, an assay was developed for detecting proliferating cells from *in vivo* somatic tissues of the eastern oyster (Chapter 2). Next, an assay was developed for detecting nuclear RNA from *in vivo* somatic tissues of the eastern oyster (Chapter 3). This assay allowed for

estimating cell proliferation by measuring cellular metabolism and conditions of *in vivo* cells in relation to environmental changes. Finally, the effects of constant and fluctuating temperatures on cell proliferation and cellular metabolism were examined, by using the two assays, on *in vivo* somatic tissues of the eastern oyster (Chapters 4 and 5). Conclusions are covered in Chapter six.

Chapters 2 and 3 will be submitted for publication in *Marine Biology*, and Chapters 4 and 5 will be submitted for publication in *Journal of Aquaculture Engineering*, all chapters are written in the format of the respective journals. A compact disc, which contains the text of this thesis and the unanalyzed data from this study, is enclosed inside the back cover.

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Ford, S. E. 1996. Range extension by the oyster parasite *Perkinsus marinus* into the northeastern United States: response to climate change? Journal of Shellfish Research 15: 45-56.

Kennedy, V. S. 1996. The ecological role of the eastern oyster, *Crassostrea virginica*, with remarks on disease. Journal of Shellfish Research 15: 177-183.

Table F.1. Conference presentations and abstracts within scope of this thesis.

Author	Title	Conference	Location
Kim-Lien T. Nguyen, Fernando Jimenez, Terrence R. Tiersch and Jerome F. La Peyre	Detection and evaluation of cell proliferation in different tissues of the eastern oyster by immunohistochemistry.	Annual meeting of the Louisiana chapter of the American Fisheries Society (2003)	Baton Rouge, LA
Fernando Jimenez, Terrence R. Tiersch, Jill A. Jenkins and Jerome F. La Peyre	Divide and conquer: control of oyster cell division	Audobon Center for Research of Endangered Species (2003)	New Orleans, LA
Fernando Jimenez, Terrence R. Tiersch, Steven G. Hall, Jill A. Jenkins and Jerome F. La Peyre	Detection of cell proliferation of somatic tissues and hemocytes of the eastern oyster by flow cytometry	Louisiana Academy of Sciences (2004)	Lake Charles, LA
Fernando Jimenez, Terrence R. Tiersch, Jill A. Jenkins and Jerome F. La Peyre	Detection of cell proliferation of somatic tissues of the eastern oyster by flow cytometry	Annual meeting of the Louisiana Chapter of the American Fisheries Society (2005)	Baton Rouge, LA
Fernando Jimenez, Terrence R. Tiersch, Jill A. Jenkins and Jerome F. La Peyre	Detection of cell proliferation of somatic tissues of the eastern oyster by flow cytometry	Annual Meeting of the National Shellfisheries Association (2005)	Philadelphia, PA

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ABSTRACT

The goal of this thesis was to evaluate temperature effects on cell proliferation of eastern oyster somatic tissues for the development of an oyster cell line. Understanding the *in vivo* cell proliferation of an organism is essential for the development of cell culture. Cell proliferation can be measured by identifying nuclear cellular proteins involved in growth regulation and cellular transformation. The primary objectives of this study were to: 1) to develop an assay to evaluate cell proliferation, 2) develop an assay to analyze nuclear RNA content, and 3) to evaluate temperature effects on cell proliferation in somatic tissues.

Proliferating cell nuclear antigen was detected in all the phases of the cell cycle of the eastern oyster. The concentrations of PCNA increased from G_0/G_1-phase into the S phase, where it peaked in mid-S-phase, and decreased in G_2/M-phase. The immunohistochemically stained slides were analyzed using image analysis and compared to the flow cytometric detection of PCNA. Overall, flow cytometry was superior to immunohistochemistry in time and cost efficiency, and in sensitivity to the detection of PCNA in oyster somatic tissues. A flow cytometric assay was developed to detect nuclear RNA in oyster somatic tissues. Nuclear RNA was detected in all the phases of the cell cycle of the eastern oyster. The amount of nuclear RNA increased from G_0/G_1-phase into the S-phase, where they peaked in mid-S-phase, and decreased in G_2/M-phase. Temperature effects on cell cycle, proliferation, and cell metabolism of oyster somatic tissues were evaluated. The cell cycle analysis of the heart and labial palps cells showed that the G_0/G_1-phase cells decreased as the S-phase and G_2/M-phase cells increased. The cell cycle analysis of both tissues also showed the opposite effect, as G_0/G_1-phase cells

increased the S-phase and G_2/M-phase cells decreased. Heart and labial palps cells for oysters held between 10°C and 25°C increased in cell proliferation after one week of temperature fluctuation. The nuclear RNA/DNA ratio at all phases of the cell cycle for heart and labial palps cells for oysters between 15°C and 20°C was higher than at all other temperature regimes. Temperature fluctuations affected the cell cycle, proliferation, and the metabolic condition of oyster somatic cells.These results support the use of flow cytometry for the analysis of cell cycle, cell proliferation, and cell metabolism. The results also suggested that temperature fluctuation has an effect on cell cycle, cell proliferation, and cell metabolism. Future research needs to focus on improving the current *in vivo* study and adapt the assays for cultured cells.

CHAPTER 1 – INTRODUCTION

In the state of Louisiana, culture of the eastern oyster, *Crassostrea virginica*, as reported in 2004 by the Louisiana Department of Wildlife and Fisheries, is a $30 million dockside industry. The state's coastal waters produce 13 million pounds or 2 to 2.5 million sacks of oysters annually. Louisiana exports 60% of the product to other states and countries. Louisiana is the largest producer of oysters in the nation. Oysters are a valuable commodity for Louisiana and must be closely regulated because of overharvesting and habitat destruction. Although these are major concerns, along the Atlantic and the Gulf coasts, diseases have plagued oyster populations since the 1950's (Burreson and Calvo 1996; Ford 1996; Kennedy 1996).

Infectious oyster diseases (Table 1.1), such as, "MSX" and "Dermo" have had significant and chronic effects on oyster populations. The combined impact of both diseases has caused hundreds of millions of dollars in losses over the last 35 to 45 years and seriously threatens natural and cultured oyster populations (Ewart and Ford 1993). To control these diseases, researchers must first understand how affected organisms respond to the disease-causing agent, as well as knowledge of the agent itself.

The development of oyster cell culture would be a crucial step in understanding oyster tissue physiology, effects of viruses and pathogens on these tissues, and would assist scientists in developing beneficial genetic traits such as disease resistance or increase growth rates. These advantages would provide a better cultured oyster product and result in higher yields.

Table 1.1. Diseases and parasites of the eastern oyster reported for the northeastern, southern and Gulf coast region of the United States.

Disease/Parasite	Description	Reference
Dermo disease *Perkinsus marinus* (protozoan)	Causes high mortalities Proliferates rapidly at temperature between 25°C and 30°C and salinity greater than 15 ppt Transmitted directly from oyster to oyster	Craig et al. 1989 Ford 1992 La Peyre et al. 1993 Volety and Chu 1995
MSX disease *Haplosporidium nelsoni* (protozoan)	Causes high mortalities Active at temperatures above 10°C and intolerant of salinities below 10 ppt Mode of transmission unknown	Ford 1988 Burreson 1991 Chintala and Fisher 1991
Cardiac edema associated with *Vibrio anguillarum* (bacteria)	Enlargement of pericardial chamber and heart	Elston et al. 1982
Shell disease caused by *Ostracoblabe implexa* (fungus)	Penetrates and proliferates in shell, causes wart-like depositions at adductor muscle attachment	Li et al. 1983
Juvenile Oyster Disease	Causes high mortalities in the northeastern U.S. Affects cultured oysters <25mm shell height Mode of transmission unknown	Bricelj et al. 1992 Davis et al. 1995 Farley et al. 1995

This chapter is intended to provide the reader with a brief background and overview of the steps required to improve the development of oyster cell cultures. Specifically, this chapter explains the history and development of cell culture, the biology of the eastern oyster, and provides brief explanations of various techniques used for detecting proliferating cells.

CELL CULTURE

In 1907 Ross Harrison, an embryologist from Yale University in Connecticut, first grew a fragment of embryonic frog tissue in a drop of lymph fluid. The fluid hung from a cover slip over a hollow slide, and the tissue was enclosed in glass (*in vitro*) and could be observed using a microscope. Harrison proved, after watching the isolated process over time that nerve fibers originated from a single cell. He showed that cells kept in aseptic conditions at the correct temperature could live outside the body (Harrison 1907; Landecker 2002). Alexis Carrel, in 1912 at the Rockefeller Institute for Medical Research in New York, introduced the idea of continuous cultures of cells. Not only did he maintain a living fragment of tissue for weeks, but used pieces of the tissue to start new cultures. Carrel concluded that scientists would never need to go back to the animal body, if only the method could be improved to keep cells alive forever. He introduced the idea of "permanent life" – or immortality – to cell culture (Carrel 1912; Landecker 2002).

Cell culture allows individual cells to act as semi-independent units, with the cells dividing by mitosis and the cell population growing until limited by nutrient depletion or by the presence of potentially toxic metabolites (Mothersill and Austin 2001). Cultured cells have several advantages: cells in culture are homogeneous in their morphological properties; experimental conditions can be controlled more rigorously than in whole

organisms (Freshney 1987); standardization of *in vitro* systems is possible; growth of obligate pathogens is possible; cellular biochemistry studies are possible (Lodish et al. 2001); studies of interactions between prokaryotic and eukaryotic cells are possible, allowing for the study of pathogenic mechanisms more naturally than the standard microbial culturing techniques; and the need for whole organism studies is reduced (Mothersill and Austin 2001).

There are three types of cell cultures: primary cultures, secondary cultures, and immortalized or transformed cultures. Primary cultures are explanted directly from a donor organism. These cells may be able to divide once or twice and survive for some time in culture, but they will not continue to grow and eventually senesce and die. These cells are cultured because they are thought to represent acceptable experimental models for *in vivo* situations, and because they may express characteristics not seen in long-term cultured cells that are more realistic to animal cells *in vivo*. Secondary cultures are explanted from a donor organism and are able to divide and grow for some time *in vitro* (50 – 100 generations). However, these cells do not continue to divide indefinitely and eventually also senesce and die. Immortalized or transformed cells continue to grow and divide indefinitely *in vitro* as long as the culture conditions are maintained (e.g., HeLa cells) (Lodish et al. 2001; Cann 1999).

There are three general concepts that are important to the development of cell cultures: basic methods and media, tissue choice, and characterization (Mothersill et al. 2001). The understanding of basic methods and media allows for flexibility in approach and a rapid response to observed changes in the cell culture. This understanding is also valuable for the optimization of each step. The choice of tissue for *in vitro* culture is

important because it will influence the developmental status, methodological factors, and eventual application of the cell culture (Freshney 1994; Evenden 2001). Cell characterization *in vitro* is essential to be able to determine differentiated cellular functions and to relate to cell behavior *in vivo* (occurring within the living body of an organism) (Freshney 1994; Doyle et. al. 1993; Lyons-Alcantara 2001).

Since Harrison and Carrel, cell culture technologies have been adapted into routine applications in medicine and industry and scientists are currently using cultured cells for research of cell membrane proteins, stem cells, and tissue engineering. Although the majority of cell cultures are still developed using higher vertebrate organisms, scientists are shifting to developing cell cultures for lower vertebrates and invertebrate organisms. The reasons for the development of these cell cultures are: the unique biological development of lower vertebrate and invertebrate organisms (e.g. tissue regeneration in amphibians and metamorphosis in insects); the need to improve pest control in agriculture (e.g. toxicity and virological studies in insects); interest in pharmaceutical compounds from the sea, and the understanding of normal biological development and pathogenesis to improve aquaculture (Freshney 1987).

BIOLOGY

A first step in the development of oyster cell cultures is to gain an understanding of the biology of the organism. The eastern oyster (Phylum Mollusca and Class Bivalvia) is ectothermic, and thus temperature affects virtually every aspect of their biology including survival, feeding and growth, gonadal development and spawning, sex determination, larval settlement, heart rate and respiration, disease outbreaks (Shumway 1996) and somatic tissue cell proliferation. Eastern oysters live in shallow and semi-

enclosed water bodies, where water temperatures can vary greatly (Fig. 1.1), along the east coast of North America from the Gulf of St. Lawrence to Key Biscayne, Florida and south through the Caribbean to the Yucatan Peninsula of Mexico and Venezuela (Osborne 1999). Adult eastern oysters are commonly found in waters where the annual temperatures range from -2°C to 36°C (Butler 1954; Galtsoff 1964). Survival of oysters is more affected by the rate in change of temperature, than actual temperatures (Fingerman and Fairbanks 1957). Growth rates of larval and adult eastern oysters are more rapid in warmer waters (Butler 1953). In the Gulf of Mexico, an oyster can grow to marketable size (90 mm) in 2 years, while in cooler waters such as Long Island Sound, it can require 4 to 5 years to attain the same size (Shumway 1996).

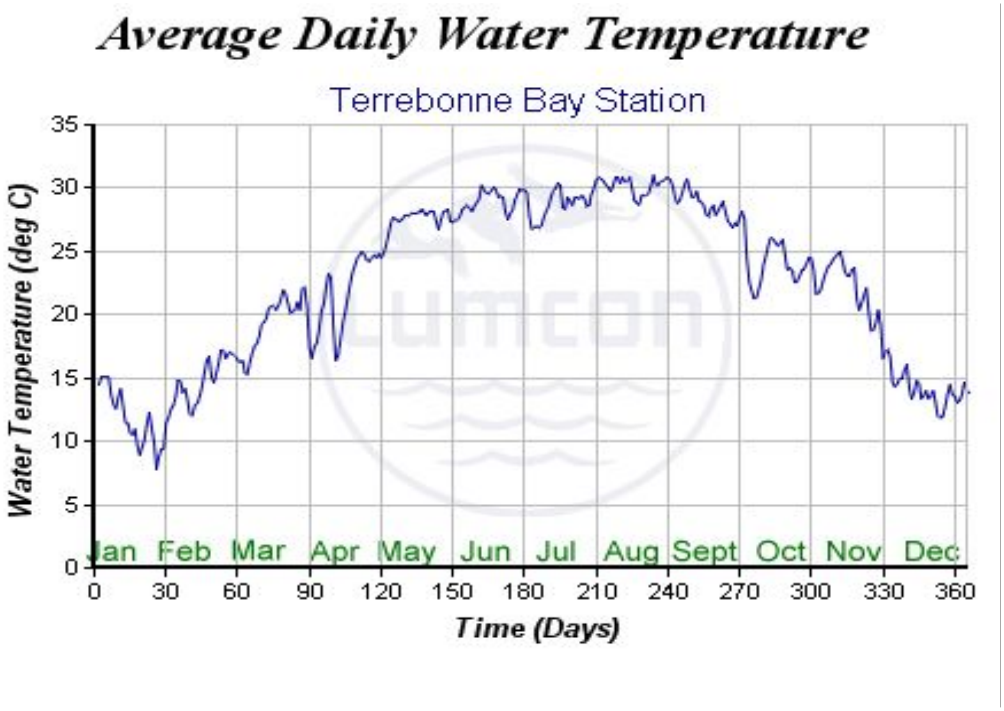

Figure 1.1. Daily average water temperatures in Terrebone Bay, Louisiana ranged from 8°C, in December and January, to about 31°C from June to August in 2003.

Eastern oysters have a tolerance for wide salinity ranges. They are found in areas were the annual salinity variations ranges from 5 to 40 parts per thousand (ppt) (Galtsoff

1964; Wallace 1966). Commercial production areas have annual salinity variations that range from 0 to 42.5 ppt (Ingle and Dawson 1950).

Eastern oysters are active suspension feeders. From the planktotrophic larval stage to the benthic life stages, they feed by removing suspended particles from the water column. The particulate food is composed of mixtures of living organisms, detritus, and inorganic particles (Berg and Newell 1986; Baldwin and Newell 1991). The suspended particles are pumped through the gills were they are trapped and sorted by size before transfer from the gills to the mouth and gut were they are ingested and digested (Beninger et al. 1991).

Eastern oysters are gonochoric or dioecious alternate hermaphrodite organisms with an annual reproductive cycle (Mackie 1984). That is, eastern oysters initially function as males and may change to females depending on environmental conditions such as water quality and temperature. Eastern oysters, in an unsuitable environment may not develop as females (Kennedy and Battle 1964). Gonad development in females demands more energy and persisting unfavorable environments would limit energy for gametogenesis (Giese and Pearse 1974). Gametogenesis is synchronized in eastern oysters, whereby eggs and sperm are released concurrently to ensure fertilization and to maximize the number of zygotes. Gamete maturation was found to begin at 15°C at a slow rate while at higher temperatures the rate of maturation increased (Sastry 1968). Manipulation of water temperature, in a hatchery environment, is a means of stimulation to ripen eastern oysters (Loosanoff and Davis 1963). Spawning occurs generally when water temperatures increase above 20°C (Hayes and Menzel 1981), it is stimulated by

environmental cues in the surrounding water and the presence of gametes from adjacent oysters (Thompson et al. 1996).

Although a number of factors have been suggested to affect larval settlement, such as food supply, oxygen supply, light, type of cultch, and waterborne chemicals, temperature has been determined to be a major factor. The most favorable temperatures for larvae to settle are between 19°C and 24°C (Ryder 1885).

The heart rate increased in the eastern oyster as temperature increased from a range of 5°C to 30°C (Menzel 1956). There is a linear relationship between the number of leucocytes in suspension in the hemolymph and the water temperature. The number of leucocytes in suspension was influenced by heart rate (Feng 1965). Similarly to heart rate, respiration decreases as temperature decreases (Shumway and Koehn 1982).

High temperatures promote disease outbreaks in eastern oysters (Ford and Tripp 1996). Temperatures above 20°C favored the development of *Perkinsus marinus* (an oyster disease caused by the protistan parasite of the same name) in the Gulf of Mexico. Eastern oysters that were infected with *P. marinus* experienced 100% mortality when held for 4 weeks at 28°C, but when the infected oysters were held at 15°C for the same amount of time, the infection ceased and mortalities were negligible (Hewatt and Andrews 1956). The incidence of oysters infected with Multinucleated Spore Unknown (MSX) disease was found to be lower at low temperatures (Hofmann et al. 2001).

Cell proliferation in somatic tissues of the eastern oyster is known to be highly variable and dependent on seasonality (Nguyen et al. Department of Veterinary Sciences). High proliferation is found in somatic tissues in the late fall to early spring, when animals are not spawning. Cell proliferation ceases in these tissues in late spring

and throughout the summer while the gonads develop (Nguyen et al. Department of Veterinary Sciences).

DETECTION OF CELL PROLIFERATION

The second step in the development of the oyster cell culture is to understand the *in vivo* cell proliferation of the organism. Understanding the cell cycle and its controls is the basis for various methods used in evaluating rates of proliferation in tissues (Yu et al. 1992). The eukaryotic cell cycle is an ordered series of events during which chromosomes are duplicated and one copy of each duplicated chromosome segregates to each of two daughter cells. The cell cycle is controlled by a number of enzymes that regulate the activities of multiple proteins involved in DNA replication and mitosis. In the adult organism, cell proliferation is essential in the repair processes and in normal cell replacement for certain tissues (Klug and Cummings 1997).

In the late 1980's, it was determined that chromosome replication and cell division processes were fundamentally similar in all eukaryotic cells. Although mitosis is the shortest component of the cell cycle, it is a period of vigorous and continual activity. It is subdivided into four discrete phases: prophase, metaphase, anaphase, and telophase. After the cell has undergone mitosis, it begins a new cycle of nuclear DNA replication during interphase, which typically accounts for about 90% of the time that elapses during each cell cycle. Interphase consists of three periods of growth: G_1-phase, S-phase, and G_2-phase. All cells follow one of two paths. The cell might enter a resting phase, (G_0), or it might initiate DNA synthesis and complete the cycle (Fig. 1.2).

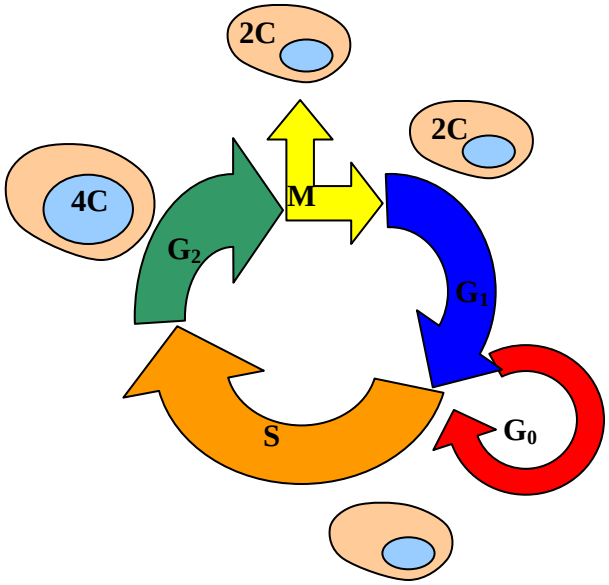

Figure 1.2. The eukaryotic cell cycle comprises four phases that proceed successively. Interphase comprise three phases: G_1, S, and G_2. DNA is synthesized in S phase, and other cellular macromolecules are synthesized throughout interphase, so the cell doubles in its size. The genetic material is evenly partitioned and the cell divides during mitosis (M). Non-dividing cells exit the normal cycle and enter a quiescent state (G_0).

The rate of cell proliferation is different among organisms and among tissues within an organism (Table 1.2)(Lodish et al. 2000).

Table 1.2. Different eukaryotic cell cycle times.

Cell type	Cell cycle times
Early frog embryo cells	30 min
Yeast cells	1.5 – 3 hours
Intestinal epithelial cells	~12 hours
Mammalian fibroblasts in culture	~20 hours
Human liver cells	1 year

Cell proliferation can be measured quantitatively by direct and indirect methods. With direct methods, proliferation is measured by the time it takes for cell populations to double in number (or rate of doubling) in culture (Scott et al. 2003). This measurement can also be used to determine the average doubling time of cell populations either *in vitro* or *in vivo*. The fraction of cells in an *in vitro* or *in vivo* population undergoing cell

division is also considered a measurement of proliferation. Although DNA synthesis can occur without cell division, use of an assay to measure the quantity of cellular DNA being synthesized enables evaluation of the rate of proliferation. In indirect methods, proliferation can be measured by calculating the number of cells undergoing mitotic progression minus the number of cells that are undergoing cell death. Cell proliferation can also be measured by determining the length of time that it takes for cells to traverse between phases of the cell cycle and the effects of the interactions between each phase. Such assays are dependent on several factors: experimental conditions, cell cycle parameters under investigation, and the source and condition of cells or tissue being assayed.

Assessment of cell proliferation can be done by counting mitosis, silver-staining of the nucleolar organizer region (AgNOR), *In situ* hybridization, of 5-bromo-deoxyuridine (BrdU) uptake, tritiated-thymidine labeling, immunohistochemical detection of cell cycle protein, and flow cytometry.

Mitotic index is a simple method of counting the number of cells visible in mitosis, but it suffers from a lack of standardization and reproducibility. Silver staining of the nucleolar organizer region is relatively easy to carry out and does not require fresh tissue; but it recognize proteins found only in early G_1 phase, and its significance and the practical applications of the AgNOR technique require further evaluation (Yu et al. 1992). *In situ* hybridization, a technique used to locate the chromosomal location of a specific DNA (or RNA) probe, for localization of histone H3 mRNA proteins is able to detect levels of these proteins only at the S-phase of the cell cycle (Crocker 1994). Staining with BrdU is an accurate method for measuring cell proliferation (Ortego et al.

1994). 5-bromo-deoxyuridine is an analogue of thymidine, and can be introduced to proliferating cells that in turn incorporate BrdU into the DNA during the S phase, prior to cell division (Lyons-Alcantara 2001). Precise timing and dosage of BrdU enables tissue-kinetic studies. Administration of BrdU may be used for labeling tissues with low proliferative activity. Incorporation of BrdU into the nucleus gives a strong and reliable signal regardless of the method of fixation and tissue processing (Mokroy and Nemecek 1995). This method measures only S phase of the cell cycle and it uses a radioactive thymidine analog that must be administered into living organisms (Ortego et al. 1995).

The main principle for evaluating cell proliferation by immunohistochemical methods is that there are cell-cycle-associated alterations in the amount or distribution of cellular proteins or other molecules that can be recognized as antigens (Hall and Woods 1990). Immunohistochemistry is one of the most effective methods to detect cell cycle proteins. Although the technology is advanced in higher vertebrates it is not well established for marine invertebrates. Most commercial antibodies are produced against human proteins only (Lyons-Alcantara et al. 1999). Antibodies that recognize a particular site on human antigens called an antigenic determinant (or epitope) have been applied to crustaceans and cross-reactivity has been demonstrated (Lyons-Alcantara et al. 2002). The assessment of cell proliferation can be done by several immunohistochemical methods, but each has inherent advantages and disadvantages. The best-known endogenous proliferating markers are Ki-67 and proliferating cell nuclear antigen (PCNA) that may be detected with specific commercially available antibodies. Ki-67 is a non-histone nuclear antigen tightly associated with the cell cycle. In cells from mammals, the Ki-67 antigen expression is found in mid-to-late G_1 phase, rises through S

phase and G_2 phase to reach a maximum in mitosis, but is not found in the resting phase (G_0) (Gerdes et al. 1984). An immunohistochemical assay for the endogenous marker, proliferating cell nuclear antigen, avoids most of these disadvantages. Proliferating cell nuclear antigen, a stable and highly conserved nuclear protein found in higher vertebrate tissues, is directly involved in DNA synthesis, and is synthesized during all phases of the cell cycle except the resting phase (G_0) (Kurki et al. 1986). Nuclear PCNA concentrations vary during the cell cycle. In mammalian cells, the PCNA concentration begins to increase from the G_1 phase, peaks at the G_1 phase/S phase interface, decreases through the G_2 phase, and reaches undetectable levels in quiescent cells (Morris and Matthews 1989). The increasing concentrations of PCNA protein can be detected by monoclonal antibodies (Erlanson et al. 1997). The development of monoclonal antibodies against PCNA has facilitated the estimation of growth activities of human malignant tumors and has allowed retrospective analysis of formalin fixed-paraffin-embedded tissues (Garcia et al. 1989; Waseem et al. 1990; Wilkins et al. 1992).

To detect immunoreactivity of antibody binding to PCNA, several factors must be considered: the type of antibody (e.g., monoclonal or polyclonal) and fixative, the fixation conditions and the staining procedure (Sasaki et al. 1993). By using antigen-retrieving procedures, PCNA can be localized in routinely fixed paraffin-embedded specimens, which allows retrospective studies (Hall et al. 1990). Although PCNA methodology was developed and applied mostly for mammalian tissues, PCNA has been identified in routinely processed, paraffin-embedded specimens of three fish species (medaka, *Oryzias latipes*; guppy, *Poecilia reticulata*; and western mosquitofish, *Gambusia affinis*)(Ortego et al. 1994). The optimum staining conditions were dependent

on fixative, primary antibody selection, antigen retrieval processing, and protein blocking reagent (Ortego et al. 1994). Proliferating cell nuclear antigen has been detected in proliferating cells of bivalves by use of an immunohistochemical procedure (Marigomez et al. 1999; Winstead 1995). Studies, conducted at the Louisiana State University Department of Veterinary Science (Nguyen et al. Department of Veterinary Sciences, Jenkins et al. unpublished), used a modified version of a commercially available PCNA staining kit (Zymed Laboratories Inc., San Francisco) to examine cell proliferation in the eastern oyster. The studies found that cell proliferation depended on tissue type (Jenkins et al. unpublished) and seasonality (Nguyen et al. Department of Veterinary Sciences). To determine the rate of cell proliferation the study used a labeling index expressed as the number of labeled cells per total cells counted for each tissue (Iatropoulos and Williams 1996; Foley et al. 1991). Although the PCNA immunohistochemical assay was reliable, it was slow and laborious, whereas flow cytometry instrumentation can quickly assess cell proliferation in individual cells.

Flow cytometry was first used in medical sciences such as oncology and hematology. Currently, flow cytometry has become a valuable tool in biology, pharmacology, toxicology, bacteriology, virology, environmental sciences, and bioprocess monitoring (Rieseberg et al. 2001). Current major applications of flow cytometry include phenotypic analysis of cells, cell sorting, and DNA content analysis. It can be used with suspensions of fresh cells, fixed cells, or cells extracted from paraffin-embedded tissues (Martinez et al. 1990). In flow cytometry, single cells or nuclei pass through a laser beam in a directed fluid stream. As they pass through the beam a variety of measurements (e.g. absorption, scattering, or fluorescence) can be simultaneously

collected for each cell. The forward-scattered light can be used to gather information on the size of the cells. The sideways-scattered light can be used to obtain information on cell granularity, size, and morphology. Intracellular components that fluoresce [e.g., nicotinamide adenosine dinucleotide phosphate (NADP)], or those stained with specific fluorescent dyes, allow for certain cell components to be selectively assayed. The fluorescent emissions are processed through photomultiplier tubes in the data-processing system, and software analyzes the resulting data. These measurements can be correlated with different cell characteristics and cell components, thus obtaining distributions of cell populations (i.e. segregated data) (Rieseberg et al. 2001).

The advantages of flow cytometry over immunohistochemistry are the ability to objectively and rapidly analyze cellular properties (DNA content, cell cycle position, or ploidy). In cell cycle analysis, flow cytometry can be used to determine the kinetics of progression through the phases of the cycle or to visualize the distribution of cells in a particular phase (Darzynkiewicz et al. 2001). Cells in the resting phase (G_0) and the G_1 phase have a DNA content that can be set equal to a relative unit typically (1.0) or expressed as a 2C or diploid ("C" refers to DNA mass; "N" would refer to chromosome number). Cells in the S-phase are synthesizing DNA that result in an increase of DNA content proportional to the progression through synthesis. In the G_2 phase, and later, mitosis, the cell has twice the amount of DNA content (or 4C) as in the resting phase (G_0) and the G_1 phase (Martinez et al. 1990). To determine the distribution of cells in a particular phase, or to quantify relative cellular DNA content, cells must be fixed or permeabilized with a detergent, and stained with a fluorescent dye. Dyes are classified into two groups: (1) those that intercalate between base pairs of DNA and RNA (e.g.,

ethidium bromide, propidium iodide, and acridine orange), and (2) those that are base-specific, binding to guanine-cytosine (e.g., mithramycine and chromomycin) or adenine-thymidine moieties (e.g., 4'-6-diamidio-2-phenylindole (DAPI) and Hoechst dyes). These dyes bind stoichiometrically to the DNA of each cell. Use of dyes from either group requires that samples be incubated with ribonuclease (RNAse) during staining to remove fluorescence caused by RNA-dye complexes so that the amount of fluorescence is proportional to the amount of DNA in each cell (Martinez et al. 1990). Thus, as the DNA content in the cells increases, so does the quantity of the dye bound, thereby increasing the fluorescent signal. The fluorescence from cells in suspension can be rapidly integrated and displayed as a DNA frequency histogram. These histograms show the proportions of cells in the respective phases of the cycle based on differences in fluorescence intensities (Fig. 1.3).

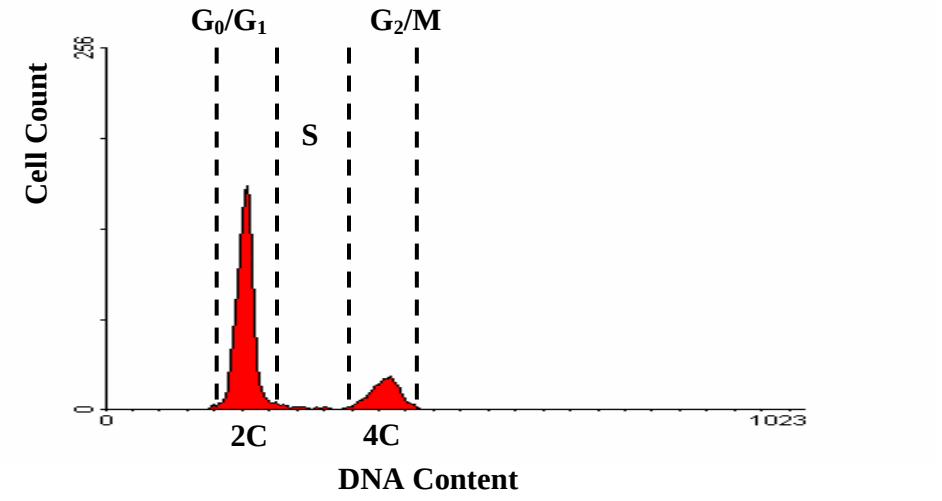

Figure 1.3. Cell cycle distribution of oyster labial palp nuclei that were labeled with propidium iodide as determined by flow cytometry. The Y-axis represents the cell or nuclei numbers and the X-axis represents the fluorescence intensity that translates to the quantity of DNA per cell. The leftmost peak represents cells at the G_1 phase and G_0 phase with 2C amounts of DNA, the rightmost peak represents cells in G_2 phase and M-phase with 4C amount of DNA, and the cells in between are in the S phase undergoing DNA synthesis with amounts of DNA between 2C and 4C.

In cell proliferation analysis, flow cytometry has been used to detect the fraction of cells that are proliferating *in vitro* (Kurki et al. 1986; Sasaki et al. 1993; Lohr et al. 1995). Proliferating cell nuclear antigen in human cells has been successfully detected by flow cytometry (Kurki et. al 1986; Kurki et al. 1988; Landberg and Roos 1991; Casasco et al. 1993; Beppu et al. 1994; Erlanson et al. 1997). The anti-PCNA monoclonal antibody (PC10) has been used as an S-phase probe in human cell lines and was determined to be an S-phase marker (Landberg and Roos 1991). The percent of PC10-positive cells was significantly correlated with cells that were in the S-phase of the cell cycle, as determined by DNA histograms (Erlanson et al. 1997). The analysis of PCNA expression and cellular DNA content showed that S-phase expression and the fraction of PC10-positive cells could be determined (Landberg and Roos 1991). A study of HeLa cells found that if cells were treated with a detergent (e.g. Triton X-100) to release nuclei and stained with anti-PCNA monoclonal antibody (PC10) it was possible to rapidly analyze the cell cycle without the need to label the cells with DNA precursors such as BrdU and ^{3}H-thymidine (Sasaki et al. 1993).

Thus with the technology available, development of an oyster cell-culture system could be possible. This would be an essential step in addressing questions and problems related to oyster physiology and pathology. There are several reports of successful short-term oyster and molluscan cell cultures, but there are fewer reports of secondary cell cultures, and there has been little standardization between laboratories (Mulcahy 2001) (Table 1.3).

Table 1.3. Primary cell cultures and cell lines of oysters and other mollusca species.

Molluscan species	Common name	Type of cultured cells	Survival time	Reference
Crassostrea virginica	eastern oyster	Mantel cells	42 days	Perkins and Menzel 1964
Crassostrea virginica	eastern oyster	Ventricles cells	10.5 months	Rosenfield 1965
Crassostrea virginica	eastern oyster	Cardiac cells	2-14 days	Li et al. 1966
Crassostrea virginica	eastern oyster	Amoebocytes	70 days	Tripp et al. 1966
Spisula solidissima	surf clams	Heart cells	2-120 days	Cecil 1969
*Biomphalaria glabrata**	freshwater snail	Trochophore larvae	>50 days	Hansen 1976
Crassostrea virginica	eastern oyster	Amoebocytes	180 days	Brewster and Nicholson 1979
Crassostrea virginica	eastern oyster	Heart, mantle, and gonad cells	7-21 days	Hetrick et al. 1981
Ruditapes decussatus	butterfish clam	Gill cells	45 days	Auzoux et al. 1993
Crassostrea gigas	Pacific oyster	Heart cells	10 days	Le Deuff et al. 1994
Mya arenaria	soft-shell clam	Heart cells	42 days	Kleinschuster et al. 1996, 1997
Nordotis discus discus	Japanese black abalone	Hemocytes	30 days	Nagai et al. 1998

* The *Bge* cell line.

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CHAPTER 2 – DEVELOPMENT OF A FLOW CYTOMETRIC ASSAY TO DETECT PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) IN OYSTER SOMATIC TISSUES

INTRODUCTION

An established cell line derived from the eastern oyster (*Crassostrea virginica*) would provide a valuable tool for pathology, molecular biology, endocrinology, cell kinetics, and cell physiology studies. Successful short-term bivalve cell cultures have been reported, including primary cultures of gill cells of the hard clam (*Meretrix lusoria*), heart cells from the Pacific oyster (*Crassostrea gigas*), and embryoic cells of the Japanese scallop (*Mizuchopecten yessoensis)* (Chen et al. 1989; Odintsova and Khomenko 1991; Le Deuff et al. 1994). *In vitro* studies of primary cell cultures from bivalve mollusks are limited by the low number of proliferating cells (Lowe and Pipe 1994; Giard et al. 1998). Effective efforts to stimulate cell division in oysters and other bivalve primary cultures have used vertebrate growth-promoting factors, but continuous mitotic activity has not been reported (Giard et al. 1998).

A crucial aspect in the establishiment of any cell line is monitoring the proliferation rate of the cells in culture (Doyle et al. 1993). Cell proliferation is an essential biological process for normal tissue growth and for the repair of damaged tissues due to pathogenic agents (Hillman 1963; Nelson and Morton 1979; Mix 1971; Awaji and Suzuki 1995; Ortego et al. 1995). Cell proliferation and mitotic activity is most commonly measured by DNA synthesis. Evidence of cell proliferation in the great scallop (*Pecten maximus*) (Le Marrec-Croq et al. 1999) and Pacific oyster (Cornet 2000) has been shown by using a combination of colchicine with Giemsa staining, and [^{3}H]-thymidine and 5-bromo-deoxyuridine (BrdU) for dynamic staining. Although this latter technique was successful, it was technically demanding and required live tissues.

Identification of nuclear proteins involved in cell proliferation helps to understand growth regulation and cellular transformation. Proliferating cell nuclear antigen (PCNA), also known as cyclin (Bravo and Celis 1980; Bravo et al. 1984; Takasaki et al. 1984), is a nuclear auxiliary protein of DNA polymerase δ (Tan et al. 1986; Bravo et al. 1987), and essential for DNA synthesis and repair, and is found at different concentrations during the cell cycle (Leonardi et al. 1992; Bravo 1986). A study of mammalian tissues found two different PCNA populations in different cellular locations (Landberg and Roos 1991). One was found in the nucleus tightly bound to intracellular structures and is closely associated with DNA replication, while the other was found in the nucleus and cytoplasm, where it is loosely bound to nuclear structures and is believed to assist in DNA repair (Landberg and Roos 1991; Erlanson 1997; Beppu 1994).

Monoclonal antibodies against PCNA have been developed for mammalian applications and used in immunohistochemical and flow cytometric assays (Kurki et. al. 1988; Robbins et. al 1987; Garcia et. al. 1989). Immunohistochemical assays have been adapted to identify proliferating cells in vertebrate aquatic organisms such as winter flounder (*Pleuronectes americanus*), English sole (*Pleuronectes vetulus*), mummichog (*Fundulus heteroclitus*) medaka (*Oryzias latipes*), guppy (*Poecilia reticulata*), and western mosquitofish (*Gambusia affinis*) (Ortego et al. 1994; Ortego et al. 1995). The immunohistochemical assay has also been adapted for invertebrate marine organisms such as the eastern oyster (*Crassostrea virginica*) (Nguyen et al. Department of Veterinary Sciences) and common marine mussel (*Mytilus galloprovincialis*) (Marigomez et al. 1999). Although this technique is effective for paraffin-embedded tissues, it is time consuming and expensive. A flow cytometric assay to detect the

monoclonal antibody anti-PCNA has not been developed for vertebrate or invertebrate aquatic organisms. This assay would provide a detailed, rapid, and accurate analysis of growth kinetics of cycling cells; it could be adapted to analyze cells from paraffin-embedded tissues.

Intracellular antigen detection by flow cytometry is dependent on optimal membrane permeabilization and fixation methods due to variable antigen characterization (Landberg and Roos 1992; Beppu et al. 1994). In this report, we discuss the development of a flow cytometric assay to detect PCNA in oyster somatic tissues. The objectives of this study were to: (1) optimize cell fixation, (2) optimize cell permeabilization, and (3) compare between flow cytometric analysis and immunochistochemical staining for the detection of PCNA. We were able to detect PCNA and found that the fluorescence was 13-times higher in S phase than in G_0/G_1 or in G_2/M phase of the cell cycle in heart cells. We determined that a paraformaldehyde fixation method could be used for fresh heart and labial palp tissues, and that flow cytometry was more sensitive in detecting PCNA than the immunohistochemical method.

MATERIALS AND METHODS

Oyster Collection

Eastern oysters were collected in August 2003 from the Louisiana Sea Grant Oyster Hatchery at Grand Isle, Louisiana, (90° 2’ 13.3” W, 29° 12’ 26” N) and were maintained for 10 weeks in an indoor temperature-controlled recirculating system until use. The oysters were kept at a water temperature of 27°C with a salinity of 18 parts per thousand. Cross sections (4-5mm) of the labial palps and the junction posterior to the labial palps and gills were collected and placed in embedding cassettes, and fixed in

4% formalin [36% concentrated formaldehyde diluted in sterilized artificial seawater (HW marine mix, Wiegandt GmbH Co., Krefeld, Germany)] for 24 hr. The salinity sterile artificial seawater was adjusted according to the water salinity of the indoor temperature-controlled recirculating system.

Cell Preparation

Oysters were opened and the heart and labial palps were surgically removed. For the analysis by flow cytometry, the hearts and labial palps were crushed using a homogenizer with a glass pestle. The cells were suspended in 0.01M-phosphate buffered saline (PBS) (1.37 M NaCl, 0.03 M KCl, 0.009 M KH_2PO_4, 0.06 M Na2HPO_4, pH 7.4) and filtered through a 35-μm mesh to remove connective tissue. Samples were placed into 1.5-mL microcentrifuge tubes at a concentration of 2 X 10^6 cell/mL and placed on ice. These samples were used to optimize a permeabilization protocol. After an optimum permeabilization method was determined; it was used to test different fixation methods.

Permeabilization

Cells were centrifuged at 400-X g for 10 min at 4ºC, and the supernatant was discarded. The cell pellet were resuspended in 750 μL of lysing buffer, modified from Langdberg and Roos (1991) containing 2% bovine serum albumin (Sigma) in PBS, 1% of the non-ionic detergent NP – 40 (Sigma), and 0.2 μg/mL of ethylenediamine tetra-acetic acid (EDTA) (Fisher Scientific Company, Pittsburgh, PA) for 15 min on ice. The lysed cells were centrifuged at 400-X g for 10 min at 4ºC. The lysis buffer was discarded and the pellet was washed twice with PBS by centrifugation. The nuclei were resuspended in 200 μL of PBS.

Fixations

Nuclei were centrifuged at 400-X g for 10 min at 4ºC. The supernatant was discarded and the nuclei resuspended using four different fixation methods: (1) 100% methanol for 10 min at -20ºC, (2) 70% ethanol for 10 min at -20ºC, (3) 70% acetone for 10 min at -20ºC, followed by washing in PBS with 70% methanol for 15 min at -20ºC (70% acetone and 70% methanol), and (4) 2% paraformaldehyde for 15 min on ice.

Monoclonal Antibody and Staining

Nuclei were centrifuged at 400-X g for 10 min at 4ºC. The fixative was discarded and the pellet was washed twice with PBS. The nuclei were resuspended in 200 µL of PBS. Fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-PCNA (PC10) (Sigma Chemical Co., St. Louis, MO) was added to the nuclei at a dilution of 1:40 (µL: µL) and the samples were held in the dark for 45 min at room temperature. The nuclei were resuspended with 200 µL of a DNA-staining solution containing 10 µg/mL propidium iodide (PI) and 1.8 Kunits/mL RNase (Sigma) and held in the dark for 30 min at room temperature until flow cytometric analysis.

Flow Cytometry

The nuclei were analyzed using a FACSCalibur flow cytometer equipped with an argon-ion laser (488 nm) (Becton-Dickinson Immunocytometry Systems, San Jose, CA) and data from at least 10^4 cells were stored in list mode. Doublets and debris were discriminated by gating in a doublet discrimination mode (DDM) using a dot plot (FL-2A vs. FL-2W). Fluorescence from FITC was detected in the FL-1 channel using a 560-nm beam splitter with a 510 to 540-nm bandpass filter and stored as a logarithmic amplification. Fluorescence from PI staining of DNA was detected in the FL-2A channel

using a 610-nm long pass filter and stored as a linear amplification. Data were analyzed using FACSCalibur software (BD CellQuest™). Permeabilization was analyzed on a dual-parameter dot plot (PCNA fluorescence vs. DNA content) (Fig. 2.1).

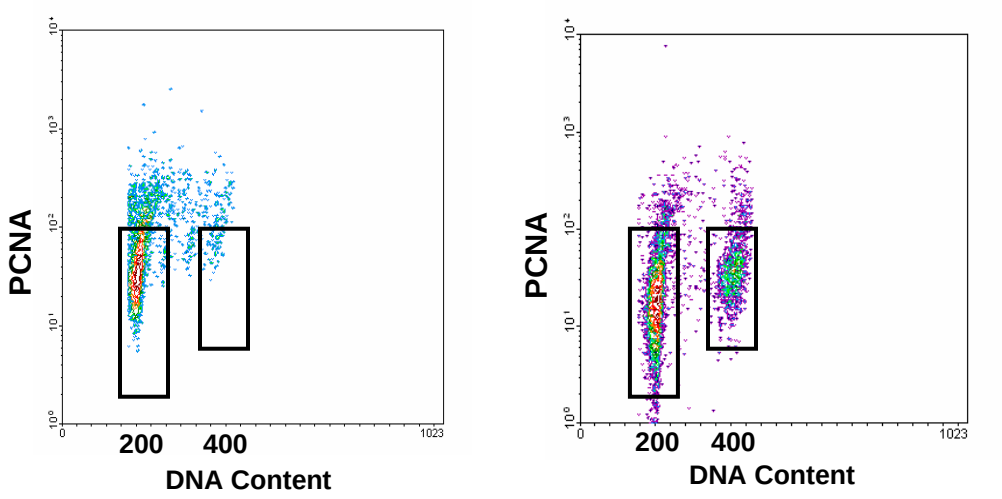

Figure 2.1. Dual-parameter contour plot (PCNA fluorescence vs. DNA content) of heart cells of eastern oysters. Showing the difference between the distributions of lysed and unlysed cells. (A) Contour plot of heart cells fixed with 2% paraformaldehyde but not permeabilized and lysed with a lysing buffer. There was a single distinguishable population of cells (left box). (B) Contour plot of heart cells fixed with 2% para-formaldehyde, and permeabilized and lysed with lysing buffer. There were two distinguishable populations of cells (boxes). The Y-axis (PCNA fluorescence) was recorded in logarithmic scale and the X-axis (DNA content) was recorded in linear scale.

Analysis of the effects of fixation methods were analyzed using DNA histograms that were divided into three regions (Martinez et al. 1990): (1) Resting /Gap 1 phase (G_0/G_1), (2) Synthesis phase (S), and (3) Gap 2/Mitosis (G_2/M); and the coefficient of variance for the G_0 phase/G_1 phase region was calculated (Fig. 2.2). The CV of the G_0/G_1 region was used to optimize fixation because the G_0/G_1 peak was prominent, with a relatively low coefficient of variance, and a low S-phase fraction (Dean 1985).

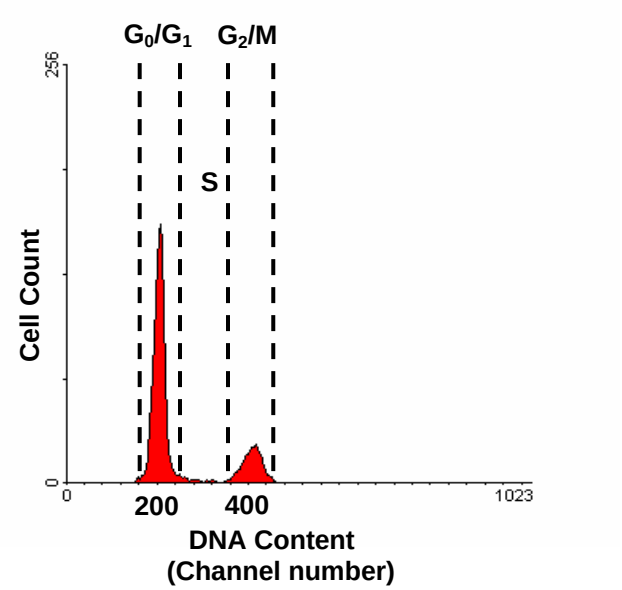

Figure 2.2. Typical flow cytometric histogram of heart nuclei from eastern oysters. The leftmost peak represents the G_0/G_1 cells with a 2C resting diploid amount of DNA, the rightmost peak represents cells in G_2/M phase with, 4C amount of DNA. The nuclei between the peaks are in the S phase, undergoing DNA synthesis, and have amounts of DNA between 2C and 4C. The coefficient of variance of the G_0/G_1 peak was calculated. The Y-axis represents the cell numbers and the X-axis represents the propidium iodide fluorescence intensity that translates to the quantity of DNA per cell (channel number is the measured value of a parameter, representing the signal intensity of an event after amplification).

The distribution of cells labeled with PCNA was analyzed on a dual-parameter dot plot (PCNA fluorescence vs. DNA content) by gating on the regions described above. The lower limit of the PCNA-positive cell gate was set close to the upper border of the labeled cells G_2/M (log 10^2) (Fig. 2.3). The ratio of fluorescence increase of PCNA during the cell cycle was calculated (geometric mean of the S-phase PCNA-labeled cells by the geometric mean of the G_0/G_1-phase PCNA-labeled cells) from the gated dual-parameter dot plot (PCNA fluorescence vs. DNA content).

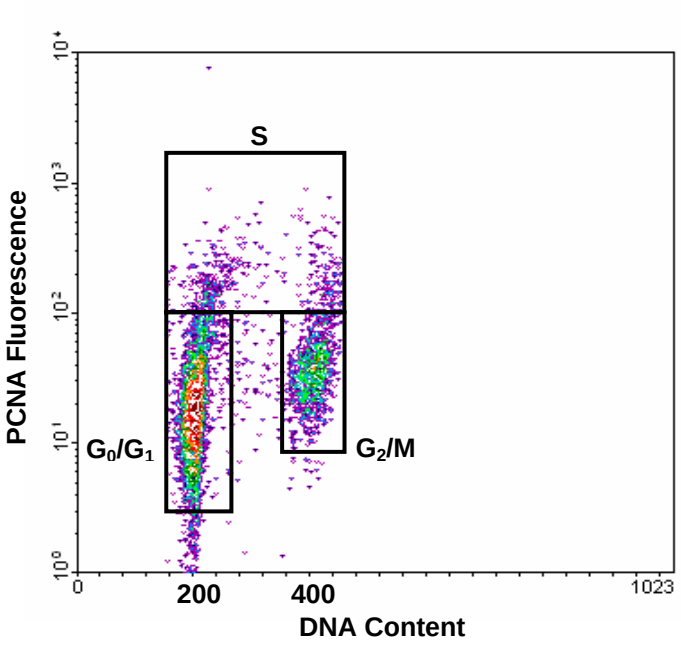

Figure 2.3. Dual-parameter contour plot (PCNA fluorescence vs. DNA content) of heart cells from eastern oysters used to determine cell cycle phases and PCNA-positive cells. Three regions were gated: Gap 0/Gap 1 (G_0/G_1), Synthesis (S), Gap 2/Mitosis (G_2/M) to determine PCNA distribution in the cell cycle. The Y-axis (PCNA fluorescence) was recorded in logarithmic scale, and the X-axis (DNA content) was recorded in linear scale. The region labeled as "S-phase" contained PCNA-positive cells.

Histopathology Processing

The fixed specimens were sent to the Louisiana State University School of Veterinary Medicine (LSU-SVM) Department of Histopathology for paraffin embedment. The embedded tissues were sectioned (3 μm thick) and mounted on poly-L-lysine-coated glass slides.

Immunohistochemistry

The presence of PCNA in the embedded oyster tissues was detected by the modified version of the PCNA Staining Kit procedure (Zymed Laboratories Inc. San Francisco, CA). This assay was adapted from Nguyen et al. (Department of Veterinary Sciences). The PCNA assay was conducted at room temperature. The slides were

deparaffinized in two changes of 96% xylenes (Fisher) for 5 min each and rehydrated in one rinse of 100% ethanol and two rinse of 95% ethanol for 2 min each. Endogenous peroxidase was blocked by incubating the slides in 3% hydrogen peroxide solution [30% hydrogen peroxide (Fisher) and 100% methanol (Fisher)] for 10 min. The slides were rinsed in three changes of phosphate buffered saline (PBS) for 2 min each to remove the hydrogen peroxide solution. The PBS (1.37 M NaCl, 0.03 M KCl, 0.009 M KH_2PO_4, 0.06 M Na_2HPO_4) was prepared at 10-X and diluted with sterilized distilled deionized water to a 1-X concentration. To reveal epitopes masked during fixation and to facilitate antigen retrieval, the slides were immersed in a 1% anhydrous zinc sulfate (Fisher) solution, and were heated in a microwave until the solution boiled for 1 min, cooled for 2 min, and finally heated again until boiling for 1 min. The slides were cooled for 6 min at room temperature, rinsed twice in distilled dionized water (ddH_2O) and once in PBS. After the PBS rinse, the blocking solution (Reagent 1) was applied to the slides for 10 min to block charged sites on proteins. The primary antibody, biotinylated mouse anti-PCNA (Reagent 2), was added to the slides. The negative control slides received the mouse isotype control solution. All slides were incubated for 1 h and were rinsed in three changes of PBS for 2 min each. The slides were incubated for 10 min with streptavidin-peroxidase (Reagent 3), and rinsed in three changes of PBS for 2 min each. Diaminobenzidine (DAB) solution (Reagents 4A-4C), the chromagen substrate, was applied to the slides for 2-5 min after the streptavidin-peroxidase reaction to biotin. The slides were rinsed with ddH_2O, counterstained with hematoxylin for 1-2 min, rinsed in tap water, washed in PBS for 30 sec or until the tissue sections turned blue, rinsed again

in ddH_2O, dehydrated by rinsing twice in 95% ethanol for 5 min each, and twice in 100% ethanol for 15 min each, and finally the tissues were overlayed.

Label Quantitation

To determine the PCNA labeling indices (LI) in the labial palps of each oyster the procedure of Foley et al. (1991) was used. Hearts were not used because the organ could not be sectioned. Positive staining by PCNA in the tissues was indicated by the occurrence of a dark brown nucleus (Fig. 2.4). A negative control was used to determine which nuclei were well stained and therefore were counted. For the labial palps, an image analysis system (Meta Imaging Series 6.1, MetaVue™, Universal Imaging Corp., Downington, PA) was used to count 1000 nuclei at random at 40-X magnification. The PCNA labeling indices were expressed as labeled nuclei per total nuclei counted.

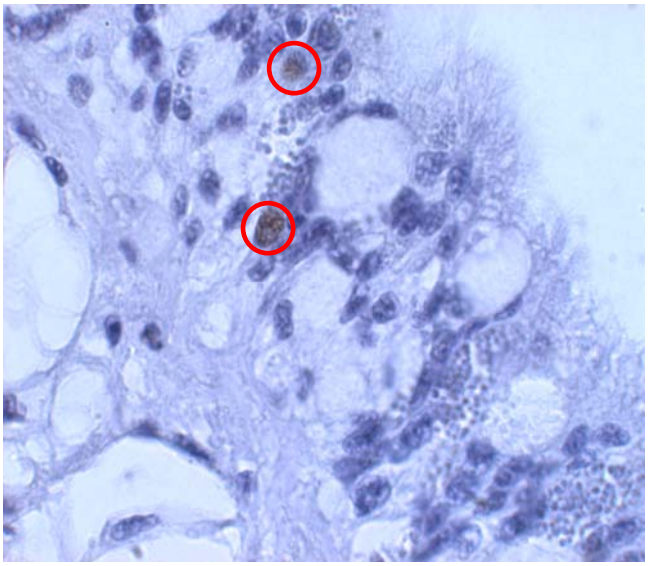

Figure 2.4. Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) in eastern oyster labial palps (brown nuclei).

Flow Cytometry vs. Immunohistochemistry

Of 110 oysters analyzed by flow cytometry, 10 were classified as having a high percentage of PCNA-positive cells (24.87 ± 4.86%) and another 10 oysters were classified as a low percentage of PCNA-positive cells (6.87 ± 1.41%) and were analyzed

by an image analysis system. The embedded tissues for these oysters were sent without reference to their PCNA status LSU-SVM for histopathology processing. At the Department of Histopathology the original labels were coded to ensure non-biased analysis. We compared the percent PCNA positive cells detected by flow cytomery analysis against the PCNA LI determined by immunohistochemical staining for labial palps.

Statistical Analysis

Kruskal-Wallis nonparametric one-way analysis of variance (ANOVA) was performed to compare the coefficient of variance (CV) of the G_0/G_1 regions among the four different fixation methods. Chi-square goodness-of-fit was used to determine if the distribution of PCNA during the cell cycle was homogeneous to the ratio of distribution of cells in the cell cycle. We used observed values for PCNA that were obtained from the gated dual-parameter dot plot (PCNA vs. DNA content) and the ratio of distribution of cells during the cell cycle was calculated using DNA histograms. Kruskal-Wallis nonparametric ANOVA was performed to compare the difference in PCNA labeling indices of "high" against "low" cell proliferation for the immunohistochemistry slides. One-way analysis of variance (ANOVA) was performed to compare the %PCNA positive cells detected by flow cytomery analysis against the PCNA LI by immunohistochemical staining.

RESULTS

The dual-parameter analysis of PCNA fluorescence vs. DNA content dot plot of cells treated with the lysing buffer revealed a reverse "U" shape. Two distinct

populations were present for lysed cells. Some S-phase nuclei labeled with PCNA were not PCNA-positive (Fig. 2.5).

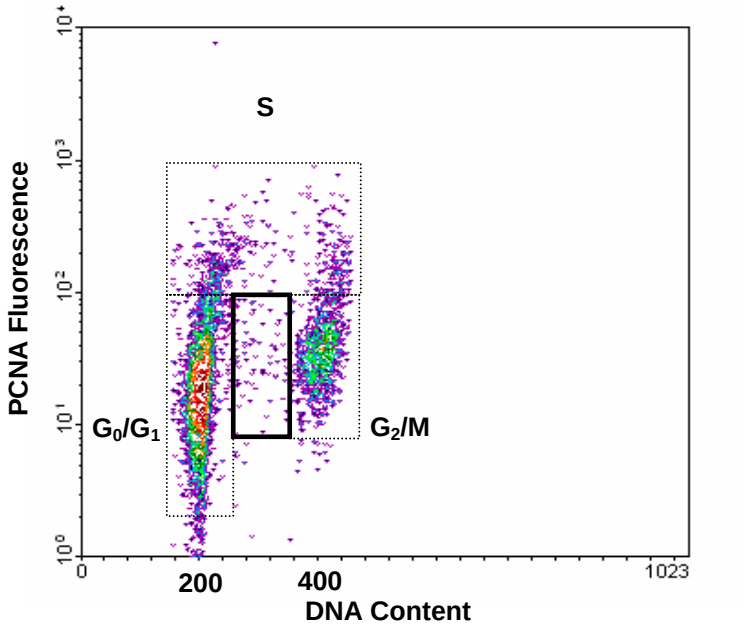

Figure 2.5. Dual-parameter contour plot (PCNA fluorescence vs. DNA content) of heart cells from eastern oysters. The solid box area represents PCNA stained cells that were recognized in the S phase of the DNA content histogram but were not considered to be PCNA-positive cells. The dotted boxes represent the phases of the cell cycle. The Y-axis (PCNA fluorescence) was recorded in logarithmic scale, and the X-axis (DNA content) was recorded in linear scale.

The mean coefficient of variance (CV) of the G_0/G_1 peak for the cells fixed with 70% acetone and 70% methanol was 6%, cells fixed with 100% methanol had a mean CV of 6%, cells fixed with 2% paraformaldehyde had a mean CV of 5%, and the cells fixed with 70% ethanol had a mean CV of 10%. There were no significant differences among the CV's of 70% acetone and 70% methanol, 100% methanol, and 2% paraformaldehyde. The CV of the 70% ethanol fixation method was significantly higher than those of other methods ($P < 0.0001$). The patterns of the flow cytometric dual-parameter dot plots (PCNA fluorescence vs. DNA content) were not different

among the fixation methods. However, methanol and paraformaldehyde fixation yielded brighter PCNA fluorescence in the S phase than did 70% acetone and 70% methanol, and 100% ethanol (Fig. 2.6).

Proliferating cell antigen was expressed throughout the cell cycle in mitotic cells. The PCNA-labeled heart nuclei and labial palp nuclei were not distributed based on the observed frequency of nuclei in the various stages of the cell cycle ($P < 0.0001$). Fourteen percent of the total PCNA-labeled heart nuclei counts were considered to be PCNA positive. Of the PCNA-positive nuclei, 30% were in the S phase while 45% were in late G_0/G_1, and 25% were in early G_2 phase/M phase (Fig. 2.7).

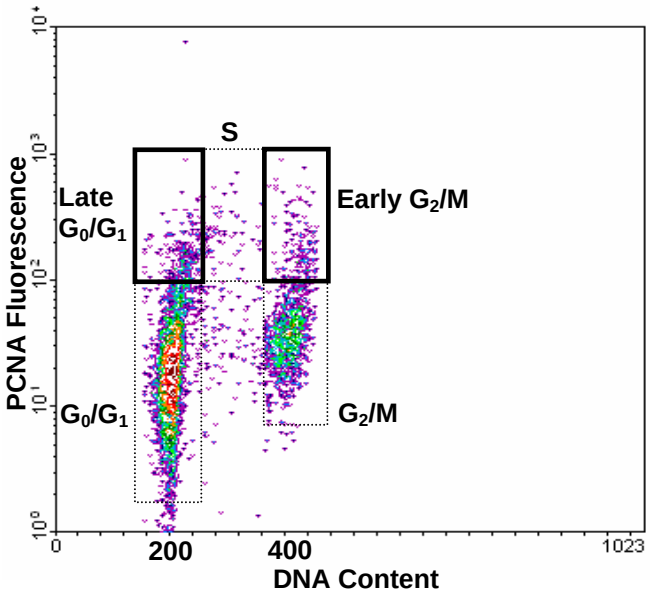

Figure 2.7. Dual-parameter contour plot (PCNA fluorescence vs. DNA content) of heart cells from eastern oysters. The solid boxes area represents PCNA stained cells that were recognized in the late G_0/G_1 and early G_2/M. The dotted boxes represent the phases of the cell cycle. The Y-axis (PCNA fluorescence) was recorded in logarithmic scale, and the X-axis (DNA content) was recorded in linear scale.

In labial palp nuclei, fourteen percent of total PCNA-labeled cells were considered to be PCNA positive. Of the PCNA-positive nuclei, 30% were in S phase while 46% were in late G_0/G_1, and 24% were in early G_2/M phase. There was no significant difference in the distribution of the S phase nuclei of the two tissues.

During the cell cycle in heart nuclei PCNA mean fluorescence increased about 13 times from the G_0/G_1 to S phase. The PCNA fluorescence decreased by half in G_2/M

phase. For labial palp nuclei, the mean fluorescence of PCNA increased about 9 times from G_0/G_1 to S phase. The PCNA fluorescence decreased by half in G_2/M phase (Table 2.1). The mean fluorescence of PCNA-positive heart nuclei in S phase was significantly higher than those of the labial palps ($P < 0.0002$).

Table 2.1. The geometric means (means ± SD) from the cell cycle phases were used to calculate the proliferating cell nuclear antigen fluorescence ratio of increase (means ± SD). There was an increase in the ratio from G_1/G_0 phase to S phase and decrease by half into the G_2/M phase for hearts and labial palps of the eastern oyster analyzed by flow cytometry.

	Geometric Mean		
Tissue	G_0/G_1	S	G_2/M
Heart	28.88 ± 8.63	355.27 ± 79.32	57.72 ± 18.08
Labial palps	27.56 ± 6.96	233.60 ± 59.15	49.46 ± 11.40

Flow cytometry was significantly more sensitive than immunohistochemistry in detecting PCNA-positive nuclei ($P < 0.0001$). When comparing flow cytometry and immunohistochemistry, there was a significant difference between the "high" and "low" PCNA-positive nuclei analyzed by flow cytometry ($P < 0.0001$). There was no significant difference detected in the labeling indices between the "high" (0.71%) and "low" (0.79%) immunohistochemically stained labial palp samples.

DISCUSSION

The development of a flow cytometric assay to detect cell proliferation in somatic tissues of the eastern oyster would provide rapid and accurate information that could assist in maximizing cell survival and promote the development of an oyster cell line. A primary consideration in cell growth is the cell cycle, the analysis of which can provide a reliable index for the prediction of growth potential and changes in cell number with time during cell culture(Leelavatcharamas et al. 1996).

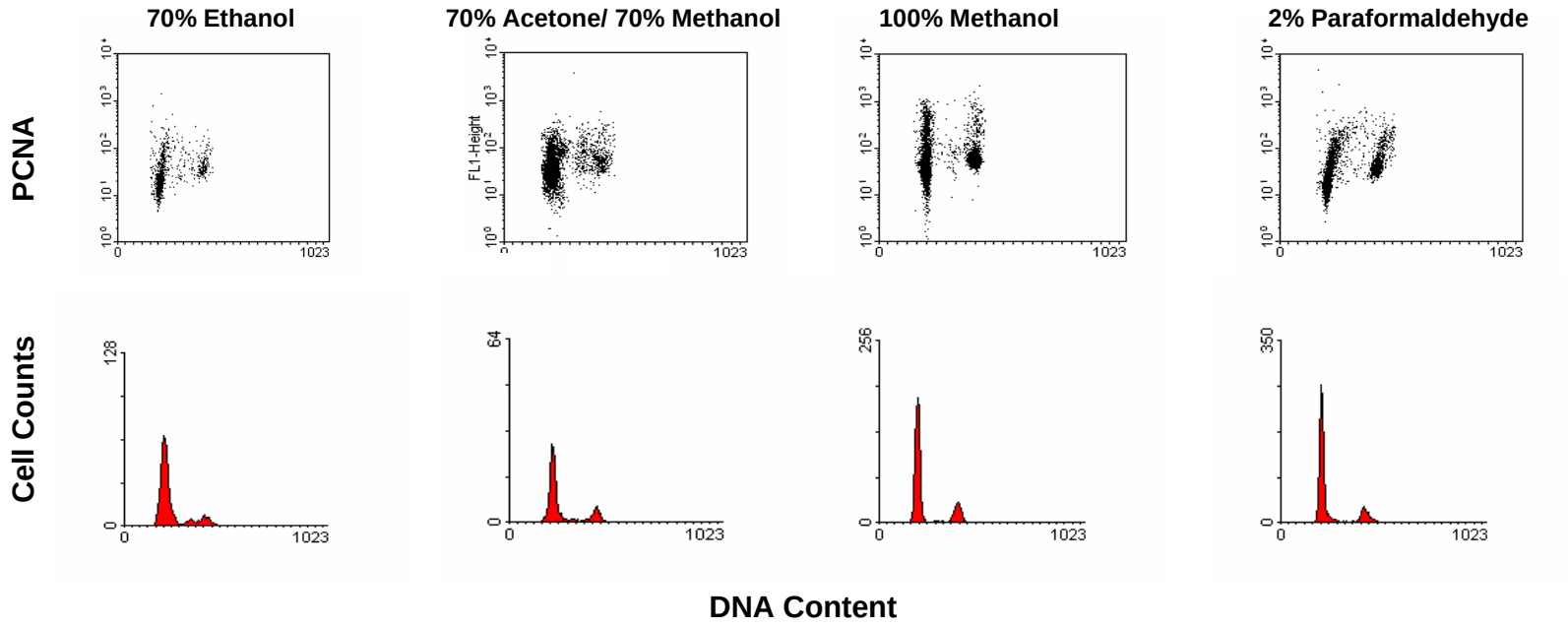

Figure 2.6. Dual-parameter dot plots of heart cells of the eastern oyster prepared using different fixation methods (Top). The Y-axis represents the monoclonal antibody (PC10) and the X-axis represents the propidium iodide fluorescence intensity that is directly proportional to the quantity of DNA per cell (channel number is the measured value of a parameter, representing the signal intensity of an event after amplification) (Bottom). DNA histograms for each fixation procedures are shown under the dual-parameter dot plot. The Y-axis (PCNA fluorescence) was recorded in logarithmic scale and the X-axis (DNA content) was on a linear scale. The Y and X axes of the DNA histograms were linear scales.

The analysis of the fraction of S-phase cells can provide reliable information on the proliferating dynamics of cell cultures. By measuring the proportion of S-phase cells, it was possible to predict the change in cell number of hybridoma cells following a disturbance of the culture conditions (Al-Rubeai et al. 1991). In Chinese hamster ovary cultures, it was found that the proportion of cells at any time in the S-phase could indicate future specific growth rates (Leelavatcharamas et al.1996).

In the present study, PCNA was detected in all the phases of the cell cycle of the eastern oyster. The concentrations of PCNA also increased from G_0/G_1 phase into the S phase, where they peaked in mid-S-phase, and decreased in G_2 phase/M. Proliferating cell nuclear antigen is found throughout the cell cycle of mammalian somatic tissues (Bravo and Macdonald-Bravo 1987; Toschi and Bravo 1988). In mouse fibroblasts and splenocytes, HeLa cells, and SP2/0 and MOLT-4 cell lines the concentration of PCNA increased as the cells progressed from G_0/G_1 phase, where it peaked in S phase, and decreased in G_2 phase/M (Bravo 1986; Morris and Mathews 1989; Kurki et. al. 1986).

In this study, oyster somatic tissue cells that were treated with a lysing buffer containing a non-ionic detergent showed three distinguishable regions that were verified using a DNA histogram to correspond to the cell cycle phases. Oyster cells not lysed and not permeabilized did not show these regions. Use of a lysing buffer containing 0.1% to 1% (in PBS) of a non-ionic detergent (e.g. Nonidet P-40 or Triton X-100), will cause the plasma membrane of cells to lyse and will permeabilize the nuclear membrane (Taylor 1980; Taylor and Milthorpe 1980; Thornthwait et al. 1980), allowing for the removal of loosely bound PCNA and an accurate measure of the S-phase cells (Landberg and Roos 1991). Dual-parameter dot plots (PCNA fluorescence vs. DNA content) of lysed

mammalian cells show distinguishable regions of the individual cell cycle phases, while unlysed cells had no defined regions of the individual cell cycle phases (Erlanson 1997).

In this study, oyster somatic cells had S-phase cells that were stained with PCNA, but were not considered to be PCNA-positive. Although these cells were included in the S-phase fraction of the DNA content histogram, these may have been quiescent S-phase cells or cellular debris. In a study of malignant lymphomas cells with S-phase DNA content but negative for the monoclonal antibody (PC10) (Landberg and Roos 1991) these cells were found to be quiescent S-phase cells, aneuploid non-S-phase cells, or debris. This problem was also encountered in material from solid tumors and especially with tumors containing large amounts of debris (Landberg and Roos 1991).

Cells were fixed to allow highly polar fluorochromes to gain access to the interior, and to polymerize, precipitate, cross-link, and denature proteins. Fixatives have been described as coagulant (e.g., acetone, ethanol, and methanol) or non-coagulant (e.g., acetic acid, formaldehyde, and paraformaldehyde) because of the observations of the behavior of plasma proteins to the fixatives. In this study methanol and paraformaldehyde fixation methods reacted similarly, but paraformaldehyde did not cause clumping of oyster somatic cells. Methanol (Elias-Jones et al. 1986; Levitt and King 1987) and paraformaldehyde (Clevenger et al. 1985) have been used for staining of intracellular antigens. Cells fixed using organic solvents caused PCNA staining to be localized at the intracellular sites where DNA synthesis was taking place (Bravo and Macdonald-Bravo 1987). The distribution of PCNA appears to change with the stage of the cell cycle (Waseem and Lane 1990). In early S-phase PCNA has a granular distribution and is absent from the nucleoli, while at late S-phase PCNA is found in the

nucleoli (Waseem and Lane 1990). Cells fixed with aldehydes showed PCNA to have a different distribution and intense nuclear staining throughout the cell cycle (Bravo 1986; Bravo and Macdonald-Bravo 1987). In previous studies of mammalian somatic tissues methanol fixation was essential for the detection of the anti-PCNA monoclonal antibody (PC10) (Kurki et. al. 1986; Hammond et. al. 1987, Ogata et. al. 1987; Landberg and Roos 1991; Casasco et al. 1993). While in other mammalian tissue studies, fixation of the cells with paraformaldehyde improved detection of the PCNA antibody (Kurki et al. 1988; Hall et al. 1990; Pollice et al.1992).

In this study, flow cytometry was more sensitive in detecting PCNA-positive nuclei than was immunohistochemistry. Flow cytometry has been shown to be more sensitive than immunohistochemistry in detecting antigens or epitopes present in low amounts (Villari et al. 1997). A cell proliferation study, using PCNA (PC10) as a prognostic indicator of human prostatic carcinomas, found no significant associations between flow cytometric and the immunohistochemical measures of cell proliferation (Visakorpi 1992). In a comparative study of flow cytometry and immunohistochemistry for the detection of human leukocyte antigens in human colorectal cancer, flow cytometry was superior to immunohistochemistry in detecting tumors identified as HLA-ABC negative by immunohistochemistry that were actually weakly positive for HLA-ABC in 33 out of 80 cases. Flow cytometry was able to detect low amounts of the antigens and epitopes (He et al. 1994; Diederichsen et al. 1998).

In the flow cytometric studies it was observed that in oyster somatic tissue cells anti-PCNA monoclonal antibody detected cells in the G_0/G_1, S and G_2/M phases. One advantage in estimating proliferating cells using anti-PCNA monoclonal antibody (PC10)

reactivity instead of DNA histograms is that it can provide an accurate value of S phase cells. In conclusion, we have shown that the anti-PCNA monoclonal antibody (PC10) is a suitable reagent for the flow cytometric evaluation of replicating (G_1, S and G_2/M phases) cells in somatic cells of the eastern oyster and that flow cytometry was more sensitive than immunohistochemistry in detecting proliferating cells.

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CHAPTER 3 – DEVELOPMENT OF A FLOW CYTOMETRIC ASSAY FOR THE DETECTION OF NUCLEAR RNA IN OYSTER SOMATIC TISSUES

INTRODUCTION

An oyster cell line would be a valuable tool for the study of pathology, toxicology, and endocrinology in oysters. An important aspect of cell culture is to monitor the growth rate of the cells (Doyle et al. 1993). Evaluation of cell growth kinetics can be done by flow cytometric measurement of RNA per cell. Flow cytometric measurement of DNA content allows for the determination of position of a cell in the cell cycle. Therefore, the simultaneous analysis of DNA and RNA content makes possible the correlation of changes in RNA content throughout the cell cycle.

In the process of transcription, the genetic information stored in DNA is copied into RNA, which has three distinct roles in protein synthesis. Messenger RNA (mRNA) specifies the order of amino acids and this information is interpreted in the cytoplasm by transfer RNA (tRNA) with the aid of ribosomal RNA (rRNA) and associated proteins. The tRNA brings the amino acids together for linkage through peptide bonds to make peptides (Lodish et al. 2000).

Ribonucleic acid in human lymphocytes was one of the first cellular constituents measured by flow cytometry (Darzynkiewicz et al. 1975; Darzynkiewicz et al. 1976). Initial flow cytometric studies of cellular RNA in human lymphocytes and 3T3-Swiss albino mouse cells assumed that because rRNA makes up about 80% of the cellular RNA (Table 3.1), the accumulation and regulation of rRNA could be used to examine cell kinetics.

Table 3.1. Cellular RNA content and the rate of cell progression of different cell types through various phases of the cell cycle. These data were published and recalculated for comparison purposes (Darzynkiewicz 1988). Unless otherwise indicated, all are human-derived cells (Adapted from Darzynkiewicz 1988).

Cell type	RNA Content	Cell cycle phase	Rate of progression	Reference
Lymphocytes	1.6	S	2.3	Darzynkiewicz et al. (1979a) Darzynkiewicz et al. (1979b)
Lymphocytes	2.4	S	3.8	Darzynkiewicz et al. (1979a) Darzynkiewicz et al. (1979b)
Lymphocytes	2.8	S	4.5	Darzynkiewicz et al. (1979a); Darzynkiewicz et al. (1979b)
CHO*	1.7	G_1	1.6	Darzynkiewicz et al. (1979a)
CHO	1.7	S	1.3	Darzynkiewicz et al. (1979a)
CHO	0.6	S	0.4	Traganos et al. (1987)
HeLa-S3	1.3	G_1	1.3	Traganos et al. (1987)
HeLa-S3	1.3	G_1, S, G_2/M	1.3	Traganos et al. (1987)
HeLa-S3	0.6	G_1, S, G_2/M	0.8	Bauer et al. (1981)
V79	1.5	S	1.3	Fujikawa-Yamamoto (1982)
Keratinocytes	3.0	G_1, S, G_2/M	3.3	Staiano-Coico et al. (1986); Kimmel et al. (1986)

*Chinese hamster ovary cells.

These studies found a correlation between rRNA content and the ability of the cell to exit from a quiescent state and enter the cell cycle (Darzynkiewicz et al. 1976; Darzynkiewicz et al. 1980). The rate of the cell cycle in human lymphocytes and Chinese hamster ovary (CHO) cells was also correlated with rRNA content (Darzynkiewicz et al. 1979a; Darzynkiewicz et al. 1979b). Exponentially growing cultured cell populations (i.e. HeLa cells), normal human cells, or tumor cells accumulate a critical amount or threshold of rRNA in the G_1 phase prior to entry into S phase (Darzynkiewicz et al. 1980).

In a flow cytometric study correlating measurements of nuclear RNA and DNA content of cultured human keratinocytes, these cells were found to enter S-phase only after all cells accumulated a threshold amount of nuclear RNA; therefore there were only

single populations of S-phase, G_2-phase, and M-phase cells. Cells with low nuclear RNA content had distinctly longer residence times in G_1 phase compared to cells with high nuclear RNA content, and cytoplasmic RNA content correlated more with differentiation while nuclear RNA content reflected primarily the kinetic properties (mechanisms that affect the rate of progression in the cell cycle) of the cell (Staiano-Coico et al. 1989). The many metabolic states related to the capacity of the cell to progress through the cell cycle can be discriminated by simultaneous measurement with an assessment of the ratio of nuclear RNA to DNA (Crissman et al. 1985). Quiescent and cycling cells, cells in different stages of differentiation, and the relation between cell growth and DNA synthesis, can be distinguished by a multiparametric analysis of RNA and DNA (Darzynkiewicz et al. 1980).

Although most of the past studies on the relationship between RNA content and cell kinetics have been done with cellular RNA, in this study development of a flow cytometric assay is reported that detects nuclear RNA in heart and labial palp tissues of the eastern oyster, *Crassostrea virginica*. Heart cells were used in this study because these cells have been found to proliferate *in vitro* for the Pacific oyster (Wen et al. 1994). Labial palps are four triangular appendages attached on either side of the mouth and are used to sort food particles and move trapped food from the gills into the mouth. Labial palps cells were used because the cells have been found to have high proliferation during the winter months (Nguyen et al. Department of Veterinary Sciences).

To my knowledge, a method has not been developed to measure DNA and nuclear RNA content simultaneously in oyster somatic tissue cells. In this study, the fluorescent dye SYBR Green II was used because of its high binding affinity for RNA, its

fluorescence enhancement and high quantum yield upon binding to RNA, and because nucleic acid-bound SYBR Green II exhibits spectral characteristics (excitation maximum ~ 497 nm) similar to those of fluorescein (excitation maximum 494 nm), making it compatible with the most common filter sets in laser-based gel scanners, confocal laser-scanning microscopy and flow cytometry (Haugland 2002). The objectives of this paper were to: (1) detect nuclear RNA by flow cytometry; (2) determine the distribution of nuclear RNA throughout the cell cycle; and (3) develop a method for the direct determination and correlation of DNA and nuclear RNA in individual oyster cells. Nuclear RNA was detected throughout the cell cycle which was tracked simultaneously using propidium iodide staining of DNA.

MATERIALS AND METHODS

Oyster Collection and Dissection

Eastern oysters (n = 30) were collected in January 2004 from the Louisiana Sea Grant Oyster Hatchery at Grand Isle, Louisiana, (90° 2' 13.3" W, 29° 12' 26" N) and were maintained for 2 d in an indoor temperature-controlled recirculating salt water (18 ppt) system at 15°C until use. The meats were shucked and the hearts and labial palps were surgically removed.

Permeabilization and Fixation

Hearts and labial palps were crushed using a glass-on-glass homogenizer. The cells were suspended in 0.01M-phosphate buffered saline (PBS) (1.37 M NaCl, 0.03 M KCl, 0.009 M KH_2PO_4, 0.06 M Na2HPO_4, pH 7.4) (Sigma Chemical Corp., St. Loius, MO) and filtered through 35-µm mesh to remove connective tissue. Heart and labial palp suspensions with a cell concentration of 2 X 10^6 cells/mL were collected and held at 4°C.

The cells were centrifuged at 400-X g (2800 rpm) for 10 min at 4ºC. The supernatant was discarded and the pellet was resuspended for 15 min at 4°C in 750 µL of lysing buffer to permeabilize and free the nucleus from the cytoplasm (modified from Langdberg and Roos 1991) containing 2% bovine serum albumin in PBS (Sigma), 1% NP-40 (Sigma), and 0.2 µg/mL of EDTA (Fisher Scientific Company, Pittsburgh, PA). Nuclei were centrifuged again at 400-X g for 10 min at 4ºC. The lysing buffer was discarded and the nuclei resuspended in 2% paraformaldehyde (Sigma) for 15 min on ice.

Nuclear RNA Staining and DNA Staining

Nuclei were centrifuged at 400- X g for 10 min at 4ºC. The fixative was discarded and the pellets were washed twice by centrifugation with PBS. The nuclei were resuspended in 200 µL of PBS, and SYBR Green II RNA gel stain (Molcular Probes, Inc., Eugene, OR) was added to the nuclei at a 1:2000 (µL:µL) concentration and the samples were incubated in the dark for 30 min at room temperature. The nuclei were resuspended with 200 µL of a DNA-staining solution containing 10-µg/mL propidium iodide (PI) (Sigma) in 10 mL of PBS and held in the dark for an additional 30 min at room temperature until flow cytometric analysis.

Flow cytometry Analysis of nuclear RNA and DNA

The nuclei were analyzed using a FACSCalibur flow cytometer equipped with an argon ion laser (488 nm) (Becton-Dickinson Immunocytometry Systems, San Jose, CA) and data from at least 10^4 nuclei were stored in list mode. Fluorescence from SYBR Green II was detected in the FL-1 channel using a 560-nm beam splitter with a 510 – 540-nm bandpass filter and stored as a logarithmic amplification. Fluorescence from PI staining of DNA was detected in the FL-2 channel using a 610-nm long pass filter and stored as a

linear amplification. Data were analyzed using FACS software (BD CellQuest™). Cell cycle DNA was analyzed using histograms that were divided into three regions: Gap 0/ Gap1 phase (G_0/G_1), Synthesis phase (S), and Gap 2/Mitosis (G_2/M) (Fig.3.1). Nuclear RNA distribution was analyzed on a dual-parameter dot plot (nuclear RNA fluorescence vs. DNA content) (Fig. 3.2). Damaged nuclei and debris were gated out from analysis on a plot of forward scatter versus side scatter, and aggregates were discriminated with doublet discrimination mode. Using this gated dual-parameter plot, nuclear RNA during S phase was compared with that in G_0/G_1 phase by generating ratios of geometric means FL-1H from these gated regions. Nuclear RNA/DNA ratio was calculated, using the gated histograms for the nuclear RNA fluorescence and DNA fluorescence, by dividing the geometric means of the nuclear RNA fluorescence (FL-1H) into the geometric means of the DNA fluorescence (FL-2H). The ratio was calculated for each phase in the cell cycle.

Statistical Analysis

Chi-square goodness-of-fit was used to determine if the distribution of nuclear RNA during the cell cycle was homogeneous with respect to the ratios of distribution of cells in the cell cycle. Observed values used for nuclear RNA were obtained from the gated dual-parameter dot plot (nuclear RNA fluorescence vs. DNA content) and the ratio of distribution of cells during the cell cycle was calculated using DNA histograms.

Kruskal-Wallis nonparametric analysis-of-variance (ANOVA) was used to determine differences between the nuclear RNA fluorescence increase from G_0/G_1 to S phase and the decrease of fluorescence from S phase to G_2/M of the heart and labial palp cells.

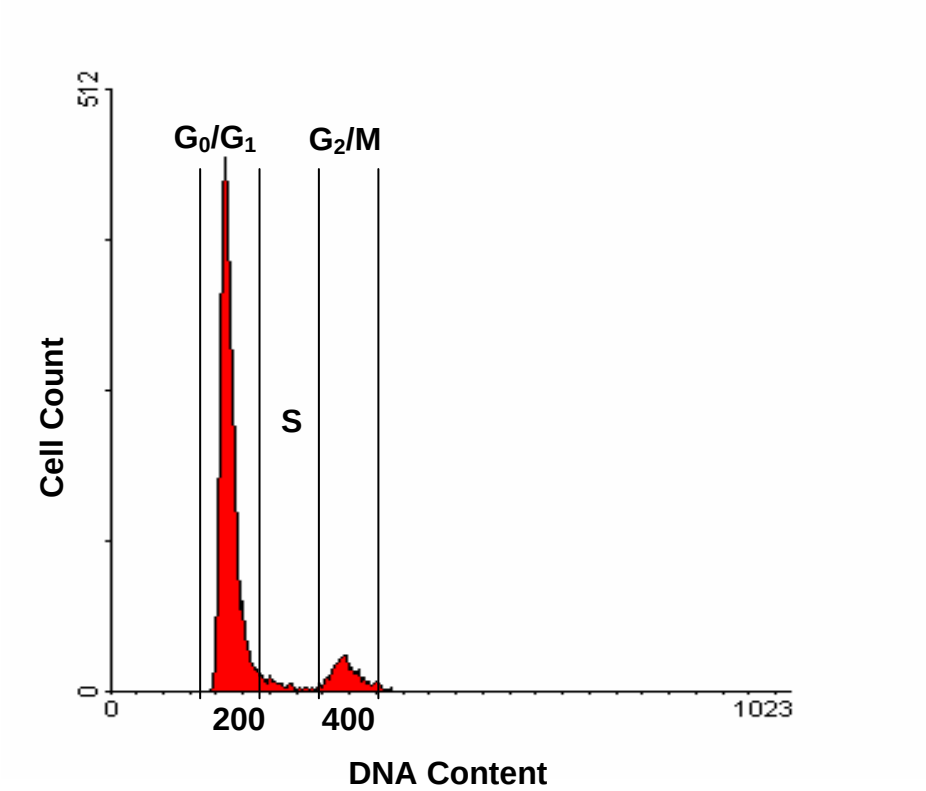

Figure 3.1. Results of flow cytometric analysis of heart cells from the eastern oyster. The leftmost peak represents the G_0/G_1 cells with a 2C (resting diploid) amount of DNA, the rightmost peak represents cells in G_2/M phase with a 4C amount of DNA, and the cells in between the peaks are in the S phase undergoing DNA synthesis that have amounts of DNA between 2C and 4C. The Y-axis represents the cell numbers and the X-axis represents the propidium iodide fluorescence intensity that is directly proportional to the quantity of DNA per cell (channel number is the measured value of a parameter, representing the signal intensity of an event after amplification).

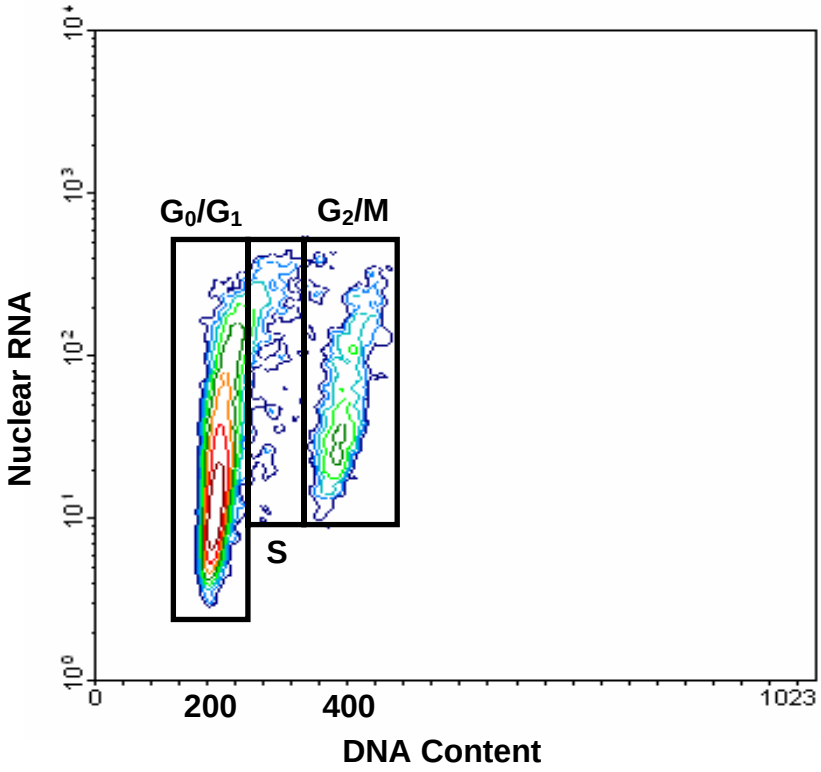

Figure 3.2. Dual-parameter contour plot (nuclear RNA vs. DNA content) of heart cells of eastern oysters used to determine cell cycle phases and nuclear RNA-positive cells. Three regions were gated to determine nuclear RNA distribution in the cell cycle: Gap 0/Gap 1 (G_0/G_1), Synthesis (S), Gap 2/Mitosis (G_2/M). The Y-axis (nuclear RNA) was recorded in logarithmic scale and the X-axis (DNA content) was recorded in linear scale. These gates allowed for calculation of the mean geometric for each phase.

Kruskal-Wallis nonparametric analysis-of-variance (ANOVA) was used to determine differences between nuclear RNA/DNA ratios in each phase of the cell cycle.

RESULTS

The stained nuclear RNA of heart and labial palp nuclei was detected at different intensities throughout the cell cycle. The nuclei with RNA staining were not distributed homogenously throughout the cell cycle. In heart an average of 71 ± 16% of the nuclei were in G_0/G_1 phase, 5 ± 3% of the nuclei were in S phase, and 24 ± 13% were in G_2/M phases. In labial palps nuclei an average of 88 ± 1% of the nuclei were in G_0/G_1 phase, 3 ± 2% of the nuclei were in S phase, and 9 ± 1% were in G_2/M phases. In the heart and labial palps nuclei, the mean fluorescence of nuclear RNA increased from G_0/G_1 phases into the S phase and decreased in the G_2/M phases. The mean fluorescence of the nuclear RNA in heart nuclei increased on average 52% from G_0/G_1 phases to S phase. The nuclear RNA fluorescence decreased on average 20% from S phase to G_2/M phases. For labial palps nuclei the nuclear RNA fluorescence increased 69% from the G_0/G_1 to S phase. The nuclear RNA fluorescence decreased on average 32% from S phase to G_2/M phases. There was no significant difference in the fluorescence increase from G_0/G_1 to S phase and the fluorescence decrease from S phase to G_2/M for the heart and the labial palps nuclei.

Analysis of the nuclear RNA/DNA ratio for heart and labial palp cells in relation to the cell cycle phases, as represented by DNA content, reveals an up-side-down "U" shape pattern of changing rates of DNA replication against transcription. During G_0/G_1 phase, when DNA content was constant, cells accumulate increasing amounts of nuclear RNA; and there was a high heterogeneity of the nuclear RNA/DNA ratio (Table 3.2).

Table 3.2. Deoxyribonucleic acid, nuclear RNA, and nuclear RNA/DNA ratios for G_1/G_0 phases, S phase, and G_2/M phases of heart and labial palps cells of eastern oyster as measured by flow cytometry. The results are expressed as geometric means (standard deviation).

Tissue	Cell cycle phase	DNA Content	Nuclear RNA Content	Nuclear RNA/DNA Ratio
Heart	G_1/G_0	201 (14)	32 (12)	0.16 (0.05)
	S	305 (32)	64 (26)	0.21 (0.06)
	G_2/M	388 (27)	55 (29)	0.14 (0.07)
Labial Palps	G_1/G_0	193 (11)	34 (12)	0.17 (0.05)
	S	291 (17)	111 (18)	0.38 (0.06)
	G_2/M	373 (14)	75 (20)	0.20 (0.05)

The nuclear RNA/DNA ratio in heart cells increased on average by 22 ± 16% from G_0/G_1 phase to S phase and decreased by 34 ± 14% in the G_2/M phases. In labial palp cells, nuclear RNA/DNA ratio increased by 47 ± 19% and decreased by 53 ± 18% in the G_2/M phases. The cells in G_2/M phases from the heart and labial palps had on average similar nuclear RNA/DNA ratios as those from G_0/G_1 phase.

DISCUSSION

Accurate analysis of cell proliferation rates would provide information of cell kinetics and assist researchers in the development of an oyster cell line. The relationship between RNA content and regulation of the cell cycle is still unclear but the accumulated evidence of cellular RNA content point out that RNA content is a marker strongly correlated with cell kinetics (the rates of change in the progression of the cell cycle) (Baserga 1984; Drazynkiewicz 1988). The analysis of DNA content in single cells by flow cytometry provides a rapid and accurate method for determining the cell cycle frequency distribution of a given cell population (Dean 1985). Cell cycle analysis by DNA content alone cannot discriminate among the many metabolic states related to the capacity to progress through the cell cycle. Distinguishing between quiescent and cycling cells, cells

in various stages of differentiation, and the relation between DNA division and cell growth can be assessed by multi-parametric analysis of DNA and cellular RNA content (Drazynkiewicz et al. 1975; Drazynkiewicz et al. 1976; Bauer et al. 1980).

Measurement of the ratio of RNA to DNA in somatic tissues in fish (Buckley 1984; Robinson and Ware 1988; McGurk and Kusser 1992), crustaceans (Sulkin et al. 1975; Anger and Hirche 1990; Juinio et al. 1992) and mollusks (Clarke et al. 1989; Kenchington 1994; Lodeiros et al. 1996; Chicharo et al. 2001) has been used to detect the response of the organism to environmental changes, growth rate, stress due to disease, and nutritional condition. This index is based on the assumption that the amount of DNA is stable under changing environmental conditions while RNA content is affected by these conditions. Therefore, the ratio of RNA to DNA is susceptible to change when the environment of the organism changes (Chicharo et al. 2001). Organisms in good conditions tend to have higher RNA/DNA ratios while organisms in a poor condition tend to have lower RNA/DNA ratios.

In this study, nuclei from somatic tissue cells to allow for the detection of nuclear RNA. Unlike fluorescence spectrophotometric analysis, which only detects total RNA and DNA in all cells, the flow cytometric analysis provided simultaneous information on the amount of nuclear RNA and DNA allows calculation of nuclear RNA/DNA ratios for each phase of the cell cycle for individual cells. In a study of nuclei isolated plant protoplasts ratios of nuclear RNA/DNA were determined throughout the cell cycle and at G_2 phase the ratio was similar to that of the G_1 phase (Bergounioux et al. 1988). In mammalian cell cultures where cells were arrested at G_0 phase and stimulated with growth factors to enter S phase, there was a surge of nuclear RNA for about 30 min. The level of nuclear RNA dropped, as inhibitors of protein synthesis blocked transcription, to a lower level that was

maintained as long as the growth factors were present in the culture medium (Reed 1997). The present study we found results similar to the plant protoplasts and mammalian cell culture in that the nuclear RNA/DNA ratio increased from G_0/G_1 phase to S phase and decreased to G_2/M phase for oyster somatic cells.

In conclusion, it was possible to detect nuclear RNA in somatic tissues of the eastern oyster by flow cytometry. Nuclear RNA content distribution could assist in the improvement of oyster cell lines by determining if cells have accumulated a threshold amount of nuclear RNA to proceed into S phase and cell division. The ability to calculate nuclear RNA/DNA ratio throughout the cell cycle can provide information of the condition of cultured cells due to environmental changes in the medium.

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CHAPTER 4 – CONSTANT TEMPERATURE EFFECT ON THE EASTERN OYSTER SOMATIC TISSUES

INTRODUCTION

The phylum Mollusca is the second largest invertebrate phylum. It comprises 80,000 extant species with an enormous diversity of form, function, and adaptation. The animals exploit marine, estuarine, freshwater and terrestrial systems. Molluscs inhabit environmental conditions varying from abyssal depths of the ocean to high altitudes (Mulcahy 2001). Physiological knowledge of molluscs is relatively limited and this narrow information is restricted to a few species of molluscs that are of commercial, medical, or ecological importance.

The development of molluscan tissue culture would be an important step in answering questions and problems related to mollusca physiology and pathology. The study of tissues isolated from molluscs has yet to proceed from tissue explants and primary cultures with limited mitosis, which may reflect the lack of understanding of the key influences on growth and proliferation of cells from these animals. The first step in acquiring this knowledge is to understand the *in vivo* factors affecting the organism and the cell proliferation in tissues (Freshney 1987).

A study of cell proliferation in somatic tissues of the eastern oyster, found that it was seasonal (Nguyen et al. Department of Veterinary Sciences). Cell proliferation increased during the late fall and winter months (November-March) and steadily decreased in the spring to summer months (April-July). Cell proliferation in somatic tissues was also determined to cease as gonadal tissue matured in during the late spring and late summer months before spawning (Frovola 1993; Leibson and Frovola 1994; Nguyen et al. Department of Veterinary Sciences).

Temperature has been determined to be a key influence in all aspects of eastern oyster biology. The goal of this study was to determine the effect of temperature on somatic tissues of the eastern oyster. In this report, the effects are disscused of constant water temperatures on the cell cycle, cell proliferation, and cell metabolism in heart and labial palp cells of the eastern oyster. Cell proliferation in somatic tissues was measured by detecting proliferating cell nuclear antigen (PCNA) by use of a flow cytometer. Proliferating cell nuclear antigen is an auxiliary protein of DNA polymerase-δ that is closely linked with DNA synthesis and repair (Tan et al. 1986; Bravo et al. 1987). Cell metabolism was measured by RNA/DNA ratios at all phases of the cell cycle. This index is based on the assumption that amount of DNA is stable under changing environmental conditions while RNA content is affected by these conditions. Therefore, the ratio of RNA to DNA is susceptible to change when the environment of the organism changes (Chicharo et al. 2001). Organisms in good conditions tend to have higher RNA/DNA ratios while organisms in a poor condition tend to have lower ratios. The objectives of this study were to determine by flow cytometry if constant water temperatures affected the: (1) cell cycle, (2) cell proliferation, and (3) cell metabolism.

METHODS AND MATERIALS

Oyster Collection and Maintenance

Eastern oysters (n = 560) were collected in March 2004 from the Louisiana Sea Grant Oyster Hatchery at Grand Isle, Louisiana, (90° 2’ 13.3” W, 29° 12’ 26” N) and were maintained in an indoor temperature-controlled recirculating system (Kolar 2004) at the Louisiana State University Aquaculture Research Station for 15 weeks.

Experimental Design

The oysters were distributed equally amongst all tanks in the recirculating system. Tanks were filled with 20 ppt of artificial saltwater (HW marine mix, Wiegandt GmbH Co., Krefeld, Germany). Oysters were fed algal paste twice daily (1800 Instant algae®) at an amount recommended by the manufacturer (Reed Mariculture, Campbell, CA). The oysters were placed in the tanks at an initial temperature of 20°C the same temperature as that of Grand Isle. Water temperatures were held at 20 ± 0.5°C for 3 weeks to acclimate the animals to the new environmental conditions. After 3 weeks the water temperatures were dropped at the rate of 1ºC per day until two tanks (9 and 11) were at 15ºC, and two tanks (14 and 16) were at 10ºC, while two tanks (1 and 7) were kept at 20ºC. The temperature in three tanks (13, 17, and 18) was increased 1ºC per day to 27ºC and were used as control treatments. Once the water reached the desired temperatures (10, 15, or 20ºC), the temperatures were maintained for 3 weeks, for temperature acclimation. After 3 weeks, fluctuation was imposed on each of the temperature regimes (Table 4.1).

Table 4.1. Temperature fluctuations imposed on tanks. Each temperature regime was replicated twice.

Tank	Average temperature (ºC)	Fluctuation (ºC)
1 & 7	20	± 1
9 & 11	15	± 1
14 & 16	10	± 1
13, 17, & 18	27	± 1

The fluctuation was diurnal and was implemented for 5 weeks (Fig. 4.1). During the span of this experiment oysters in the system were sampled at various times (Table 4.2). Oysters were kept in Grand Isle as a standard to determine the natural effects of temperature and were sampled at the same time as those from the system.

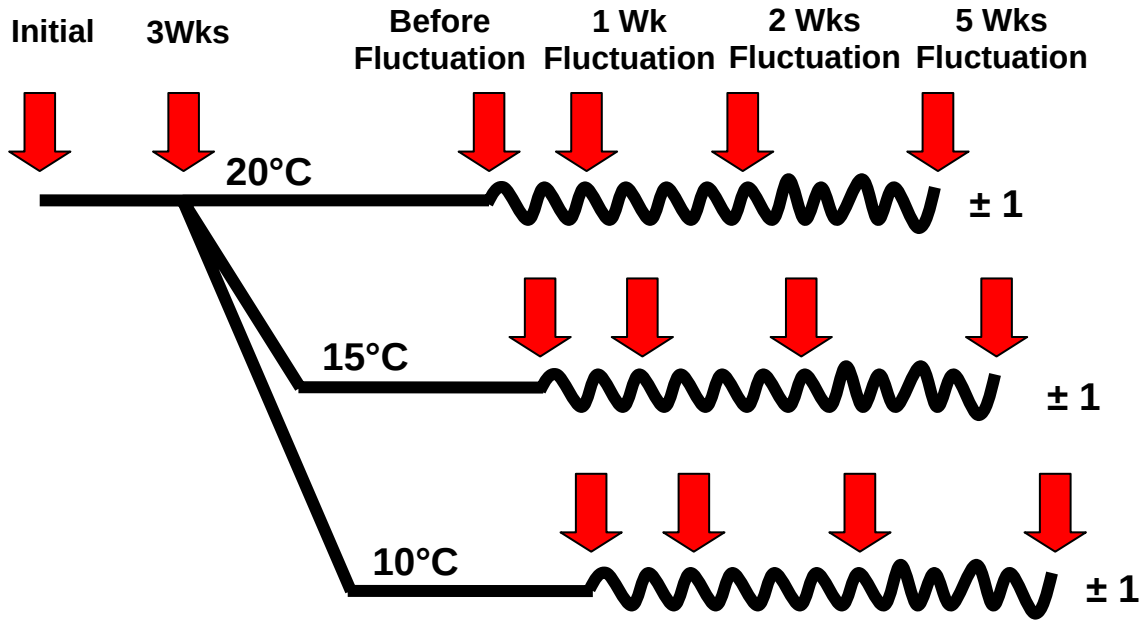

Figure 4.1. Schematic of the temperature regimes imposed in this study.

Cell Preparation

Oysters were opened and the heart and labial palps were surgically removed. Hearts and labial palps from individual oysters were crushed using a homogenizer with a glass-on-glass pestle. The cells were suspended in 0.01M-phosphate buffered saline (PBS) (1.37 M NaCl, 0.03 M KCl, 0.009 M KH_2PO_4, 0.06 M Na_2HPO_4, pH 7.4) and filtered through a 35-µm mesh to remove connective tissue. Samples were placed into 1.5-mL micro-centrifuge tubes at a concentration of 2 X 10^6 cells/mL and placed on ice.

PCNA Labeling and DNA Staining

Cells were centrifuged at 400-X g for 10 min at 4ºC. The supernatant was discarded and the cells resuspended in 750 µL of lysing buffer, modified from Langdberg and Roos (1991) containing 2% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in PBS, 1% NP-40 (Sigma), and 0.2 µg/mL of ethylenediamine tetra-acetic acid (EDTA) (Fisher Scientific Company, Pittsburgh, PA) for 15 min on ice.

Table 4.2. Times at which the animals were sampled to determine temperature effects in heart and labial palps cells. Total number of oysters sampled (number of oysters per temperature regime).

Time	Number of oysters	Temperature regime (°C)
Initial collection from Grand Isle	15	20
3 Weeks after initial sampling	15	20
Before fluctuation	75 (15)	20, 15, 10, 27, and Grand Isle
1 Week of fluctuation	60 (12)	20 ± 1, 15 ± 1, 10± 1, 27 ± 1, and Grand Isle
2 Weeks of fluctuation	60 (12)	20 ± 1, 15 ± 1, 10 ± 1, 27 ± 1, and Grand Isle
5 Weeks of fluctuation	60 (12)	20 ± 1, 15 ± 1, 10 ± 1, 27 ± 1, and Grand Isle

Nuclei were centrifuged at 400-X g for 10 min at 4ºC, and the supernatant was discarded. The pelleted nuclei were resuspended and fixed in 1 mL of 2% paraformaldehyde for 15 min on ice. Cells were centrifuged at 400-X g for 10 min at 4ºC. The fixative was discarded and the pellet was washed twice with PBS. The cells were resuspended in 200 µL of PBS. Fluorescein isothiocyanate (FITC) conjugated monoclonal anti-proliferating cell nuclear antigen (PC10) (Sigma) was added to the cells at a dilution of 1:40 (µL: µL) and the samples were held in the dark for 45 min at room temperature. The cells were resuspended with 200 µL of a DNA-staining solution containing 10 µg/mL propidium iodide (PI) and 1.8 Kunits/mL RNase (Sigma) and held in the dark for 30 min at room temperature until flow cytometric analysis.

Cell Cycle Analysis and PCNA Detection by Flow Cytometry

The cells were analyzed using a FACSCalibur flow cytometer equipped with an argon laser (488 nm) (Becton-Dickinson Immunocytometry Systems, San Jose, CA) and

data from at least 10^4 cells were stored in list mode. Doublets and debris were discriminated by gating on doublet discrimination modes (FL-2A vs. FL-2W) dot plot. Fluorescein isothiocyanate fluorescence was detected in the FL-1H channel using a 560-nm beam splitter with a 510 – 540-nm bandpass filter and stored as a logarithmic amplification. Fluorescence from PI staining of DNA was detected in the FL-2A channel using a 610-nm long pass filter and stored as a linear amplification. Data were analyzed using FACS software (BD CellQuest™). The cell cycle was analyzed using DNA histograms which were divided into three regions: (1) Resting /Gap 1 phase (G_0/G_1), (2) Synthesis phase (S), and (3) Gap 2/Mitosis (G_2/M) (Fig. 4.1). The distribution of PCNA stained cells was analyzed on a dual-parameter dot plot (PCNA fluorescence vs. DNA content) by gating on the regions described above. The lower limit of PCNA-positive cells gate was set on top of the upper border of the labeled cells G_2/M. The ratio of fluorescence increase of PCNA during the cell cycle was calculated (geometric mean of the S-phase PCNA stained cells by the geographic mean of the G_0/G_1-phase PCNA stained cells) from the gated dual-parameter dot plot (PCNA fluorescence vs. DNA content).

Nuclear RNA and DNA Staining

The supernatant was discarded and the pellet was resuspended for 15 min on ice in 750 µL of lysing buffer, modified from Langdberg and Roos (1991) containing 2% bovine serum albumin in PBS (Sigma) 1% NP – 40 (Sigma), and 0.2 µg/mL of EDTA (Fisher). Cells were centrifuged again at 400-X g for 10 min at 4ºC. The lysing buffer was discarded and the cells resuspended in 2% paraformaldehyde (Sigma) for 15 min on ice. Cells were centrifuged at 400-X g for 10 min at 4ºC. The fixative was discarded and the pellets were washed twice by centrifugation with PBS. The cells were resuspended in 200 µL of PBS. SYBR Green II RNA gel stain (Molcular Probes, Inc., Eugene, OR) was

added to the cells at a 1:2000 (μL:μL) ratio, and the samples were held in the dark for 30 min at room temperature. The cells were resuspended with 200 μL of a DNA-staining solution containing 10-μg/mL of PI (Sigma) in 10 mL of PBS and held in the dark for an additional 30 min at room temperature until flow cytometric analysis.

Flow Cytometry Analysis of Nuclear RNA and DNA Ratio

The cells were analyzed using the FACSCalibur flow cytometer described above and data from at least 10^4 cells were stored in list mode. Damaged cells and debris were excluded from the analysis by gating on a forward and side scatter dot plot. Doublets and debris were discriminated by gating on an area integral vs. a pulse width measurement dot plot. Fluorescence from SYBR Green II was detected in the FL-1H channel as described above. Fluorescence from PI staining of DNA was detected in the FL-2A channel using a 610-nm long pass filter and stored as a linear amplification. Data were analyzed using FACS software (BD CellQuest™). DNA content was analyzed as described above. Nuclear RNA distribution was analyzed on a dual-parameter dot plot (nuclear RNA fluorescence vs. DNA content) by gating on the regions described above. Nuclear RNA:DNA ratio was calculated, using the gated dual-parameter dot plot (nuclear RNA fluorescence vs. DNA content), by dividing the nuclear RNA fluorescence (geometric means) into the DNA fluorescence (geometric means). The ratio was calculated for each phase of the cell cycle.

Statistical Analysis

Friedman's nonparametric two-way analysis of variance (ANOVA) was used to compare the effect of temperature regime over time and the effect of time over each temperature regime for each cell cycle phase (G_0/G_1, S, and G_2/M phases). Friedman's nonparametric two-way ANOVA was used to compare the effect of temperature regime

over time and the effect of time over each temperature regime for the percent PCNA positive nuclei. Friedman's nonparametric two-way ANOVA was used to compare the effect of temperature regime over time and the effect of time over each temperature regime for the RNA/DNA ratios of each cell cycle phase (G_0/G_1, S, and G_2/M phases).

RESULTS

Water Temperature

Water temperatures in the tanks were found to be within ± 0.5ºC of the target temperatures (Kolar 2004). Because the mean of the constant temperatures were similar to those of the 1°C fluctuation temperatures, they were grouped them together for statistical analysis. The system held the water temperature at 20°C for the initial 3 weeks of acclimation (Fig. 4.2). Because the water was held at the different temperature regimes for 3 weeks before imposing fluctuations, plotting of data became difficult (approximately 19000 observations). Hence, for high resolution, only a 48-hr period of data at the beginning of each temperature regime was plotted. When the temperatures were dropped a 1°C per day tanks with the target temperature of 15°C were able to maintain the temperature constant for 3 weeks. The 10°C tanks did not attain the target temperature, except for the last two weeks of acclimation. The 27°C tanks were able to maintain the temperature constant throughout the study. During fluctuation of water temperature, the tanks with temperature regimes of 10 ± 1°C (Fig. 4.3), 15 ± 1°C (Fig. 4.4), and 20 ± 1°C (Fig. 4.5) attained the desired range of temperatures.

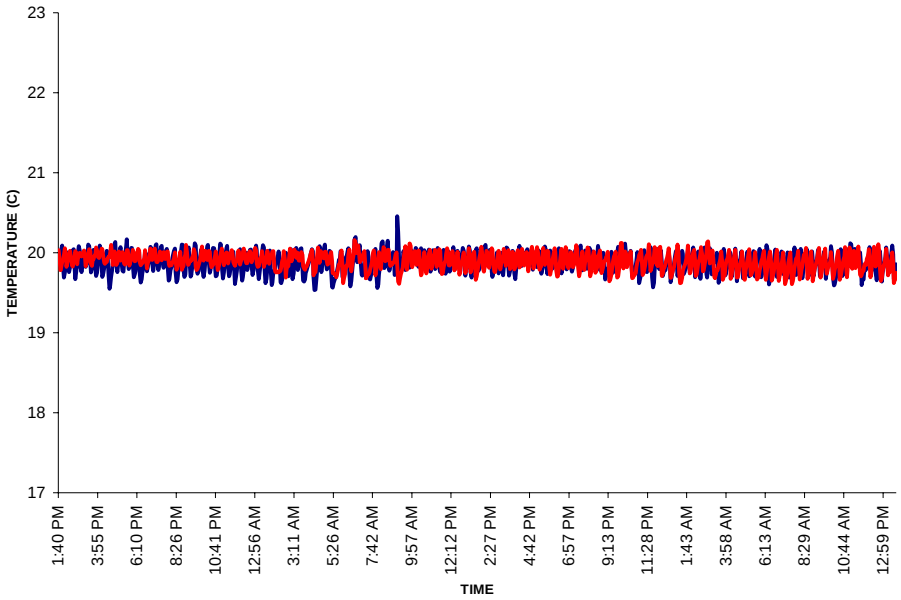

Figure 4.2. Water temperatures in tanks 1 and 7 for a 48-hr period. Initial temperatures were maintained around 20°C for 3 weeks before imposing a variation of ± 5°C.

Means and variances of the differences between expected and actual temperatures for all regimes for a 48-hr period are provided (Table 4.3).

Table 4.3. Mean of differences between expected and actual temperatures for a 48-hr period. Positive mean denotes that heat is required to maintain water at expected temperatures. Negative mean denotes the mead of cooling to maintain the expected water temperature.

Regime	Mean of difference between expected and actual water temperatures (°C)	Variance of differences between expected and actual water temperatures (°C)
20 ± 1°C	0.11	0.015
15 ± 1°C	-0.03	0.004
10 ± 1°C	N/A	N/A

Temperature Effects on Cell Cycle Phases

In heart and labial palp nuclei, there were no significant differences on the cell cycle phases between all temperature regimes.

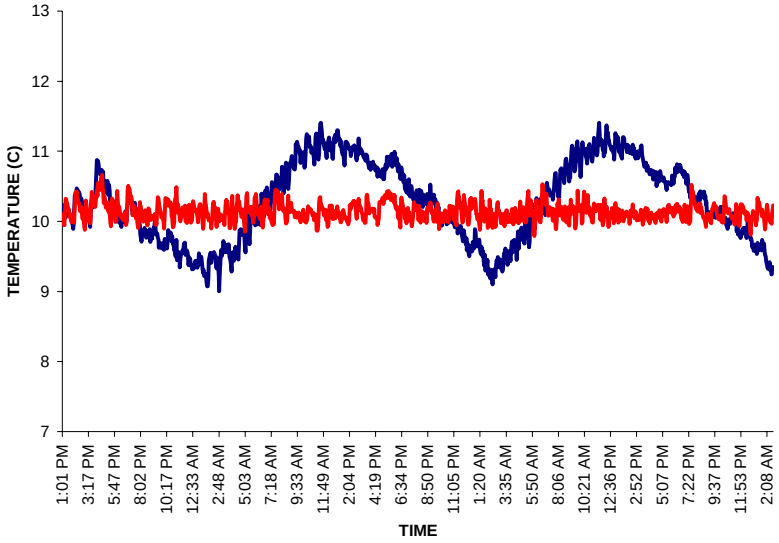

Figure 4.3. Water temperatures in tanks 14 and 16 for a 2-day period. Temperatures were maintained at 10 ± 1°C for 5 weeks in tank 14 while temperature was kept at 10°C for 5 weeks in tank 16.

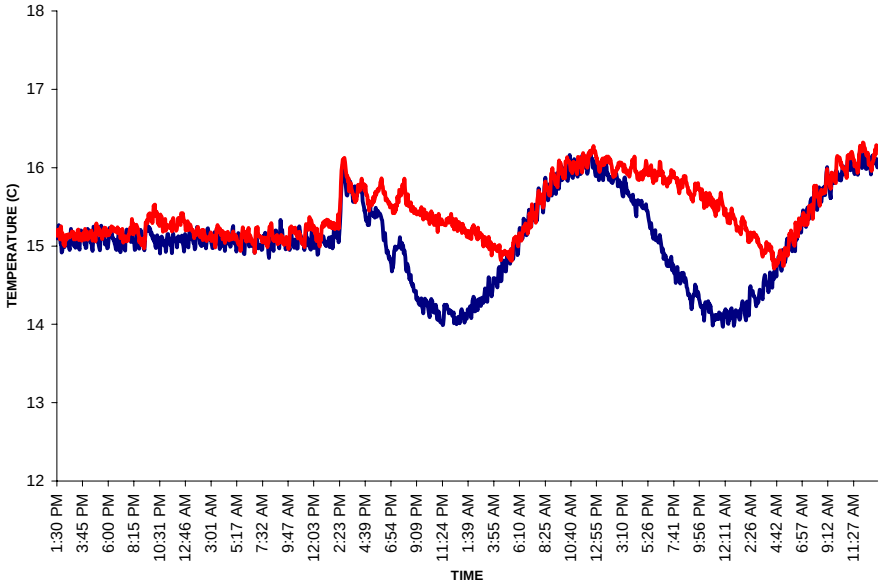

Figure 4.4. Water temperatures in tanks 3 and 10 for a 2-day period. Temperatures were maintained at 15 ± 1°C for 5 weeks. The water could not be cooled to the desired temperatures in tank 10.

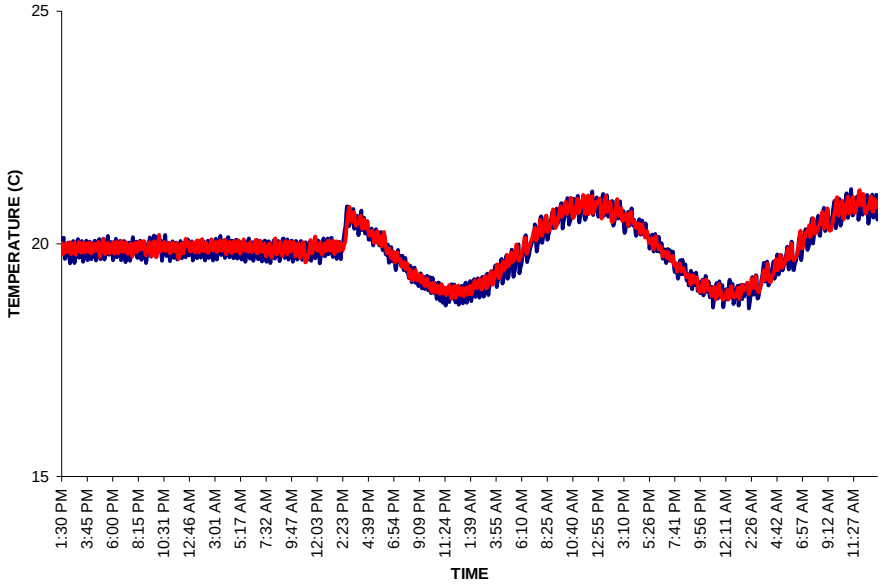

Figure 4.5. Water temperatures in tanks 1 and 7 for a 2-day period. Temperatures were maintained at 20 ± 1°C for 5 weeks. The water was able to attain the desired temperatures.

Temperature Effects on PCNA Concentrations

There were no significant differences in the levels of PCNA detected for heart and labial palp nuclei among temperature regimes.

Temperature Effects on Nuclear RNA/DNA Ratio for each Cell Cycle Phase

All temperatures had a significant effect on heart nuclear RNA/DNA ratio at the G_0/G_1 ($P < 0.0001$), S ($P < 0.0001$), and G_2/M phases ($P < 0.0001$). Oysters held at 10°C had a significantly lower G_0/G_1 nuclear RNA/DNA ratio than those at 15°C ($P < 0.0001$), 20°C ($P < 0.0001$), control ($P < 0.001$), and Grand Isle ($P < 0.0141$). These oysters had a significantly lower S-phase nuclear RNA/DNA ratio than did those at 15°C ($P = 0.0002$), 20°C ($P = 0.0169$), and control ($P = 0.0009$). These oysters also had a significantly lower G_2/M phase nuclear RNA/DNA ratio than did those at 15°C ($P < 0.0001$), 20°C ($P < 0.0001$), and control ($P < 0.0001$).

Oysters collected from Grand Isle had a significantly higher G_0/G_1 nuclear RNA/DNA ratio than did those of 10°C ($P < 0.0141$), but were significantly lower than 15°C ($P < 0.0001$), 20°C ($P < 0.0001$), and control ($P = 0.0002$). These oysters had significantly lower S-phase labial palp nuclear RNA/DNA ratio than did those at 15°C ($P < 0.0001$), 20°C ($P = 0.004$), and control ($P = 0.0002$). These oysters also had a significantly lower G2/M phase nuclear RNA/DNA than did those at 15°C ($P < 0.0001$), 20°C ($P < 0.0001$), and control ($P = 0.0002$).

Effect of Time on Cell Cycle Phase

Through time the proportions of cells in the G_0/G_1 ($P < 0.0001$), S ($P = 0.0156$), and G_2/M phases ($P < 0.0001$) of the cell cycle changed significantly for heart nuclei. As the number of cells in the G_0/G_1 phase increased significantly, there were significant decreases in the S and the G_2/M phases. There was a significant increase in G_0/G_1 nuclei from before fluctuation to 5 weeks of fluctuation ($P = 0.0030$). There were also significant increases in G_0/G_1 nuclei from 1 week of fluctuation to 2 weeks ($P = 0.0069$) and 5 weeks ($P < 0.0001$) of fluctuation. In S-phase nuclei, there was a significant decrease from before fluctuation to after 2 weeks ($P = 0.0245$) and 5 weeks ($P = 0.003$) of fluctuation. For G_2/M-phase nuclei, there was a significant increase from before fluctuation to 1 week ($P = 0.0054$) and 2 weeks ($P < 0.0001$) of fluctuation, and there was a significant decrease after 5 weeks of fluctuation ($P = 0.0385$). There was a significant decrease of G_2/M nuclei from 1 week to 2 weeks ($P = 0.0154$) and 5 weeks ($P < 0.0001$) of fluctuation. There was also a significant decrease in G_2/M nuclei from 2 weeks to 5 weeks ($P < 0.0001$) of fluctuation.

Through time the proportions of cells in the G_0/G_1 ($P < 0.0001$), S ($P < 0.0001$), and G_2/M phases ($P < 0.0001$) of the cell cycle changed significantly for labial palp nuclei.

As the number of cells in the G_0/G_1 phase increased significantly, there were significant decreases in the S and the G_2/M phases. There was a significant decrease in G_0/G_1 nuclei from before to after 1 week ($P = 0.0007$) of fluctuation. However there was a significant increase of G_0/G_1 nuclei from before fluctuation to after 2 weeks ($P = 0.0101$) and 5 weeks ($P < 0.0001$) of fluctuation. There was a significant decrease of S-phase nuclei from before to 2 weeks ($P < 0.0001$) and 5 weeks of fluctuation ($P < 0.0001$). There was also a significant decrease in S-phase nuclei from 1 week to 2 weeks ($P < 0.0001$) and 5 weeks ($P < 0.0001$) of fluctuation. There was a significant increase of G_2/M nuclei from before to 1 week ($P = 0.0005$) of fluctuation. However there was a significant decrease of G_2/M nuclei from before to 5 weeks ($P = 0.0101$) of fluctuation. There was a significant decrease of G_2/M nuclei from 1 week to 2 weeks ($P = 0.0014$) and 5 weeks ($P < 0.0001$) of fluctuation. There was a significant decrease of G2/M nuclei from 2 weeks to 5 weeks ($P = 0.0093$) of fluctuation.

Effect of Time on PCNA Concentration

There were significant differences in the levels of PCNA concentrations for heart ($P < 0.0001$) and labial palp nuclei ($P < 0.0001$) through time. As the number of cells in the S phase in heart and labial palp nuclei decreased significantly, the PCNA concentration decreased significantly. For the heart ($P < 0.0001$) and labial palp ($P < 0.0006$) nuclei there was a significant increase in PCNA levels from the sampling period before fluctuation of temperature to after 1 week of fluctuation. After 2 weeks of fluctuation there was no significant difference in PCNA levels for heart and labial palp nuclei. After 5 weeks of fluctuation there was significant increase in PCNA leves in heart nuclei ($P = 0.0217$).

Effect of Time on Nuclear RNA/DNA ratio for each Cell Cycle Phase

Time had a significant effect on the heart nuclear RNA/DNA ratio at the G_0/G_1 ($P < 0.0001$), S ($P < 0.0001$), and G_2/M ($P < 0.0001$) phases of the cell cycle. The nuclear RNA/DNA ratio for all phases of the cell cycle was significantly higher after 1 week of fluctuation. The ratio decreased after 5 weeks of fluctuation at all cell cycle phases to the same ratio as before fluctuation. There was a significant increase of the G_0/G_1 nuclear RNA/DNA ratio from before fluctuation to 1week ($P < 0.0001$) of fluctuation. There was a significant decrease from 1 week to 2 weeks ($P < 0.0001$) and 5 weeks ($P < 0.0001$) of fluctuation. The number of cells in S-phase significantly increased from before fluctuation to 1 week ($P < 0.0001$), 2 weeks ($P < 0.0001$), and 5 weeks ($P < 0.0001$) of fluctuation. There was also a significant decrease from 1 week of fluctuation to 5 weeks ($P = 0.0051$) of fluctuation. The G_2/M-phase heart nuclei significantly increased from before fluctuation to 1 week ($P < 0.0001$), 2 weeks ($P < 0.0001$), and 5 weeks ($P < 0.0001$) of fluctuation. There was also a significant decrease from 1 week of fluctuation to 2 weeks ($P = 0.0016$) and 5 weeks of fluctuation ($P = 0.0001$).

Time had a significant effect on the labial palp nuclear RNA/DNA ratio at the G_0/G_1 ($P < 0.0001$), S ($P < 0.0001$), and G_2/M ($P < 0.0001$) phases of the cell cycle. The nuclear RNA/DNA ratio for all phases of the cell cycle was significantly higher after 1 week of fluctuation. There was a significant increase of the G_0/G_1 nuclear RNA/DNA ratio from before fluctuation to 1week ($P < 0.0001$) of fluctuation. There was a significant decrease from 1 week to 2 weeks ($P < 0.0001$) and 5 weeks ($P < 0.0001$) of fluctuation. The number of cells in S-phase significantly increased from before fluctuation to 1 week ($P < 0.0001$), 2 weeks ($P < 0.0001$), and 5 weeks ($P < 0.0001$). There was a significant decrease from 1 week to 5 weeks ($P = 0.0051$) of fluctuation. The G_2/M-phase labial palp

nuclei significantly increased from before fluctuation to 1 week ($P < 0.0001$), 2 weeks ($P < 0.0001$), and 5 weeks ($P < 0.0001$). There was a significant decrease from 1 week to 2 weeks ($P = 0.0016$) and 5 weeks ($P = 0.0051$) of fluctuation.

DISCUSSION

An improved understanding of the key influences on growth and proliferation of somatic tissue cells of the eastern oyster would assist in the development of tissue cultures. Temperature is the most important factor affecting almost all aspects of oyster biology (Shumway 1996), and cell proliferation in somatic tissues of the eastern oyster was affected by seasonal temperature changes, with cell proliferation increasing in the winter (Nguyen et al. Department of Veterinary Sciences). To fully understand the effects of temperature on somatic tissues, it is necessary to first consider the effects of temperature on the cell cycle.

The major consideration of growth is the cell cycle, the analysis of which can provide a reliable index for the prediction of growth potential and changes in cell number with time (Leelavatcharamas et al. 1996). The cell cycle is a series of biochemical and structural events involving the growth, replication, and division of eukaryotic cells. The cell cycle is divided into four parts: mitosis (cell divison), period between mitosis and the beginning of DNA synthesis (Gap 1 or G_1 phase), period of DNA synthesis (S phase), and period between the end of DNA synthesis and the beginning of mitosis (Gap 2 or G_2) (Klug and Cummings 1994). Temperature affects all the phases of the cell cycle in ways characteristic of each period (Sisken et al. 1965).

In this study, constant temperature had no effect on the cell cycle phases and cellular proliferation of heart and labial palp cells. It is possible that this occurred because temperature was decreased at a slow rate (1ºC per day for 5-10 days) and the cells were

able to acclimate to the change. The cell cycle phases could have lengthened to equal proportions due to slowing down of biochemical reactions as suggested by studies in phytoplankton growth (Vaulot 1994). In studies of mammalian cell cultures and human amnionic cells it was found that constant temperatures did not affect the G_1-phase cells, but when temperature was increased or decreased, the cells in G_1-phase were affected (Sisken et al. 1965; Watanabe and Okada 1967).

Measurement of the ratio of RNA to DNA in somatic tissues in crustaceans (Sulkin et al. 1975; Anger and Hirche 1990; Juinio et al. 1992) and mollusks (Clarke et al. 1989; Kenchington 1994; Lodeiros et al. 1996; Chicharo et al. 2001) has been used to detect responses to environmental changes, growth rate, stress due to disease, and nutritional condition. Although this study evaluated nuclear RNA/DNA ratios, our results are similar to studies on temperature effects using RNA/DNA ratios derived from tissues on a per gram basis. In a study of seasonal variation of muscle and liver RNA/DNA ratios in bluegill *Lepomis macrochirus*, it was found that at warmer temperatures (20°C – 25°C) the muscle ratios were higher and at lower temperatures (10°C – 20°C) the liver ratios were higher (Bulow et al. 1981). In a study of *Gadus morhua* larvae and juveniles of Atlantic cod, the RNA/DNA ratio also increased as temperature increased (Clemmesen et al. 2003).

Constant temperature had an affect on metabolic condition of somatic tissue cells in the eastern oyster. In the heart and labial palps cells temperatures between 15°C and 20°C might have decreased the amount of RNA present in cells because of increasing RNAase activity. Although this study showed that temperature is a factor in cell metabolism of *in vivo* cells of the eastern oyster, future research should include water temperature fluctuations to determine if cell cycle rates and cellular proliferation could be affected.

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CHAPTER 5 – DIURNAL TEMPERATURE EFFECTS ON CELL PROLIFERATION OF EASTERN OYSTER SOMATIC TISSUES

INTRODUCTION

Eastern oysters, *Crassostrea virginica*, are found along the Atlantic and Gulf coasts of the United States, in shallow and semi-enclosed water bodies, where water temperatures can vary greatly. Oysters are ectothermic organisms, having a body temperature that fluctuates with the temperature of its surroundings. Temperature is the most important factor that affects almost all aspects of oyster biology including survival, feeding and growth, gonadal development and spawning, larval settlement, heart rate and respiration, disease outbreaks (Shumway 1996), and somatic tissue cell proliferation (Nguyen et al. Department of Veterinary Sciences).

A study of cell proliferation in somatic tissues of the eastern oyster, found that it was seasonal (Nguyen et al. Department of Veterinary Sciences). Cell proliferation increased during the late fall and winter months (November-March) and steadily decreased in the spring to summer months (April-July). Cell proliferation in somatic tissues was also determined to cease as gonadal tissue matured in during the late spring and late summer months before spawning (Frovola 1993; Leibson and Frovola 1994; Nguyen et al. Department of Veterinary Sciences).

The goal of this study was to determine the effect of fluctuating temperature on the somatic tissues of the eastern oyster. In this report, the effects of fluctuating water temperatures are evaluated for the cell cycle, cell proliferation, and cell metabolism in heart and labial palps cells of the eastern oyster. Cell proliferation in somatic tissues was measured by detection of proliferating cell nuclear antigen (PCNA) by use of a flow cytometer. Proliferating cell nuclear antigen, an auxiliary protein of DNA polymerase-δ, is closely linked with DNA synthesis and repair (Tan et al. 1986; Bravo et al. 1987). Cell

metabolism was measured by nuclear RNA/DNA ratios at all phases of the cell cycle. This index is based on the assumption that amount of DNA is stable under changing environmental conditions while nuclear RNA content is affected by environmental fluctuation conditions. Therefore, the ratio of nuclear RNA to DNA is susceptible to change when the environment of the organism changes (Chicharo et al. 2001). The objectives of this study were to determine by flow cytometry if fluctuating water temperatures affected the: (1) cell cycle, (2) cell proliferation, and (3) cell metabolism. Temperature fluctuation had an effect on all phases of the cell cycle for the heart and labial palps. We determined that temperature fluctuation increased cell proliferation and in turn increased cell metabolism.

METHODS AND MATERIALS

This study was conducted in conjunction with the study from Chapter 4 of this thesis. The materials and methods (oyster collection, oyster maintenance, cell preparation, PCNA labeling and DNA staining, nuclear RNA and DNA staining, flow cytometric analysis of the cell cycle and PCNA, and flow cytometry analysis of nuclear RNA and DNA ratio) are similar, but the temperature fluctuations were of 5 or 10°C.

Experimental Design

After 3 weeks the water temperatures were dropped at the rate of 1ºC per day until four tanks (2, 3, 4, and 10) were at 15ºC, and two tanks (12 and 15) were at 10ºC, while two tanks (6 and 8) were kept at 20ºC. The temperature in three tanks (13, 17, and 18) was increased 1ºC per day to 27ºC and the tanks were labeled as the control. Once the water reached the target temperatures (10, 15 or 20ºC) they were maintained for 3 weeks, for temperature acclimation. After 3 weeks, fluctuation was imposed on each of the temperature regimes (As described in table 5.1) (Fig. 5.1).

Table 5.1. Temperature fluctuations imposed on tanks. Each temperature regime was replicated twice.

Tank	Average Temperature (ºC)	Fluctuation (ºC)
6 & 8	20	± 5
3 & 10	15	± 5
2 & 4	15	± 10
12 & 15	10	± 5
13, 17, & 18	27	± 1

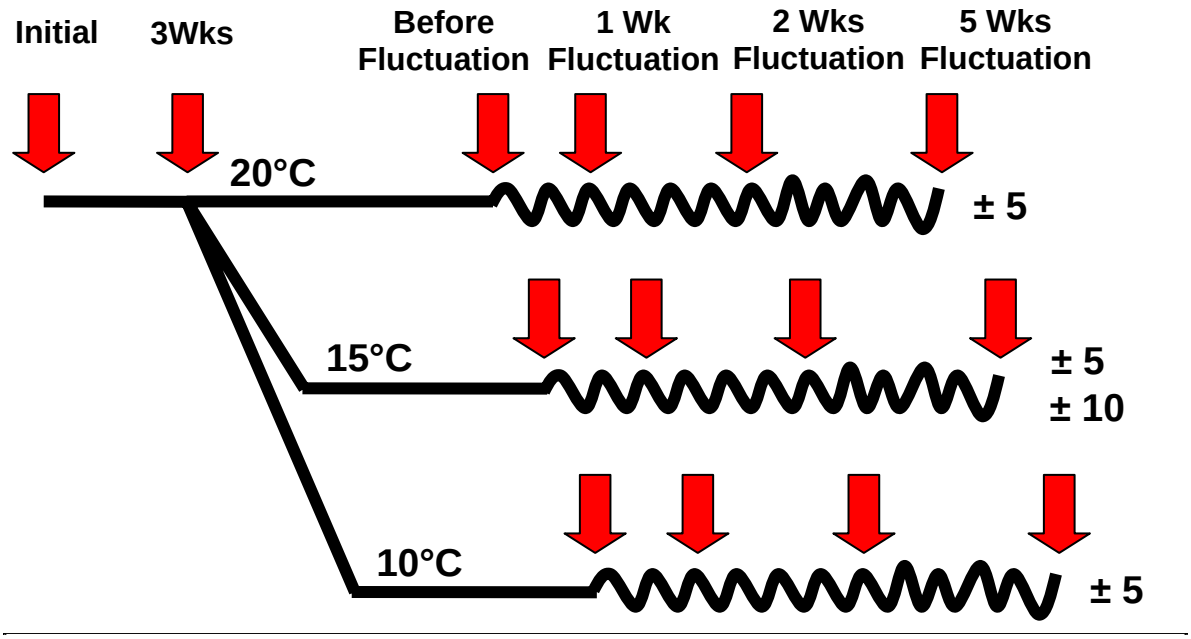

Figure 5.1. Schematic of the temperature regimes imposed in this study.

The fluctuation was diurnal and was implemented for 5 weeks. During the span of this experiment oysters in the system were sampled at various times (Table 5.2). Oysters were kept in Grand Isle as a standard to determine the natural effects of temperature and were sampled at the same time as those in the system.

Table 5.2. Times at which the animals were sampled to determine temperature effects in heart and labial palps cells. Total number of oysters sampled (number of oysters per temperature regime).

Time	Number of Oysters	Temperature Regime (°C)
1 Week of fluctuation	60 (12)	20 ± 5, 15 ± 10, 10± 5, 27, and Grand Isle
2 Weeks of fluctuation	60 (12)	20 ± 5, 15 ± 10, 10 ± 5, 27, and Grand Isle
5 Weeks of fluctuation	60 (12)	20 ± 5, 15 ± 10, 10 ± 5, 27, and Grand Isle

Statistical Analysis

Friedman's nonparametric two-way analysis of variance (ANOVA) was used to compare the effect of temperature regime over time and the effect of time over each temperature regime for each cell cycle phase (G_0/G_1, S, and G_2/M phases). Friedman's nonparametric two-way ANOVA was used to compare the effect of temperature regime over time and the effect of time over each temperature regime for the percent PCNA positive nuclei. Friedman's nonparametric two-way ANOVA was used to compare the effect of temperature regime over time and the effect of time over each temperature regime for the nuclear RNA/DNA ratios of each cell cycle phase (G_0/G_1, S, and G_2/M phases).

RESULTS

Water Temperature

Water temperatures in the tanks were found to be within ± 0.5ºC from the expected temperatures. The system held the water temperature at 20°C for the initial 3 weeks of acclimation (Fig. 5.2).

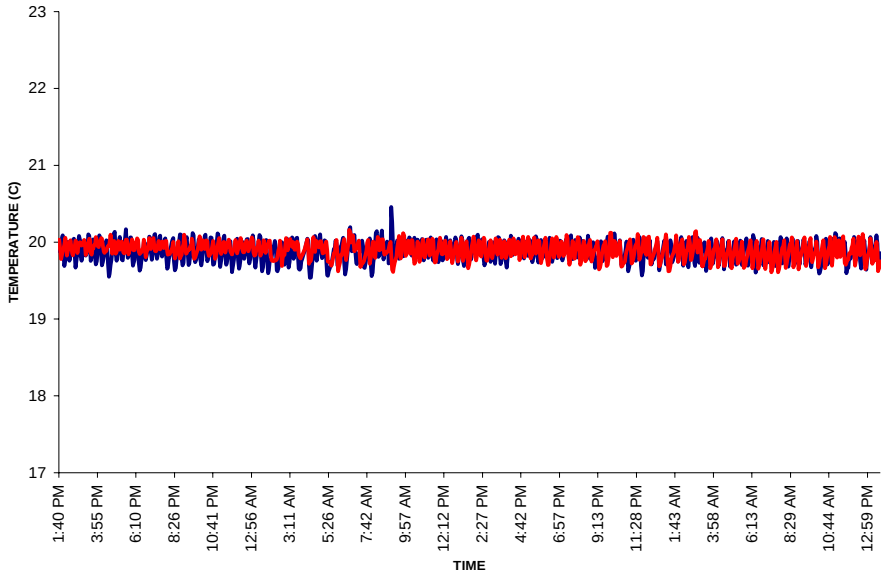

Figure 5.2. Water temperatures in tanks 6 and 8 for a 48-hr period. Initial temperatures were maintained around 20°C for 3 weeks before imposing a variation of ± 5°C.

Because the water was held at the different temperature regimes for 3 weeks before imposing fluctuations, plotting of data became difficult (approximately 19000 observations). Hence, for high resolution, only a 48-hr period of data at the beginning of each temperature regime was plotted. When the temperatures were dropped 1°C per day tanks with the target temperature of 15°C were able to maintain the temperature constant for 3 weeks (Fig. 5.3). The 10°C tanks did not attain the target temperature, except for the last two weeks of acclimation (Fig. 5.4). The control tanks were able to maintain the temperature constant throughout the study. During fluctuation of water temperature, the tanks with temperature regimes of 10 ± 5°C (Fig. 5.5), 15 ± 5°C (Fig. 5.6), and 15 ± 10°C (Fig. 5.7) did not attain the complete range of temperatures.

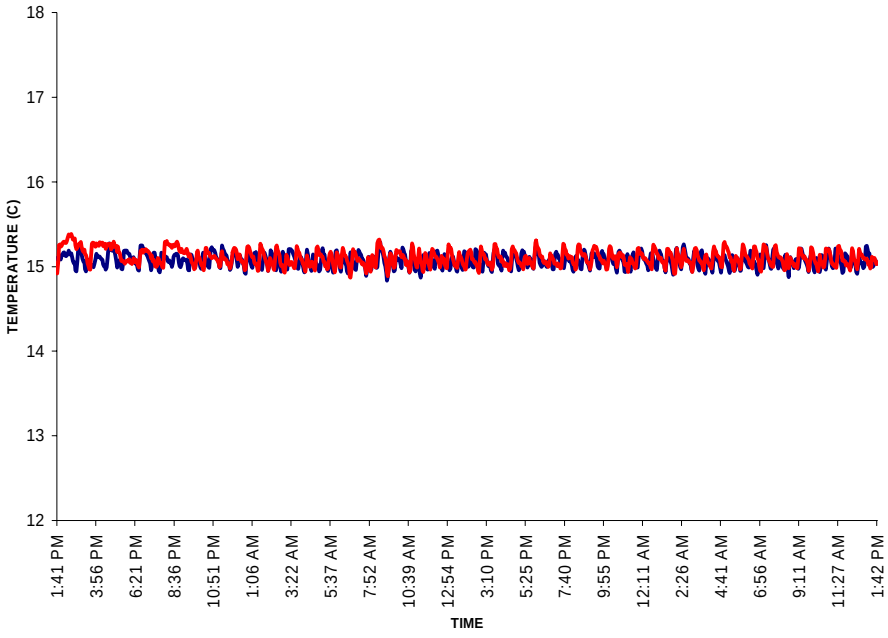

Figure 5.3. Water temperatures in tanks 3 and 10 for a 2-day period. Temperatures were maintained around 15°C for 3 weeks before imposing a fluctuations of ± 5°C and ± 10°C.

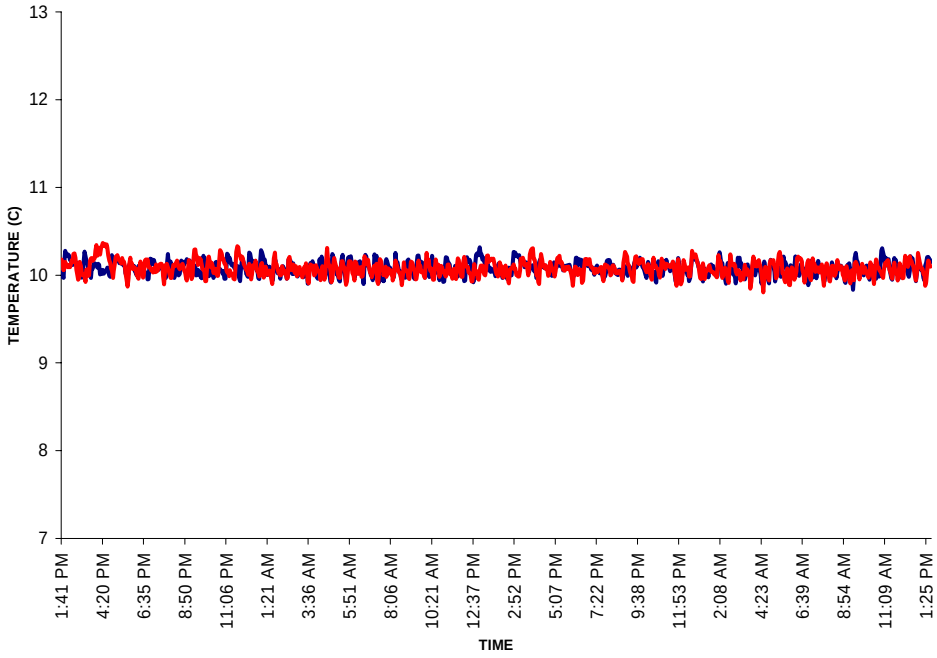

Figure 5.4. Water temperatures in tanks 12 and 15 for a 2-day period. Temperatures were maintained around 10°C for 3 weeks before imposing a fluctuations of ± 5°C.

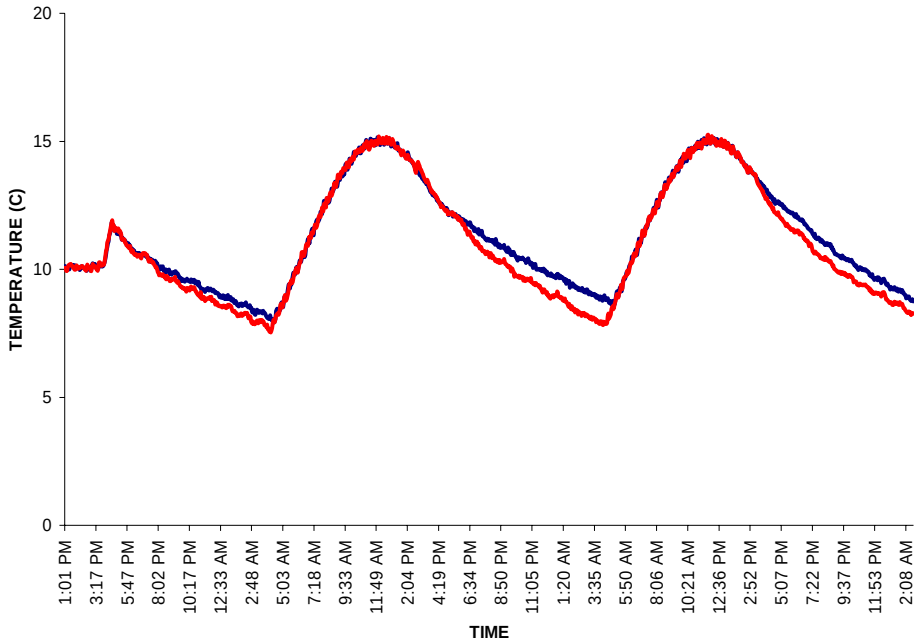

Figure 5.5. Water temperatures in tanks 12 and 15 for a 2-day period. Temperatures were maintained at 10°C ± 5°C for 5 weeks. The water was not able to be cooled to the desired temperatures.

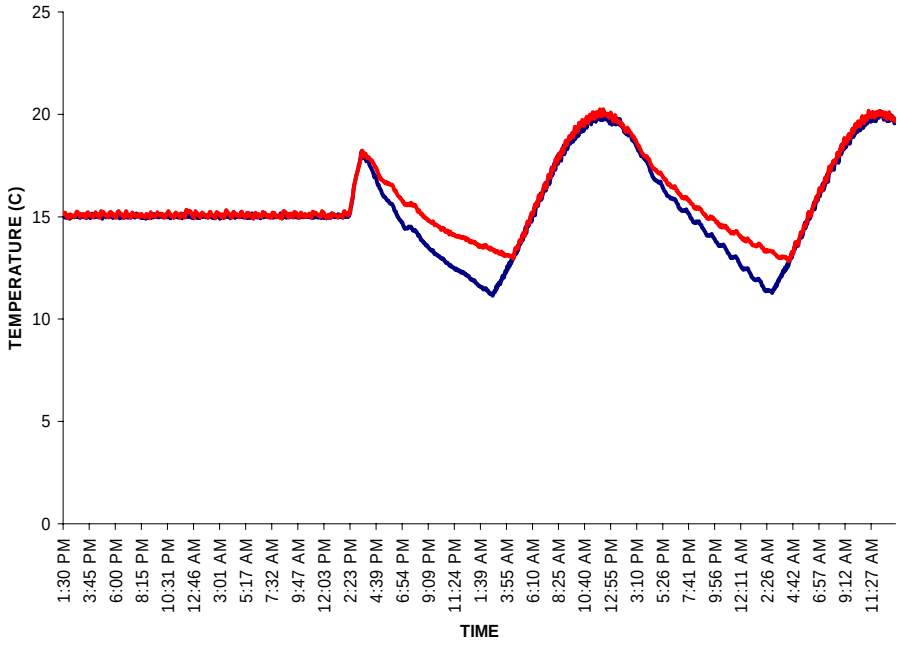

Figure 5.6. Water temperatures in tanks 3 and 10 for a 2-day period. Temperatures were maintained at 15 ± 5°C for 5 weeks. The water was not able to be cooled to the desired temperatures.

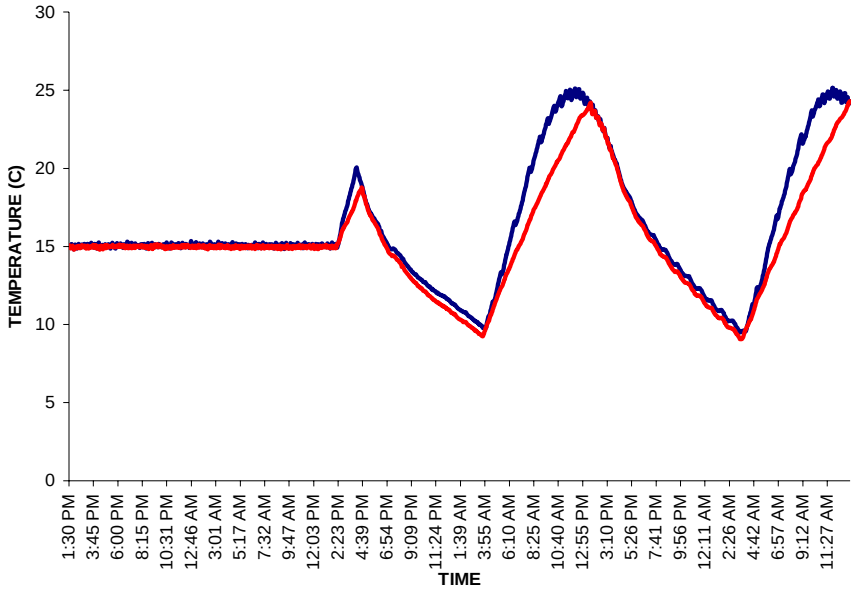

Figure 5.7. Water temperatures in tanks 2 and 4 for a 2-day period. Temperatures were maintained at 15 ± 10°C for 5 weeks. The water was not able to be warmed or cooled to the desired temperatures.

Means and variances of the differences between expected and actual temperatures for all regimes for a 48-hr period are provided (Table 5.3). Tanks with fluctuations of 20 ± 5°C attained the desire temperatures (Fig 5.8).

Table 5.3. Mean of differences between expected and actual temperatures for a 48-hr period. Positive mean denotes that heat if required into the system to maintain water at expected temperatures. Regimes 15 ± 5°C, and 15 ± 10°C needed to be cooled further to be maintained at expected temperatures.

Regime	Mean of difference between expected and actual water temperatures (°C)	Variance of differences between expected and actual water temperatures (°C)
20 ± 5°C	0.19	0.088
15 ± 5°C	-0.43	0.556
15 ± 10°C	-1.78	6.610
10 ± 5ºC	N/A	N/A

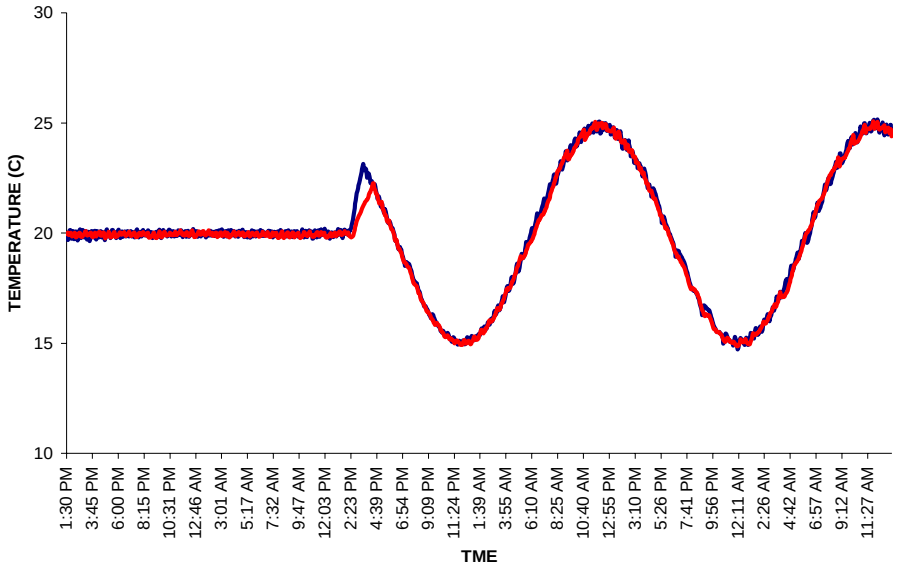

Figure 5.8. Water temperatures in tanks 6 and 8 for a 2-day period. Temperatures were maintained at 20 ± 5°C for 5 weeks. The water was able to attain the desired temperatures.

Temperature Effects on Cell Cycle Phases

The temperature regimes had a significant effect on the proportions of cells in the heart G_0/G_1-phase ($P = 0.009$) and S-phase ($P < 0.0001$) nuclei. Temperature regimes had no significant effects on the proportions of cells in the heart G_2/M phase nuclei. Oysters in 10 ± 5°C had significantly greater proportion of cells in the heart G_0/G_1 nuclei than 15 ± 10°C ($P = 0.0427$), control ($P = 0.0014$), and Grand Isle ($P = 0.0171$). Oysters in 15 ± 5°C had significantly greater proportions of cells in the heart G_0/G_1 nuclei than control ($P = 0.0033$), and Grand Isle ($P = 0.0347$). Oysters in 10 ± 5°C had significantly lower proportions of cells in the heart S-phase nuclei than 15 ± 10°C ($P < 0.0001$), 20 ± 5°C ($P = 0.0001$), control ($P < 0.0001$), and Grand Isle ($P = 0.0091$). Oysters in 15 ± 5°C had significantly lower proportions of cells in the heart S-phase nuclei than 15 ± 10°C ($P = 0.0013$), 20 ± 5°C ($P = 0.0026$), and control ($P < 0.0001$). Oysters collected from

Grand Isle had significantly lower proportions of cells in the S-phase heart nuclei than oysters at control ($P = 0.0141$).

The temperature regimes had a significant effect on the proportion of labial palps in S-phase ($P < 0.0001$) and G_2/M-phase ($P < 0.0001$) nuclei. Temperature regimes had no significant effects on the proportions of cells in the heart G_0/G_1 phase nuclei. Oysters in 10 ± 5°C had significantly lower proportions of cells in the labial palp S phase nuclei than 15 ± 5°C ($P = 0.0036$), 15 ± 10°C ($P = 0.001$), 20 ± 5°C ($P < 0.0001$), and control ($P < 0.0001$). Control oysters had significantly lower proportions of cells in the labial palp S phase nuclei than 10 ± 5°C ($P < 0.0001$), 15 ± 5°C ($P = 0.0003$), 15 ± 10°C ($P = 0.0006$), 20 ± 5°C ($P < 0.0001$), and those collected from Grand Isle ($P < 0.0001$).

Temperature Effects on PCNA Concentrations

Temperature regimes had a significant effect ($P = 0.0007$) on the levels of PCNA detected in the heart nuclei. Oysters in 10 ± 5°C had significantly lower levels of PCNA concentrations than those in 15 ± 10°C ($P = 0.0005$), 20 ± 5°C ($P = 0.0004$), control ($P < 0.0001$), and those collected from Grand Isle ($P = 0.0102$).

Temperature regimes had a significant effect ($P < 0.0001$) on the levels PCNA detected in labial palp nuclei. Oysters in 10 ± 5°C had significantly lower levels of PCNA concentrations than those in 15 ± 5°C ($P = 0.0087$), 15 ± 10°C ($P = 0.0007$), 20 ± 5°C ($P = 0.0005$), control ($P < 0.0001$), and those collected from Grand Isle ($P = 0.005$). Control oysters had significantly higher levels of PCNA concentrations than those in 15 ± 5°C ($P = 0.011$), 15 ± 10°C ($P = 0.0012$), 20 ± 5°C ($P = 0.0144$), and those collected from Grand Isle ($P = 0.001$).

Temperature Effects on Nuclear RNA/DNA Ratio for each Cell Cycle Phase

The temperature regimes had a significant effect on nuclear RNA/DNA ratio of heart G_0/G_1 phase ($P < 0.0001$) and G_2/M phase ($P = 0.0007$) nuclei. We found that in all phases of the cell cycle, the nuclear RNA/DNA ratios were the highest between 15°C and 20°C. Oysters in 10 ± 5°C had significantly lower nuclear RNA/DNA ratio of heart G_0/G_1 nuclei than 15 ± 5°C ($P < 0.0001$), 15 ± 10°C ($P < 0.0001$), 20 ± 5°C ($P < 0.0001$), and control ($P < 0.0001$). Oysters collected from Grand Isle had significantly lower nuclear RNA/DNA ratio of heart G_0/G_1 nuclei than 15 ± 5°C ($P = 0.0357$). Oysters in 10 ± 5°C had significantly lower nuclear RNA/DNA ratio of heart G_0/G_1 nuclei than 15 ± 5°C ($P < 0.0001$), 15 ± 10°C ($P < 0.0001$), 20 ± 5°C ($P = 0.0262$), and control ($P = 0.0032$).

The temperature regimes had a significant effect on nuclear RNA/DNA ratio of labial palp G_0/G_1 phase ($P = 0.001$) and G_2/M phase ($P = 0.0132$) nuclei. We found that in all phases of the cell cycle, the nuclear RNA/DNA ratios were the highest between 15°C and 20°C. Oysters in 10 ± 5°C had significantly lower nuclear RNA/DNA ratio of labial palp G_0/G_1 nuclei than 15 ± 5°C ($P = 0.0001$), 15 ± 10°C ($P = 0.0022$), 20 ± 5°C ($P = 0.0003$), and control ($P = 0.0169$). Oysters in 10 ± 5°C had significantly lower nuclear RNA/DNA ratio of labial palp G_2/M nuclei than 15 ± 5°C ($P = 0.0012$), 15 ± 10°C ($P = 0.0253$), and 20 ± 5°C ($P = 0.0049$).

Effect of Time on Cell Cycle Phase

Through time the proportions of cells in the heart G_0/G_1 ($P < 0.0001$), S ($P = 0.0003$), and G_2/M ($P < 0.0001$) phase nuclei changed significantly. As the number of cells in the G_0/G_1 phase increased significantly, the S and G_2/M phase significantly decreased. There were significantly lower proportions of cells in the G_0/G_1 phase heart nuclei after 1 week of fluctuation than after 2 weeks ($P < 0.0001$) and 5 weeks

($P < 0.0001$) of fluctuation. There was also significantly lower proportions of cells in the G_0/G_1 phase heart nuclei after 2 weeks of fluctuation than after 5 weeks ($P = 0.0022$) of fluctuation. The number of cells in the S-phase were significantly higher after 2 weeks ($P < 0.0001$) and 5 weeks ($P = 0.0034$) of fluctuation. There were significantly higher proportions of cells in the G_2/M phase heart nuclei after 1 week of fluctuation than after 2 weeks ($P < 0.0001$) and 5 weeks ($P < 0.0001$) of fluctuation. There were also significantly higher proportions of cells in the G_0/G_1 phase heart nuclei after 2 weeks of fluctuation than after 5 weeks ($P < 0.0001$) of fluctuation.

Through time the proportions of cells in the labial palp G_0/G_1 ($P < 0.0001$), S ($P < 0.0001$), and G_2/M ($P < 0.0001$) phase nuclei change significantly. As the number of cells in the G_0/G_1 phase significantly increased the S and G_2/M phases significantly decreased. There were significantly lower proportions of cells in the G_0/G_1 phase heart nuclei after 1 week of fluctuation than after 2 weeks ($P < 0.0001$) and 5 weeks ($P < 0.0001$) of fluctuation. The number of cells in the S-phase labial palp nuclei were significantly higher after 2 weeks ($P < 0.0001$) and 5 weeks ($P < 0.0001$) of fluctuation. There were significantly higher the proportions of cells in the G_2/M phase labial palp nuclei after 1 week of fluctuation than after 2 weeks ($P < 0.0001$) and 5 weeks ($P < 0.0001$) of fluctuation.

Effect of Time on PCNA Concentration

Through time the levels of PCNA in the heart ($P < 0.0001$) changed significantly. As the proportions of cells in the S-phase decreased significantly, the PCNA levels decreased significantly. There were significantly higher PCNA levels after 1 week of fluctuation than that of 2 weeks ($P < 0.0001$) and 5 weeks ($P = 0.0033$) of fluctuation.

Through time the levels of PCNA in the labial palps ($P < 0.0001$) changed significantly. As the proportions of cells in the S-phase decreased significantly, the PCNA levels decreased significantly. There were significantly higher PCNA concentrations after 1 week of fluctuation than after 2 weeks ($P < 0.0001$) and 5 weeks ($P < 0.0001$) of fluctuation.

Effect of Time on Nuclear RNA/DNA ratio for Each Cell Cycle Phase

Through time the nuclear RNA/DNA ratio of heart G_0/G_1 ($P < 0.0001$), S ($P = 0.0149$), and G_2/M ($P = 0.0002$) phase nuclei changed significantly. After 5 weeks of fluctuation the conditions of the cells were similar to those as before fluctuation. There was a significantly higher nuclear RNA/DNA ratio of the G_0/G_1 phase in heart nuclei after 1 week of fluctuation than after 2 weeks ($P < 0.0001$) and 5 weeks ($P < 0.0001$) of fluctuation. There was a significantly higher nuclear RNA/DNA ratio of the S phase in heart nuclei after 1 week of fluctuation than after 2 weeks ($P = 0.0131$) and 5 weeks ($P = 0.0064$) of fluctuation. There was a significantly higher nuclear RNA/DNA ratio of the G_2/M phase in heart nuclei after 1 week of fluctuation than after 2 weeks ($P = 0.0008$) and 5 weeks ($P < 0.0001$) of fluctuation.

Through time the nuclear RNA/DNA ratio of labial palps G_0/G_1 ($P < 0.0001$), S ($P = 0.0002$), and G_2/M ($P = 0.0017$) phase nuclei changed significantly. After 5 weeks of fluctuation the conditions of the cells were similar as those from before fluctuation of temperatures. There was a significantly higher nuclear RNA/DNA ratio of the G_0/G_1 phase in labial palp nuclei after 1 week of fluctuation than after 2 weeks ($P = 0.0201$) and 5 weeks ($P < 0.0001$) of fluctuation. There was a significantly higher nuclear RNA/DNA ratio of the S-phase in labial palp nuclei after 2 weeks of fluctuation than after 1 week ($P = 0.0007$) and 5 weeks ($P = 0.0002$) of fluctuation. There was a significantly lower

nuclear RNA/DNA ratio of the G_2/M phase in labial palp nuclei after 5 weeks of fluctuation than after 1 week ($P = 0.014$) and 2 weeks ($P = 0.0005$) of fluctuation.

DISCUSSION

In Chapter 4 of this thesis, the initial decrease of water temperature and the consequent constant temperatures had no effect on phases of the cell cycle or cellular proliferation in the heart and labial palps cells. Once temperature fluctuations were implemented, it was found that the cell cycle and cellular proliferation were affected. These results agree with a report of temperature effects on mammalian cell cultures. A study of a recombinant Chinese hamster ovary (rCHO) cell line (CL-11G) producing pro-urokinase (pro-UK) in batch cultures for temperature optimization, found that a decrease of the optimal temperature of 37°C to 31°C led to an increase of the number of cells at G_0/G_1 phase from 56% to 83%, and cells in the S phase decreased from 35% to 12%. The cell growth rate decreased from 0.024/h at 37°C to 0.006/h at 31°C (Chen et al. 2004).

Although this study did not address cell cycle rates, the decrease of nuclei in the G_0/G_1 phase and the subsequent increase in the nuclei of S-phase and G_2/M-phase after 5 weeks of fluctuations suggests that the duration times in the G_0/G_1 phase might have increased with decrease in the other two phases. A report on the effects of temperature on the kinetics of the mitotic cycle of mammalian cell cultures, found that below optimum temperatures (37-39°C) the progression through all phases of the cell cycle decreased (Sisken et al. 1965). The progression through G_1 phase was the slowest below 34°C, the optimum temperature. Growth kinetics and antibody formation of an anti-interleukin-2 producing hybridoma line were studied in a suspension culture at temperatures ranging from 34°C to 39°C (Bloemkolk et al. 1997). When temperatures were lowered the cells stayed longer at the G_1 phase of the cell cycle.

Measurement of the ratio of RNA to DNA in somatic tissues in fishes (Buckley 1984; Robinson and Ware 1988; McGurk and Kusser 1992), crustaceans (Sulkin et al. 1975; Anger and Hirche 1990; Juinio et al. 1992) and mollusks (Clarke et al. 1989; Kenchington 1994; Lodeiros et al. 1996; Chicharo et al. 2001) has been used to detect the responses to environmental changes, growth rate, stress due to disease, and nutritional condition. Although the present study evaluated cell-specific nuclear RNA/DNA ratios, the results are similar to studies on temperature effects on RNA/DNA ratios derived on a per gram basis from tissues. In eggs and larvae of walleye pollock *Theragra chalcogramma* cooler temperatures increased the RNA/DNA ratios (Canino 1994). A study that investigated the effect of time of day on RNA/DNA ratios on starved and fed Pacific oysters, *Crassostrea gigas*, and juvenile clams, *Ruditapes decussates*, also found higher RNA/DNA ratios with cooler water temperatures in the holding tanks (Chicharo et al. 2001). The high RNA/DNA ratios at lower temperatures have been found to be associated with the lower activity rate of the RNA, an increase in the amount of RNA present in cells, and the reduction of RNAase activity (Buckley 1982; Goolish 1984; Clemmesen 1994).

In the present study, temperature fluctuation played a key role in the cell cycle, proliferation, and metabolic condition of somatic tissue cells in the eastern oyster. Temperatures below 15°C had an increase in the amount of cells in the G_0/G_1 phase for heart and labial palps nuclei, perhaps because of the decrease in progression rate through this phase. Fluctuating temperatures with a mean of 15°C and 20°C increased the amount of cells in the S phase and the concentrations of PCNA in both tissues suggesting that progression of heart and labial palp cells through these phases might have increased. The metabolic conditions in the heart and labial palp cells for oysters at 10 ± 5°C suggests that

the low temperatures increased the amount of RNA present in cells because of the reduction in RNAase activity. Although this study showed that temperature is a factor in the growth and proliferation of *in vivo* cells of the eastern oyster, future research should include more sampling times and water temperatures higher than 27°C to determine if cell cycle rates and cellular proliferation at these temperatures are affected.

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CHAPTER 6 – SUMMARY AND CONCLUSIONS

The overall goal of these studies was to evaluate temperature effects on cell proliferation of eastern oyster somatic tissues for the development of an oyster cell line. Understanding the *in vivo* cell proliferation of an organism is essential for the development of cell culture. Cell proliferation can be measured by identifying nuclear cellular proteins involved in growth regulation and cellular transformation. To evaluate cell proliferation, analysis of nuclear RNA content can provide information on cell kinetics at each phase of the cell cycle. The techniques developed herein include two flow cytometric methods: (1) detection of proliferating cells and (2) detection of nuclear RNA throughout the cell cycle for cells *in vivo*. These techniques were used to evaluate the effects of constant and fluctuating temperatures on somatic tissues of the eastern oyster.

In the first part of this work, (Chapter 3) a flow cytometric assay was developed and optimized to detect proliferating cell nuclear antigen (PCNA) in oyster somatic tissues. Oysters were collected in August from the Louisiana Sea Grant Oyster Hatchery at Grand Isle, Louisiana and were maintained in an indoor temperature-controlled recirculating system until use. The hearts and labial palps of the oysters were surgically removed and for comparison of immunohistological methods and flow cytometry labial palps were embedded in paraffin, fixed, and sectioned for immunohistochemical analysis. To optimize the flow cytometric assay, different cellular and nuclear permeabilization and fixation methods were tested and once optimized, the protocol was used throughout subsequent studies. To evaluate proliferating cells, monoclonal anti-PCNA (PC10) conjugated with fluorescein isothiocyanate (FITC) was added to the cells, which were analyzed using flow cytometry and data from at least 10^4 cells were analyzed using FACSCalibur software (BD CellQuest™). Proliferating cell nuclear antigen was detected

in all the phases of the cell cycle of the eastern oyster. The levels of PCNA increased from G_0/G_1-phase into the S-phase, where it peaked in mid-S phase, and decreased in G_2/M phase.

The fixed labial palps cross sections were processed and mounted onto glass slides. The presence of PCNA in the embedded oyster tissues was detected by immunohistochemical staining using a modified version of procedures for a commercially available PCNA Staining Kit (Zymed). The PCNA levels of somatic cells on stained slides were analyzed using image analysis and compared to the flow cytometric detection of PCNA. Twice as many samples could be analyzed in 1 h by use of flow cytometry. The cost per sample using flow cytometry was half that of image analysis. Tissues analyzed by flow cytometry and classified as "high" and "low" PCNA-positive cells could not be distinguished by image analysis. Overall, flow cytometry was superior to immunohistochemistry in time and cost efficiency, and in sensitivity to the detection of PCNA in oyster somatic tissues.

To study another cellular parameter reflective of cell growth, (Chapter 3) a flow cytometric assay was developed to detect nuclear RNA in oyster somatic tissues. Oysters were collected in January from the Louisiana Sea Grant Oyster Hatchery at Grand Isle, Louisiana and were maintained in a temperature-controlled recirculating system until use. The hearts and labial palps of the oysters were surgically removed, crushed to single cell suspensions, permeabilized, and fixed. The fixed cells were stained with SYBR Green II to evaluate nuclear RNA throughout the cell cycle. The cells were analyzed using flow cytometer and data from at least 10^4 cells were analyzed using FACSCalibur software (BD CellQuest™) and stored in list mode. Nuclear RNA was detected in all the phases of the cell cycle of the eastern oyster. The concentrations of nuclear RNA increased from G_0/G_1

phase into the S phase, where they peaked in mid-S-phase, and decreased in G_2 phase/M. The ability to calculate the amount of nuclear RNA and the nuclear RNA/DNA ratio throughout the cell cycle can provide information on cellular kinetics and conditions of cultured cells due to environmental changes in the medium.

In the final part of this work, (Chapters 4 and 5) temperature effects on cell cycle, proliferation, and cell metabolism of oyster somatic tissues were evaluated. Oysters were collected in March from the Louisiana Sea Grant Oyster Hatchery at Grand Isle, Louisiana, and were maintained at different temperature regimes in an indoor temperature-controlled recirculating system. The hearts and labial palps of the oysters were surgically removed and the two flow cytometric assays were used to analyze the cell cycle, proliferation, and metabolic condition for these cells. Cell cycle analysis of the heart and labial palp cells showed that the proportions of G_0/G_1 phase cells decreased, the S phase and G_2/M phase cells increased. Heart and labial palp cells for oysters held between 15°C and 25°C increased in PCNA-positive cells after 1 week of temperature fluctuation. The nuclear RNA/DNA ratio at all phases of the cell cycle for heart and labial palps cells for oysters between 10°C and 20°C was higher than at all other temperature regimes indicating increased metabolic activity at the lower temperatures. Temperature variations affected the cell cycle, proliferation, and the metabolic condition of oyster somatic cells.

Future research to improve the development of oyster cell lines include: (1) use other species of oysters, (2) use other markers that can determine temperature effects, (3) use of temperature regimes more reflective of the natural environment, (4) study of the effects of temperatures above 25°C, (5) study of the long-term effects of temperature fluctuation, and (6) study of the short-term effects of temperature fluctuation.

APPENDIX A – TEMPERATURE-CONTROL SYSTEM

The system contained nineteen independently functioning cylindro-conical food-grade holding tanks that had a capacity of 250 L (Aquatic Eco-Systems, Inc, Apopka, FL). The tanks were filled with 200 L of artificial seawater at a concentration of 20 ppt (HW marine mix, Wiegandt GmbH Co., Krefeld, Germany). All the tanks were insulated with encapsulated fiberglass insulation (~10 cm) (Johns Manville, Denver, CO) to prevent the loss of heat (Fig. A.1). Each tank had separate heating and cooling mechanisms that consisted of: two 300-W electric glass immersion heater (Model # 300, Commodity Axis, San Gabriel, CA) and a submersible water pump (Model # 306, Commodity Axis, San Gabriel, CA) that recirculated the water in the tank through a heat exchanger at 2 Lpm (liters per min) for cooling. Two perforated plastic sacs filled with plastic bio balls (5 cm dia) [with a void space of approximately 94% and geometric surface area 31 ft^2/ft^3] (Keeton Industries, Inc., Wellington, CO) were placed at the bottom of each tank, which functioned as biological filters. Aeration and circulation of the water were done by an airlift. The airlift also functioned in water mixing to provide homogeneity of temperature. A T-type thermocouple (Omega Engineering Inc., Stamford, CT) was placed in each tank as a temperature sensor (Fig. A.2).

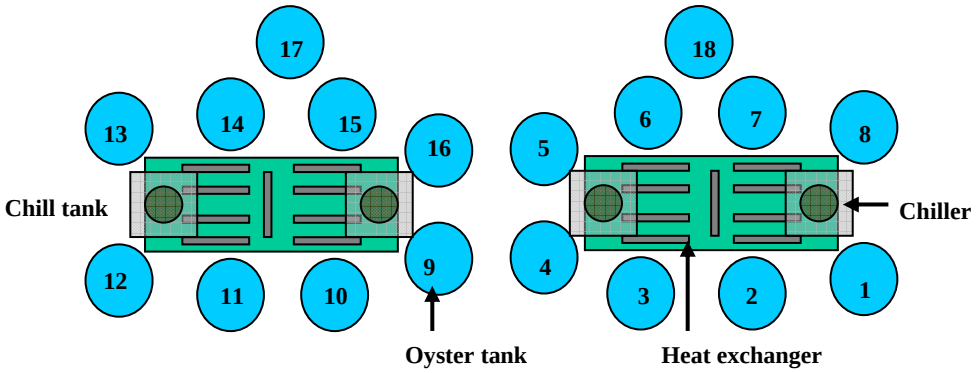

Figure A.1. General schematic of the indoor temperature controlled system. The heat exchangers are placed in the chill tank were the water is cooled to ~ 4°C by two electric chillers.

Nine tanks were connected to one rectangular chill tank (Frigid Units, Toledo, OH). The chill tank was filled with fresh water and the water cooled to 4°C by a pair of electrically operated chillers (120 V AC and 2000 W approximately) (Fig. 1). The heat exchangers were submerged in the chilled water and the submersible pump recirculated water in the oyster tank through the heat exchanger. The heat exchangers were built from stainless steel trays (54 x 33 x 6 cm) with the lids sealed with silicone and held in place with stainless steel screws.

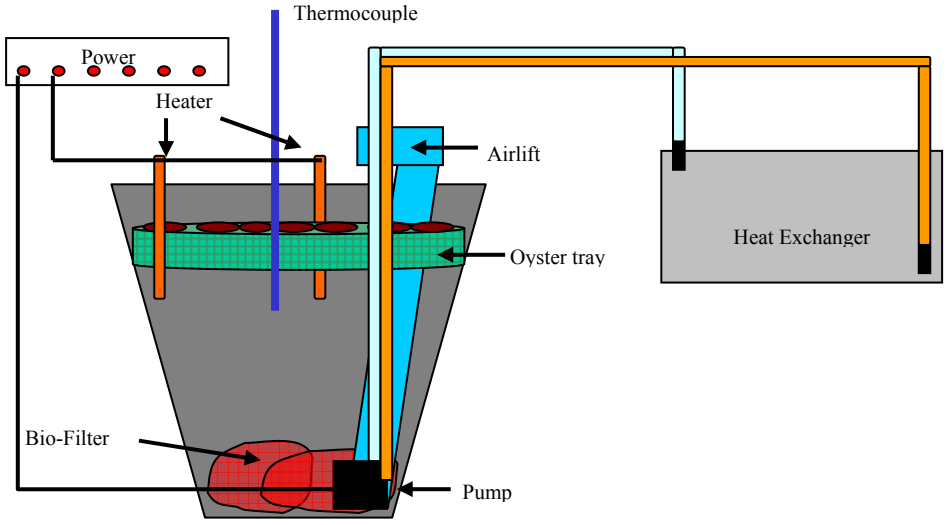

Figure A.2. Tank components including: an airlift to aerate and circulate water in the tank, an immersion heater, a water pump used for temperature control, heat exchanger to cool water and a thermocouple to sense the water temperature. Biological filters placed at the bottom of the tank to treat wastes generated by oysters.

The temperatures were monitored and recorded by a personal computer. The computer processed the signals from the thermocouples that were connected to a 32-channel CIO EXP-32 multiplexer (Measurement Computing Inc., Middleboro, MA.). The multiplexer converted the signal from analog to digital so that the personal computer could process the data and relayed the output signals to the solid-state relays (P/N 611489, Eastman Kodak Co) that actuated the pumps and heaters (Fig. A.3).

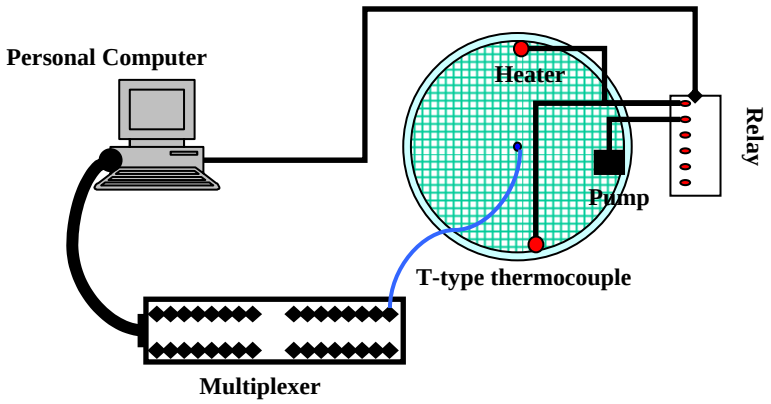

Figure A.3. Thermocouples acted as temperature sensors and all thermocouples were connected to a multiplexer. The personal computer processed the signals and relayed the output signals to the relays that actuated the pumps and heaters.

APPENDIX B – UNANALYZED DATA

Table B.1. Data for hearts of initial experimentation of cell cycle analysis and PCNA detection (Chapter 2).

Tissue	%G0/G1	%S	%G2/M	% PCNA
Heart	79.29	3.70	17.01	11.62
Heart	74.90	5.43	19.67	15.64
Heart	72.74	6.87	20.39	18.07
Heart	78.88	3.68	17.44	13.18
Heart	84.72	1.75	13.53	6.58
Heart	76.91	4.17	18.92	15.20
Heart	79.33	3.46	17.21	14.95
Heart	82.53	2.55	14.92	12.82
Heart	80.31	3.21	16.48	9.06
Heart	78.75	3.93	17.32	10.58
Heart	77.61	4.05	18.34	16.67
Heart	80.75	2.43	16.82	12.66
Heart	77.75	4.03	18.22	17.46
Heart	72.60	6.73	20.67	18.85
Heart	75.02	5.67	19.31	18.44
Heart	75.60	5.38	19.02	17.85
Heart	75.72	5.61	18.67	17.23
Heart	79.10	3.57	17.33	11.50
Heart	72.76	6.41	20.83	23.05
Heart	80.96	2.13	16.91	9.58
Heart	77.43	4.10	18.47	17.35
Heart	80.69	2.53	16.78	10.61
Heart	77.32	4.57	18.11	18.00
Heart	80.85	2.43	16.72	7.91
Heart	81.70	2.20	16.10	11.22
Heart	78.34	3.67	17.99	18.48
Heart	78.95	3.42	17.63	13.46
Heart	72.15	7.20	20.65	17.60
Heart	77.30	4.38	18.32	11.96
Heart	73.78	6.21	20.01	14.44
Heart	76.16	4.99	18.85	11.89
Heart	77.77	4.24	17.99	11.20
Heart	78.87	4.20	16.93	12.60
Heart	78.08	4.38	17.54	11.56
Heart	75.93	5.44	18.63	12.73
Heart	77.95	4.23	17.82	10.05
Heart	80.46	3.18	16.36	11.00
Heart	81.27	2.85	15.88	9.81

Tissue	%G0/G1	%S	%G2/M	% PCNA
Heart	86.11	0.46	13.43	4.33
Heart	79.68	3.65	16.67	10.95
Heart	76.50	5.22	18.28	14.06
Heart	74.89	6.06	19.05	17.43
Heart	74.65	6.01	19.34	16.43
Heart	79.18	3.28	17.54	14.30
Heart	81.42	2.60	15.98	12.34
Heart	61.20	14.36	24.44	32.26
Heart	71.44	7.80	20.76	15.76
Heart	69.89	8.72	21.39	15.12
Heart	76.15	5.04	18.81	10.78
Heart	74.48	6.48	19.04	11.34
Heart	76.07	5.25	18.68	13.94
Heart	68.82	9.20	21.98	21.00
Heart	76.39	5.22	18.39	10.87
Heart	75.61	5.75	18.64	11.20
Heart	74.48	6.49	19.03	13.59
Heart	72.29	7.02	20.69	11.37
Heart	73.36	7.12	19.52	14.64
Heart	80.21	3.07	16.72	7.89
Heart	75.62	5.60	18.78	9.25
Heart	80.53	3.06	16.41	8.80
Heart	77.58	4.83	17.59	11.48
Heart	81.74	2.58	15.68	6.55
Heart	80.83	3.89	15.28	7.50
Heart	82.10	2.56	15.34	6.96
Heart	85.96	0.93	13.11	7.56
Heart	84.79	1.26	13.95	9.30
Heart	81.76	2.63	15.61	10.53
Heart	81.50	3.32	15.18	13.20
Heart	76.55	5.19	18.26	17.32
Heart	76.76	5.88	17.36	14.71
Heart	68.43	9.89	21.68	13.68
Heart	78.36	4.09	17.55	9.71
Heart	78.47	4.01	17.52	8.44
Heart	80.48	3.27	16.25	6.58
Heart	73.75	7.47	18.78	12.41

Table B.2. Data for labial palps of initial experimentation of cell cycle analysis and PCNA detection (Chapter 2).

Tissue	%G0/G1	%S	%G2/M	% PCNA
Labial Palp	76.24	5.85	17.91	17.79
Labial Palp	69.19	10.22	20.59	21.88
Labial Palp	79.42	4.56	16.02	13.59
Labial Palp	79.17	4.36	16.47	14.23
Labial Palp	76.29	5.72	17.99	15.93
Labial Palp	76.14	5.99	17.87	16.83
Labial Palp	79.40	4.21	16.39	13.09
Labial Palp	82.58	2.61	14.81	11.38
Labial Palp	82.79	2.98	14.23	10.88
Labial Palp	84.47	1.89	13.64	12.90
Labial Palp	84.41	1.67	13.92	10.42
Labial Palp	77.14	5.53	17.33	18.59
Labial Palp	81.27	3.71	15.02	18.07
Labial Palp	79.29	4.46	16.25	16.62
Labial Palp	79.09	4.04	16.87	15.35
Labial Palp	85.27	1.64	13.09	10.19
Labial Palp	84.93	1.96	13.11	12.49
Labial Palp	83.19	2.02	14.79	14.13
Labial Palp	83.06	2.37	14.57	11.66
Labial Palp	73.04	7.31	19.65	20.39
Labial Palp	78.99	4.81	16.20	16.49
Labial Palp	83.51	2.36	14.13	13.07
Labial Palp	84.96	1.18	13.86	9.72
Labial Palp	75.46	6.25	18.29	20.83
Labial Palp	81.09	3.24	15.67	14.89
Labial Palp	79.10	4.09	16.81	14.79
Labial Palp	77.14	5.83	17.03	16.63
Labial Palp	83.23	2.24	14.53	10.75
Labial Palp	79.61	4.05	16.34	14.19
Labial Palp	72.83	7.44	19.73	20.63
Labial Palp	79.18	4.19	16.63	18.40
Labial Palp	81.02	3.56	15.42	18.01
Labial Palp	73.47	7.44	19.09	23.26
Labial Palp	78.87	4.65	16.48	18.54
Labial Palp	76.84	5.87	17.29	18.91
Labial Palp	82.63	2.69	14.68	11.00
Labial Palp	83.31	2.27	14.42	9.09
Labial Palp	81.86	3.03	15.11	12.12
Labial Palp	82.59	2.63	14.78	7.89

Tissue	%G0/G1	%S	%G2/M	% PCNA
Labial Palp	84.68	1.76	13.56	7.04
Labial Palp	85.37	1.15	13.48	5.75
Labial Palp	79.47	4.25	16.28	13.00
Labial Palp	81.14	3.13	15.73	9.38
Labial Palp	75.40	6.41	18.19	17.95
Labial Palp	86.46	0.60	12.94	5.74
Labial Palp	78.95	4.58	16.47	9.80
Labial Palp	73.15	7.02	19.83	15.79
Labial Palp	80.84	3.40	15.76	9.67
Labial Palp	81.70	3.08	15.22	8.95
Labial Palp	76.93	5.12	17.95	10.60
Labial Palp	78.82	5.16	16.02	9.74
Labial Palp	78.58	4.64	16.78	11.11
Labial Palp	78.63	5.04	16.33	13.38
Labial Palp	71.07	8.02	20.91	11.95
Labial Palp	72.23	7.02	20.75	11.20
Labial Palp	71.34	7.89	20.77	12.65
Labial Palp	70.23	8.41	21.36	14.49
Labial Palp	78.24	4.80	16.96	8.28
Labial Palp	76.60	5.56	17.84	11.39
Labial Palp	79.27	4.29	16.44	10.63
Labial Palp	68.62	9.17	22.21	13.76
Labial Palp	77.49	5.02	17.49	10.46
Labial Palp	82.90	2.33	14.77	9.30
Labial Palp	81.73	3.23	15.04	9.68
Labial Palp	84.65	1.82	13.53	5.45
Labial Palp	87.72	0.59	11.69	1.18
Labial Palp	82.81	2.38	14.81	4.76
Labial Palp	81.74	3.17	15.09	7.67
Labial Palp	74.08	6.45	19.47	12.90
Labial Palp	80.77	3.51	15.72	10.53
Labial Palp	80.11	4.00	15.89	8.00
Labial Palp	84.04	2.33	13.63	9.3
Labial Palp	74.71	6.95	18.34	12.07

Table B.3. Data for heart and labial palps for the analysis of PCNA distribution in relation to the cell cycle (Chapter 1).

	Cell Cycle			Positive PCNA			Positive PCNA		
Tissue	**%G0/G1**	**%S**	**%G2/M**	**%G0/G1**	**%S**	**%G2/M**	**%Late G0/G1**	**%S**	**%Early G2/M**
Heart	84.71	1.67	13.62	79.43	9.17	11.40	57.01	20.34	22.65
Heart	81.33	2.54	16.13	74.66	15.45	9.89	38.77	35.29	25.94
Heart	83.89	2.01	14.10	78.52	9.78	11.70	56.36	21.11	22.53
Heart	85.22	1.27	13.51	81.92	8.56	9.52	60.32	19.67	20.01
Heart	82.47	2.56	14.97	75.28	15.12	9.60	38.11	34.05	27.84
Heart	78.96	3.98	17.06	72.77	20.23	7.00	32.42	39.21	28.37
Heart	80.77	2.97	16.26	76.89	16.14	6.97	37.94	35.68	26.38
Heart	80.49	2.37	17.14	79.72	9.46	10.82	59.03	19.17	21.80
Heart	77.62	4.34	18.04	71.02	20.13	8.85	34.92	38.52	26.56
Heart	84.97	2.29	12.74	76.17	17.13	6.70	32.98	36.76	30.26
Labial Palps	85.98	1.76	12.26	78.14	12.17	9.69	37.18	33.49	29.33
Labial Palps	80.21	2.87	16.92	75.31	16.45	8.24	43.62	36.52	19.86
Labial Palps	84.78	2.21	13.01	77.61	13.78	8.61	38.71	32.67	28.62
Labial Palps	86.21	1.39	12.40	79.39	11.56	9.05	41.85	27.56	30.59
Labial Palps	80.75	2.01	17.24	76.28	14.12	9.60	53.17	26.99	19.84
Labial Palps	79.10	3.65	17.25	74.37	17.23	8.40	45.82	36.76	17.42
Labial Palps	80.67	2.69	16.64	76.78	13.14	10.08	50.36	29.45	20.19
Labial Palps	80.82	2.04	17.14	78.36	12.46	9.18	45.26	24.98	29.76
Labial Palps	78.86	3.21	17.93	74.77	16.13	9.10	55.23	25.78	18.99
Labial Palps	83.53	2.47	14.00	76.89	15.13	7.98	53.44	26.11	20.45

Table B.4. Data of heart nuclei for the analysis of temperature effects on cell cycle and PCNA (Chapter 4 and 5).

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	initial	10	75.16	11.14	13.70	16.23
Heart	initial	10	75.79	4.45	19.76	7.61
Heart	initial	10	76.32	9.06	14.62	10.06
Heart	initial	10	74.12	10.51	15.37	14.70
Heart	initial	10	81.59	4.81	13.61	6.54
Heart	initial	10	76.96	9.11	13.93	15.46
Heart	initial	10	75.32	9.31	15.37	12.29
Heart	initial	10	77.38	8.59	14.02	12.61
Heart	initial	10	75.56	8.47	15.97	11.91
Heart	initial	10	79.95	3.51	16.54	7.27
Heart	initial	10	80.87	4.58	14.55	7.76
Heart	initial	10	78.68	5.22	16.10	10.09
Heart	initial	10	78.68	6.36	14.96	10.16
Heart	3 weeks after	10	81.55	7.26	11.19	10.21
Heart	3 weeks after	10	80.96	6.12	12.92	7.17
Heart	3 weeks after	10	76.87	4.05	19.09	8.34
Heart	3 weeks after	10	72.96	10.66	16.38	12.96
Heart	3 weeks after	10	71.82	10.97	17.21	14.12
Heart	3 weeks after	10	70.68	6.69	22.63	10.92
Heart	3 weeks after	10	77.76	7.93	14.30	13.76
Heart	3 weeks after	10	73.61	9.73	16.66	15.79
Heart	3 weeks after	10	73.60	8.46	17.94	14.09
Heart	3 weeks after	10	79.25	7.13	13.62	12.05
Heart	3 weeks after	10	78.20	7.17	14.63	13.73
Heart	3 weeks after	10	77.95	4.76	17.29	8.44
Heart	3 weeks after	10	75.33	9.64	15.03	16.06

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	3 weeks after	10	78.42	4.24	17.34	8.12
Heart	3 weeks after	10	78.66	9.73	11.62	17.64
Heart	before	10	82.80	6.48	10.71	8.72
Heart	before	10	80.84	5.64	13.51	8.32
Heart	before	10	75.30	5.86	18.85	8.54
Heart	before	10	75.23	8.26	16.52	13.72
Heart	before	10	72.36	9.46	18.19	21.13
Heart	before	10	71.61	9.54	18.85	15.85
Heart	before	10	79.47	5.13	15.40	11.67
Heart	before	10	74.70	8.28	17.02	15.39
Heart	before	10	72.51	10.71	16.78	22.01
Heart	before	10	78.48	7.78	13.74	13.17
Heart	before	10	76.87	10.03	13.10	22.26
Heart	before	10	78.49	6.06	15.46	14.21
Heart	before	10	79.71	8.14	12.16	16.37
Heart	before	10	77.52	5.08	17.40	9.31
Heart	before	10	78.83	7.73	13.43	15.08
Heart	1week	10±1	73.16	9.93	16.91	20.74
Heart	1week	10±1	80.33	3.09	16.58	13.49
Heart	1week	10±1	82.43	3.34	14.23	14.62
Heart	1week	10±1	81.81	3.24	14.95	13.75
Heart	1week	10±1	69.76	14.00	16.23	31.41
Heart	1week	10±1	81.91	2.52	15.57	7.23
Heart	1week	10±1	77.47	3.72	18.81	15.93
Heart	1week	10±1	80.26	2.64	17.09	12.80
Heart	1week	10±1	81.38	3.82	14.80	15.26
Heart	1week	10±1	85.21	2.03	12.76	9.85

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	2weeks	10±1	78.26	6.36	15.39	22.81
Heart	2weeks	10±1	78.87	4.08	17.05	18.30
Heart	2weeks	10±1	74.76	7.25	17.98	25.62
Heart	2weeks	10±1	76.26	5.33	18.41	18.75
Heart	2weeks	10±1	83.37	2.68	13.94	9.61
Heart	2weeks	10±1	80.71	3.78	15.51	14.63
Heart	2weeks	10±1	78.26	3.54	18.19	14.53
Heart	2weeks	10±1	80.18	2.54	17.28	10.79
Heart	2weeks	10±1	81.48	3.58	14.94	15.38
Heart	2weeks	10±1	78.54	5.27	16.20	15.40
Heart	2weeks	10±1	77.90	4.26	17.84	15.18
Heart	2weeks	10±1	81.34	3.49	15.17	17.38
Heart	5weeks	10±1	80.52	5.09	14.39	20.32
Heart	5weeks	10±1	82.23	5.13	12.64	19.26
Heart	5weeks	10±1	81.13	6.65	12.23	22.68
Heart	5weeks	10±1	76.66	8.11	15.24	24.80
Heart	5weeks	10±1	78.65	7.16	14.19	23.48
Heart	5weeks	10±1	82.77	6.16	11.08	20.35
Heart	5weeks	10±1	78.55	7.23	14.22	23.88
Heart	5weeks	10±1	80.35	8.33	11.32	25.73
Heart	5weeks	10±1	82.24	5.29	12.47	15.13
Heart	5weeks	10±1	74.37	8.74	16.89	31.99
Heart	5weeks	10±1	79.53	6.14	14.33	21.92

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	initial	15	75.16	11.14	13.70	16.23
Heart	initial	15	75.79	4.45	19.76	7.61
Heart	initial	15	76.32	9.06	14.62	10.06
Heart	initial	15	74.12	10.51	15.37	14.70
Heart	initial	15	81.59	4.81	13.61	6.54
Heart	initial	15	76.96	9.11	13.93	15.46
Heart	initial	15	75.32	9.31	15.37	12.29
Heart	initial	15	77.38	8.59	14.02	12.61
Heart	initial	15	75.56	8.47	15.97	11.91
Heart	initial	15	79.95	3.51	16.54	7.27
Heart	initial	15	80.87	4.58	14.55	7.76
Heart	initial	15	78.68	5.22	16.10	10.09
Heart	initial	15	78.68	6.36	14.96	10.16
Heart	3 weeks after	15	81.55	7.26	11.19	10.21
Heart	3 weeks after	15	80.96	6.12	12.92	7.17
Heart	3 weeks after	15	76.87	4.05	19.09	8.34
Heart	3 weeks after	15	72.96	10.66	16.38	12.96
Heart	3 weeks after	15	71.82	10.97	17.21	14.12
Heart	3 weeks after	15	70.68	6.69	22.63	10.92
Heart	3 weeks after	15	77.76	7.93	14.30	13.76
Heart	3 weeks after	15	73.61	9.73	16.66	15.79
Heart	3 weeks after	15	73.60	8.46	17.94	14.09
Heart	3 weeks after	15	79.25	7.13	13.62	12.05
Heart	3 weeks after	15	78.20	7.17	14.63	13.73
Heart	3 weeks after	15	77.95	4.76	17.29	8.44
Heart	3 weeks after	15	75.33	9.64	15.03	16.06
Heart	3 weeks after	15	78.42	4.24	17.34	8.12

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	3 weeks after	15	78.66	9.73	11.62	17.64
Heart	before	15	76.15	9.68	14.17	16.32
Heart	before	15	86.62	4.63	8.75	7.54
Heart	before	15	81.06	7.01	11.93	15.20
Heart	before	15	83.76	4.47	11.77	8.83
Heart	before	15	81.30	5.40	13.31	11.24
Heart	before	15	80.99	5.06	13.96	10.39
Heart	before	15	79.65	7.93	12.42	14.34
Heart	before	15	77.50	5.25	17.25	9.93
Heart	before	15	81.99	6.61	11.41	12.38
Heart	before	15	76.12	6.26	17.62	12.11
Heart	before	15	79.60	5.17	15.23	9.88
Heart	before	15	80.29	9.20	10.51	16.00
Heart	before	15	79.86	7.47	12.66	14.46
Heart	before	15	83.15	6.79	10.06	13.36
Heart	before	15	80.37	8.65	10.98	16.46
Heart	1week	15±1	78.07	5.44	16.49	16.70
Heart	1week	15±1	65.22	11.92	22.86	30.80
Heart	1week	15±1	78.54	6.74	14.72	22.99
Heart	1week	15±1	72.76	7.67	19.57	23.44
Heart	1week	15±1	71.42	8.73	19.85	22.86
Heart	1week	15±1	70.80	8.98	20.23	24.60
Heart	1week	15±1	72.39	8.58	19.04	24.30
Heart	1week	15±1	82.07	5.82	12.10	14.66
Heart	1week	15±1	76.87	5.64	17.48	16.70
Heart	1week	15±1	76.75	6.29	16.96	17.86
Heart	1week	15±1	80.44	6.20	13.36	14.06

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	2weeks	15±1	75.42	6.12	18.46	17.90
Heart	2weeks	15±1	72.41	10.65	16.94	22.07
Heart	2weeks	15±1	66.94	12.18	20.88	23.59
Heart	2weeks	15±1	79.54	5.89	14.57	16.86
Heart	2weeks	15±1	73.58	9.89	16.53	24.41
Heart	2weeks	15±1	81.66	7.10	11.24	16.91
Heart	2weeks	15±1	81.64	4.30	14.06	14.03
Heart	2weeks	15±1	78.18	4.49	17.33	15.64
Heart	2weeks	15±1	81.55	4.76	13.69	18.52
Heart	2weeks	15±1	84.36	3.98	11.66	12.79
Heart	2weeks	15±1	82.33	3.85	13.82	16.10
Heart	2weeks	15±1	80.33	3.96	15.71	16.26
Heart	5weeks	15±1	85.44	2.62	11.94	10.56
Heart	5weeks	15±1	76.90	7.16	15.94	17.95
Heart	5weeks	15±1	82.85	4.50	12.65	13.31
Heart	5weeks	15±1	89.18	3.84	6.98	11.13
Heart	5weeks	15±1	80.40	4.54	15.06	12.57
Heart	5weeks	15±1	76.59	10.65	12.76	22.53
Heart	5weeks	15±1	80.93	4.81	14.26	9.62
Heart	5weeks	15±1	76.26	9.68	14.06	24.65
Heart	5weeks	15±1	82.96	3.78	13.26	15.24
Heart	5weeks	15±1	75.68	8.52	15.80	26.77

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	initial	20	75.16	11.14	13.70	16.23
Heart	initial	20	75.79	4.45	19.76	7.61
Heart	initial	20	76.32	9.06	14.62	10.06
Heart	initial	20	74.12	10.51	15.37	14.70
Heart	initial	20	81.59	4.81	13.61	6.54
Heart	initial	20	76.96	9.11	13.93	15.46
Heart	initial	20	75.32	9.31	15.37	12.29
Heart	initial	20	77.38	8.59	14.02	12.61
Heart	initial	20	75.56	8.47	15.97	11.91
Heart	initial	20	79.95	3.51	16.54	7.27
Heart	initial	20	80.87	4.58	14.55	7.76
Heart	initial	20	78.68	5.22	16.10	10.09
Heart	initial	20	78.68	6.36	14.96	10.16
Heart	3 weeks after	20	73.80	7.26	18.52	10.21
Heart	3 weeks after	20	65.65	6.12	25.17	7.17
Heart	3 weeks after	20	65.57	4.05	24.46	8.34
Heart	3 weeks after	20	58.92	10.66	31.88	12.96
Heart	3 weeks after	20	63.97	10.97	26.07	14.12
Heart	3 weeks after	20	74.58	6.69	21.31	10.92
Heart	3 weeks after	20	72.40	7.93	20.75	13.76
Heart	3 weeks after	20	70.52	9.73	19.03	15.79
Heart	3 weeks after	20	75.88	8.46	17.61	14.09
Heart	3 weeks after	20	71.37	7.13	17.82	12.05
Heart	3 weeks after	20	66.57	7.17	25.51	13.73
Heart	3 weeks after	20	68.72	4.76	16.74	8.44
Heart	3 weeks after	20	72.98	9.64	18.33	16.06
Heart	3 weeks after	20	75.20	4.24	14.36	8.12

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	before	20	84.64	4.28	11.08	12.51
Heart	before	20	87.27	3.66	9.08	12.75
Heart	before	20	83.70	6.00	10.30	14.94
Heart	before	20	85.23	4.20	10.57	11.56
Heart	before	20	83.06	5.05	11.89	13.43
Heart	before	20	82.44	4.46	13.10	11.90
Heart	before	20	84.27	4.95	10.78	12.90
Heart	before	20	81.54	5.14	13.32	13.68
Heart	before	20	82.12	6.52	11.36	14.43
Heart	before	20	79.56	4.44	16.00	12.95
Heart	before	20	76.40	7.98	15.62	19.50
Heart	before	20	79.14	6.59	14.28	17.10
Heart	before	20	80.64	5.23	14.13	13.07
Heart	before	20	75.13	10.47	14.40	22.47
Heart	1week	20±1	77.53	7.40	15.07	27.69
Heart	1week	20±1	77.41	6.59	16.00	25.84
Heart	1week	20±1	75.61	6.00	18.39	25.38
Heart	1week	20±1	70.35	15.15	14.50	37.22
Heart	1week	20±1	73.66	12.58	13.75	33.13
Heart	1week	20±1	70.72	10.79	18.49	31.54
Heart	1week	20±1	72.61	5.94	21.46	21.34
Heart	1week	20±1	84.49	3.36	12.14	19.94
Heart	1week	20±1	75.10	10.46	14.45	26.66
Heart	1week	20±1	76.45	8.15	15.41	27.36
Heart	1week	20±1	73.68	7.73	18.59	27.33
Heart	1week	20±1	73.26	7.25	19.49	21.56
Heart	2weeks	20±1	77.70	5.49	16.81	15.00

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	2weeks	20±1	79.00	5.00	16.00	17.22
Heart	2weeks	20±1	73.48	14.11	12.40	21.39
Heart	2weeks	20±1	71.92	12.36	15.71	22.77
Heart	2weeks	20±1	77.87	4.29	17.84	14.89
Heart	2weeks	20±1	72.05	13.07	14.88	26.92
Heart	2weeks	20±1	70.56	13.26	16.18	27.85
Heart	2weeks	20±1	73.10	7.65	19.25	19.97
Heart	2weeks	20±1	77.78	6.97	15.26	15.12
Heart	2weeks	20±1	85.46	6.06	8.48	11.49
Heart	2weeks	20±1	78.29	7.43	14.28	18.29
Heart	2weeks	20±1	77.83	8.00	14.17	18.78
Heart	5weeks	20±1	79.33	3.45	17.22	16.72
Heart	5weeks	20±1	78.19	7.83	13.98	14.20
Heart	5weeks	20±1	86.42	1.79	11.80	5.51
Heart	5weeks	20±1	82.91	5.53	11.56	13.43
Heart	5weeks	20±1	78.74	8.47	12.79	25.95
Heart	5weeks	20±1	80.55	6.92	12.53	21.66
Heart	5weeks	20±1	82.50	5.83	11.66	17.01
Heart	5weeks	20±1	82.46	7.73	9.81	23.15
Heart	5weeks	20±1	80.85	6.27	12.88	18.58

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	1week	10±5	83.30	3.95	16.91	14.90
Heart	1week	10±5	81.40	3.37	16.58	10.46
Heart	1week	10±5	82.53	3.64	14.23	11.59
Heart	1week	10±5	77.60	5.14	14.95	17.83
Heart	1week	10±5	84.34	3.70	16.23	13.24
Heart	1week	10±5	79.81	5.37	15.57	18.09
Heart	1week	10±5	84.52	3.16	18.81	12.10
Heart	1week	10±5	81.31	4.64	17.09	14.99
Heart	1week	10±5	85.21	4.34	14.80	14.04
Heart	1week	10±5	79.41	6.25	12.76	16.93
Heart	1week	10±5	79.48	4.07	16.45	16.18
Heart	1week	10±5	81.23	3.31	15.46	13.19
Heart	2weeks	10±5	78.41	8.89	12.70	22.69
Heart	2weeks	10±5	79.73	4.96	15.31	14.09
Heart	2weeks	10±5	76.16	8.19	15.65	20.14
Heart	2weeks	10±5	78.45	5.57	15.98	15.13
Heart	2weeks	10±5	74.00	5.61	20.38	18.10
Heart	2weeks	10±5	78.61	2.76	18.63	13.66
Heart	2weeks	10±5	79.52	4.41	16.06	16.00
Heart	2weeks	10±5	79.82	3.84	16.33	13.80
Heart	2weeks	10±5	80.09	4.05	15.86	13.06
Heart	2weeks	10±5	79.40	3.99	16.61	15.82
Heart	2weeks	10±5	75.40	5.80	18.80	19.46
Heart	2weeks	10±5	79.21	4.09	16.70	15.82
Heart	5weeks	10±5	78.87	5.77	15.36	18.86
Heart	5weeks	10±5	80.53	3.27	16.19	9.42
Heart	5weeks	10±5	84.23	4.54	11.23	14.64

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	5weeks	10±5	85.23	3.22	11.55	10.44
Heart	5weeks	10±5	82.04	4.30	13.66	18.51
Heart	5weeks	10±5	84.73	3.95	11.33	12.92
Heart	5weeks	10±5	82.69	3.94	13.37	17.42
Heart	5weeks	10±5	82.93	2.76	14.31	11.42
Heart	5weeks	10±5	77.15	5.01	17.84	24.10
Heart	5weeks	10±5	83.60	3.25	13.15	7.40
Heart	5weeks	10±5	77.77	3.98	18.25	15.95
Heart	5weeks	10±5	83.39	2.72	13.89	8.42
Heart	1week	15±5	77.26	5.62	17.12	21.39
Heart	1week	15±5	68.83	12.28	18.89	41.36
Heart	1week	15±5	69.86	13.80	16.34	45.61
Heart	1week	15±5	74.95	8.33	16.72	25.55
Heart	1week	15±5	72.38	9.73	17.89	22.45
Heart	1week	15±5	72.98	5.47	21.56	16.90
Heart	1week	15±5	79.13	3.86	17.01	18.39
Heart	1week	15±5	83.15	2.31	14.54	16.99
Heart	1week	15±5	83.49	2.96	13.54	15.36
Heart	2weeks	15±5	79.79	5.15	15.05	17.21
Heart	2weeks	15±5	77.99	4.40	17.62	16.43
Heart	2weeks	15±5	87.46	2.48	10.06	8.84
Heart	2weeks	15±5	78.89	3.97	17.15	16.98
Heart	2weeks	15±5	77.95	2.30	19.75	9.63
Heart	2weeks	15±5	79.07	3.18	17.75	14.40
Heart	2weeks	15±5	81.85	3.93	14.22	15.21
Heart	2weeks	15±5	80.15	3.91	15.94	14.43

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	2weeks	15±5	83.21	3.46	13.34	14.54
Heart	2weeks	15±5	85.88	4.67	9.45	19.56
Heart	2weeks	15±5	84.56	2.05	13.39	6.93
Heart	2weeks	15±5	83.42	2.90	13.68	12.14
Heart	5weeks	15±5	86.95	3.24	9.81	12.81
Heart	5weeks	15±5	82.62	3.93	13.46	11.89
Heart	5weeks	15±5	78.25	8.54	13.21	24.66
Heart	5weeks	15±5	90.05	3.12	6.83	13.86
Heart	5weeks	15±5	89.65	5.02	5.33	17.33
Heart	5weeks	15±5	85.74	5.35	8.91	15.66
Heart	5weeks	15±5	84.30	4.19	11.51	18.25
Heart	5weeks	15±5	84.48	2.27	13.25	13.79
Heart	5weeks	15±5	83.95	5.17	10.88	17.15
Heart	5weeks	15±5	75.22	9.02	15.76	26.68
Heart	5weeks	15±5	81.12	6.00	12.88	21.88
Heart	5weeks	15±5	79.01	4.52	16.47	14.37
Heart	1week	15±10	69.08	11.53	19.39	27.70
Heart	1week	15±10	67.66	12.83	19.51	29.60
Heart	1week	15±10	71.43	11.06	17.51	26.01
Heart	1week	15±10	70.48	8.92	20.60	18.65
Heart	1week	15±10	76.80	6.93	16.27	18.67
Heart	1week	15±10	65.51	17.36	17.12	34.37
Heart	1week	15±10	70.06	9.20	20.74	20.49
Heart	1week	15±10	76.48	10.76	12.76	25.07
Heart	1week	15±10	74.45	9.41	16.14	18.69
Heart	2weeks	15±10	77.75	5.86	16.40	19.86

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	2weeks	15±10	81.43	2.60	15.97	10.15
Heart	2weeks	15±10	77.67	6.25	16.09	22.48
Heart	2weeks	15±10	84.80	4.15	11.04	12.50
Heart	2weeks	15±10	83.48	3.92	12.59	14.96
Heart	2weeks	15±10	79.21	1.58	19.21	11.06
Heart	2weeks	15±10	80.82	4.08	15.10	16.00
Heart	2weeks	15±10	82.03	3.01	14.96	12.75
Heart	2weeks	15±10	76.15	7.05	16.80	19.33
Heart	2weeks	15±10	83.10	4.75	12.15	12.24
Heart	2weeks	15±10	80.59	4.79	14.62	17.98
Heart	2weeks	15±10	81.34	3.21	15.45	13.70
Heart	5weeks	15±10	78.28	6.33	15.39	22.59
Heart	5weeks	15±10	85.16	3.58	11.26	14.33
Heart	5weeks	15±10	78.51	5.81	15.69	20.25
Heart	5weeks	15±10	86.27	5.86	7.87	17.75
Heart	5weeks	15±10	87.00	5.80	7.20	18.07
Heart	5weeks	15±10	82.42	5.62	11.96	17.45
Heart	5weeks	15±10	80.04	7.35	12.61	23.59
Heart	5weeks	15±10	81.96	5.30	12.74	16.05
Heart	5weeks	15±10	79.33	8.45	12.22	22.09
Heart	5weeks	15±10	76.57	7.68	15.76	28.37
Heart	1week	20±5	75.81	10.06	14.12	35.14
Heart	1week	20±5	70.94	7.15	21.91	25.01
Heart	1week	20±5	76.83	8.48	14.69	31.42
Heart	1week	20±5	74.23	9.67	16.10	31.36
Heart	1week	20±5	73.62	8.54	17.84	30.73

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	1week	20±5	72.65	4.99	22.36	11.99
Heart	1week	20±5	77.45	3.91	18.64	12.33
Heart	1week	20±5	84.14	2.59	13.27	8.21
Heart	1week	20±5	54.67	6.40	38.93	16.97
Heart	1week	20±5	56.70	8.59	34.71	21.37
Heart	2weeks	20±5	81.27	5.03	13.70	16.97
Heart	2weeks	20±5	76.15	7.23	16.62	20.50
Heart	2weeks	20±5	80.52	5.90	13.58	15.37
Heart	2weeks	20±5	74.80	11.17	14.03	33.29
Heart	2weeks	20±5	83.90	4.97	11.13	13.76
Heart	2weeks	20±5	79.75	8.40	11.85	18.68
Heart	2weeks	20±5	79.16	6.45	14.39	20.72
Heart	2weeks	20±5	80.60	5.54	13.86	21.02
Heart	2weeks	20±5	78.51	6.93	14.56	19.41
Heart	2weeks	20±5	88.61	2.91	8.48	13.45
Heart	2weeks	20±5	79.67	5.54	14.79	19.50
Heart	2weeks	20±5	79.07	6.37	14.56	17.68
Heart	5weeks	20±5	84.15	3.14	12.72	12.36
Heart	5weeks	20±5	80.26	5.13	14.61	17.02
Heart	5weeks	20±5	82.59	4.56	12.85	14.88
Heart	5weeks	20±5	84.04	6.54	9.42	25.30
Heart	5weeks	20±5	80.59	6.89	12.52	20.97
Heart	5weeks	20±5	85.18	5.41	9.41	21.36
Heart	5weeks	20±5	81.47	5.50	13.02	20.38
Heart	5weeks	20±5	81.64	5.70	12.66	21.47
Heart	5weeks	20±5	84.29	3.09	12.63	10.55

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	initial	27	75.16	11.14	13.70	16.23
Heart	initial	27	75.79	4.45	19.76	7.61
Heart	initial	27	76.32	9.06	14.62	10.06
Heart	initial	27	74.12	10.51	15.37	14.70
Heart	initial	27	81.59	4.81	13.61	6.54
Heart	initial	27	76.96	9.11	13.93	15.46
Heart	initial	27	75.32	9.31	15.37	12.29
Heart	initial	27	77.38	8.59	14.02	12.61
Heart	initial	27	75.56	8.47	15.97	11.91
Heart	initial	27	79.95	3.51	16.54	7.27
Heart	initial	27	80.87	4.58	14.55	7.76
Heart	initial	27	78.68	5.22	16.10	10.09
Heart	initial	27	78.68	6.36	14.96	10.16
Heart	3 weeks after	27	81.55	7.26	11.19	10.21
Heart	3 weeks after	27	80.96	6.12	12.92	7.17
Heart	3 weeks after	27	76.87	4.05	19.09	8.34
Heart	3 weeks after	27	72.96	10.66	16.38	12.96
Heart	3 weeks after	27	71.82	10.97	17.21	14.12
Heart	3 weeks after	27	70.68	6.69	22.63	10.92
Heart	3 weeks after	27	77.76	7.93	14.30	13.76
Heart	3 weeks after	27	73.61	9.73	16.66	15.79
Heart	3 weeks after	27	73.60	8.46	17.94	14.09
Heart	3 weeks after	27	79.25	7.13	13.62	12.05
Heart	3 weeks after	27	78.20	7.17	14.63	13.73
Heart	3 weeks after	27	77.95	4.76	17.29	8.44
Heart	3 weeks after	27	75.33	9.64	15.03	16.06
Heart	3 weeks after	27	78.42	4.24	17.34	8.12

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	3 weeks after	27	78.66	9.73	11.62	17.64
Heart	before	27	84.87	4.95	10.18	14.66
Heart	before	27	81.90	5.12	12.98	17.83
Heart	before	27	77.99	9.58	12.43	22.56
Heart	before	27	82.33	6.34	11.33	16.45
Heart	before	27	82.04	9.25	8.70	20.78
Heart	before	27	83.48	2.92	13.60	12.49
Heart	before	27	75.46	6.39	18.15	16.05
Heart	before	27	75.73	5.90	18.37	16.90
Heart	before	27	72.28	8.76	18.96	19.49
Heart	before	27	68.93	12.33	18.74	27.04
Heart	before	27	79.42	4.18	16.40	14.91
Heart	before	27	76.08	7.25	16.67	18.27
Heart	before	27	76.87	10.19	12.94	19.08
Heart	before	27	71.37	11.33	17.30	23.27
Heart	before	27	80.26	8.61	11.13	20.09
Heart	1week	27	73.05	9.29	17.66	30.62
Heart	1week	27	67.82	9.49	22.68	26.33
Heart	1week	27	67.82	13.99	18.19	33.43
Heart	1week	27	69.63	12.95	17.42	34.74
Heart	1week	27	72.53	10.87	16.60	31.32
Heart	1week	27	70.40	15.57	14.03	36.39
Heart	1week	27	74.38	6.01	19.61	20.27
Heart	2weeks	27	83.39	2.58	14.03	6.02
Heart	2weeks	27	74.62	8.34	17.04	19.82
Heart	2weeks	27	79.19	5.71	15.10	16.74
Heart	2weeks	27	80.66	5.31	14.02	14.19

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	2weeks	27	75.79	6.60	17.61	15.07
Heart	2weeks	27	79.26	7.48	13.26	22.72
Heart	2weeks	27	77.06	5.75	17.19	14.65
Heart	2weeks	27	69.18	16.49	14.33	34.07
Heart	2weeks	27	82.25	4.18	13.57	15.50
Heart	2weeks	27	80.91	4.69	14.40	14.34
Heart	2weeks	27	84.04	5.63	10.33	18.23
Heart	2weeks	27	73.29	11.47	15.24	24.70
Heart	5weeks	27	79.60	6.37	14.04	19.32
Heart	5weeks	27	79.08	6.13	14.79	18.82
Heart	5weeks	27	85.99	3.77	10.24	11.05
Heart	5weeks	27	85.21	3.31	11.47	9.81
Heart	5weeks	27	80.75	5.80	13.45	17.31
Heart	5weeks	27	79.10	4.91	15.99	19.63
Heart	5weeks	27	83.67	3.96	12.38	14.68
Heart	5weeks	27	70.82	10.53	18.65	25.17
Heart	5weeks	27	78.86	5.94	15.20	19.94
Heart	5weeks	27	78.53	6.33	15.14	17.90
Heart	initial	GI	75.16	11.14	13.70	16.23
Heart	initial	GI	75.79	4.45	19.76	7.61
Heart	initial	GI	76.32	9.06	14.62	10.06
Heart	initial	GI	74.12	10.51	15.37	14.70
Heart	initial	GI	81.59	4.81	13.61	6.54
Heart	initial	GI	76.96	9.11	13.93	15.46
Heart	initial	GI	75.32	9.31	15.37	12.29
Heart	initial	GI	77.38	8.59	14.02	12.61

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	initial	GI	75.56	8.47	15.97	11.91
Heart	initial	GI	79.95	3.51	16.54	7.27
Heart	initial	GI	80.87	4.58	14.55	7.76
Heart	initial	GI	78.68	5.22	16.10	10.09
Heart	initial	GI	78.68	6.36	14.96	10.16
Heart	3 weeks after	GI	81.55	7.26	11.19	10.21
Heart	3 weeks after	GI	80.96	6.12	12.92	7.17
Heart	3 weeks after	GI	76.87	4.05	19.09	8.34
Heart	3 weeks after	GI	72.96	10.66	16.38	12.96
Heart	3 weeks after	GI	71.82	10.97	17.21	14.12
Heart	3 weeks after	GI	70.68	6.69	22.63	10.92
Heart	3 weeks after	GI	77.76	7.93	14.30	13.76
Heart	3 weeks after	GI	73.61	9.73	16.66	15.79
Heart	3 weeks after	GI	73.60	8.46	17.94	14.09
Heart	3 weeks after	GI	79.25	7.13	13.62	12.05
Heart	3 weeks after	GI	78.20	7.17	14.63	13.73
Heart	3 weeks after	GI	77.95	4.76	17.29	8.44
Heart	3 weeks after	GI	75.33	9.64	15.03	16.06
Heart	3 weeks after	GI	78.42	4.24	17.34	8.12
Heart	3 weeks after	GI	78.66	9.73	11.62	17.64
Heart	before	GI	71.47	14.48	14.05	26.43
Heart	before	GI	78.11	8.49	13.40	21.40
Heart	before	GI	75.04	11.72	13.25	26.24
Heart	before	GI	73.38	8.26	18.36	20.72
Heart	before	GI	77.81	7.83	14.36	18.23
Heart	before	GI	72.05	10.50	17.45	25.61
Heart	before	GI	74.26	10.30	15.44	22.76

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	before	GI	69.79	10.34	19.87	25.26
Heart	before	GI	73.35	8.54	18.11	19.80
Heart	before	GI	74.61	7.15	18.24	17.11
Heart	before	GI	72.67	7.77	19.56	20.31
Heart	before	GI	77.74	7.95	14.31	15.29
Heart	before	GI	76.74	6.58	16.68	17.97
Heart	before	GI	74.18	10.18	15.64	21.28
Heart	before	GI	76.00	7.84	16.16	21.89
Heart	1week	GI	70.25	7.29	22.45	30.40
Heart	1week	GI	74.99	5.88	19.13	31.00
Heart	1week	GI	70.74	5.56	23.70	25.00
Heart	1week	GI	72.17	7.66	20.18	31.45
Heart	1week	GI	75.32	5.94	18.74	22.54
Heart	1week	GI	84.80	3.56	11.64	14.02
Heart	1week	GI	77.71	4.28	18.00	16.94
Heart	1week	GI	80.31	4.80	14.88	15.37
Heart	1week	GI	83.94	4.10	11.96	9.79
Heart	1week	GI	76.37	4.08	19.55	19.33
Heart	2weeks	GI	70.06	14.57	15.38	28.93
Heart	2weeks	GI	80.11	3.91	15.98	14.03
Heart	2weeks	GI	77.47	4.43	18.10	13.66
Heart	2weeks	GI	75.65	8.86	15.49	30.04
Heart	2weeks	GI	81.01	2.99	16.00	10.88
Heart	2weeks	GI	78.08	4.61	17.30	11.46
Heart	2weeks	GI	83.66	4.33	12.01	12.42
Heart	2weeks	GI	80.78	5.05	14.17	19.58
Heart	2weeks	GI	78.97	2.27	18.76	7.71

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Heart	2weeks	GI	77.55	6.45	16.00	22.10
Heart	2weeks	GI	80.82	4.67	14.50	13.89
Heart	2weeks	GI	78.27	3.77	17.97	16.29
Heart	5weeks	GI	78.47	9.55	11.98	21.33
Heart	5weeks	GI	74.17	9.74	16.08	21.60
Heart	5weeks	GI	80.38	6.91	12.71	20.70
Heart	5weeks	GI	91.22	3.25	5.53	7.80
Heart	5weeks	GI	90.65	2.94	6.41	8.16
Heart	5weeks	GI	79.24	7.85	12.91	20.22
Heart	5weeks	GI	79.42	6.53	14.05	18.44
Heart	5weeks	GI	80.99	6.46	12.55	21.17
Heart	5weeks	GI	77.60	4.63	17.77	18.17
Heart	5weeks	GI	80.02	5.21	14.77	15.75
Heart	5weeks	GI	77.06	5.61	17.33	16.93

Table B.5. Data of labial palp nuclei for the analysis of temperature effects on cell cycle and PCNA (Chapter 4 and 5).

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	initial	10	89.23	2.03	8.74	9.96
Labial palps	initial	10	80.89	8.61	10.51	19.92
Labial palps	initial	10	83.30	6.15	10.54	17.07
Labial palps	initial	10	84.17	5.81	10.02	15.53
Labial palps	initial	10	87.36	3.88	8.76	13.11
Labial palps	initial	10	91.54	1.80	6.66	11.22
Labial palps	initial	10	90.89	2.03	7.08	12.92
Labial palps	initial	10	89.75	1.87	8.38	10.53
Labial palps	initial	10	89.61	3.54	6.85	11.45
Labial palps	initial	10	84.38	6.15	9.47	17.79
Labial palps	initial	10	80.98	4.39	14.64	11.18
Labial palps	initial	10	81.80	4.82	13.38	13.12
Labial palps	initial	10	85.69	4.15	10.16	10.12
Labial palps	initial	10	69.03	9.33	21.64	26.02
Labial palps	initial	10	76.33	7.50	16.17	14.51
Labial palps	3 weeks after	10	73.80	7.68	18.52	12.31
Labial palps	3 weeks after	10	65.65	9.18	25.17	14.44
Labial palps	3 weeks after	10	65.57	9.97	24.46	15.81
Labial palps	3 weeks after	10	58.92	9.20	31.88	15.76
Labial palps	3 weeks after	10	63.97	9.96	26.07	16.11
Labial palps	3 weeks after	10	74.58	4.11	21.31	8.18
Labial palps	3 weeks after	10	72.40	6.85	20.75	12.40
Labial palps	3 weeks after	10	70.52	10.44	19.03	22.15
Labial palps	3 weeks after	10	75.88	6.51	17.61	11.96
Labial palps	3 weeks after	10	71.37	10.81	17.82	20.28
Labial palps	3 weeks after	10	66.57	7.92	25.51	14.35

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	3 weeks after	10	68.72	14.54	16.74	23.26
Labial palps	3 weeks after	10	72.98	8.69	18.33	16.97
Labial palps	3 weeks after	10	75.20	10.44	14.36	20.27
Labial palps	before	10±1	74.03	4.65	21.32	9.96
Labial palps	before	10±1	69.15	5.34	25.51	11.34
Labial palps	before	10±1	69.29	5.81	24.91	11.17
Labial palps	before	10±1	78.55	9.34	12.12	17.82
Labial palps	before	10±1	63.07	10.20	26.73	18.20
Labial palps	before	10±1	74.68	3.09	22.22	9.14
Labial palps	before	10±1	73.48	8.14	18.38	14.26
Labial palps	before	10±1	69.43	11.52	19.05	20.70
Labial palps	before	10±1	78.50	4.46	17.04	8.91
Labial palps	before	10±1	72.02	8.56	19.42	16.29
Labial palps	before	10±1	66.02	13.03	20.95	19.30
Labial palps	before	10±1	67.46	14.34	18.20	21.51
Labial palps	before	10±1	72.79	8.44	18.76	13.71
Labial palps	before	10±1	77.26	10.69	12.05	16.47
Labial palps	1week	10±1	71.18	7.22	21.60	20.37
Labial palps	1week	10±1	74.77	5.35	19.88	15.82
Labial palps	1week	10±1	71.77	6.12	22.10	14.75
Labial palps	1week	10±1	80.20	4.11	15.69	8.90
Labial palps	1week	10±1	72.57	6.95	20.48	15.41
Labial palps	1week	10±1	82.24	2.52	15.24	9.86
Labial palps	1week	10±1	69.86	5.61	24.53	16.22
Labial palps	1week	10±1	80.04	1.88	18.09	5.37
Labial palps	1week	10±1	79.42	2.61	17.97	13.62
Labial palps	1week	10±1	76.78	3.80	19.42	10.11

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	1week	10±1	83.62	1.70	14.68	4.76
Labial palps	1week	10±1	74.42	2.96	22.63	10.38
Labial palps	2weeks	10±1	76.93	3.03	20.05	8.93
Labial palps	2weeks	10±1	78.65	2.39	18.96	9.16
Labial palps	2weeks	10±1	73.60	5.80	20.59	16.35
Labial palps	2weeks	10±1	77.17	3.83	19.00	8.84
Labial palps	2weeks	10±1	75.92	4.12	19.95	13.44
Labial palps	2weeks	10±1	72.61	6.31	21.07	17.44
Labial palps	2weeks	10±1	76.92	2.03	21.04	8.27
Labial palps	2weeks	10±1	71.42	6.55	22.04	17.69
Labial palps	2weeks	10±1	74.74	3.41	21.85	15.27
Labial palps	2weeks	10±1	70.84	7.81	21.35	20.38
Labial palps	2weeks	10±1	82.74	3.74	13.51	9.20
Labial palps	2weeks	10±1	80.92	2.85	16.23	8.74
Labial palps	5weeks	10±1	74.82	6.86	18.32	15.20
Labial palps	5weeks	10±1	73.26	5.50	21.24	17.21
Labial palps	5weeks	10±1	72.45	7.46	20.09	22.33
Labial palps	5weeks	10±1	74.58	5.06	20.36	14.67
Labial palps	5weeks	10±1	79.08	3.07	17.85	12.27
Labial palps	5weeks	10±1	74.72	6.76	18.52	16.64
Labial palps	5weeks	10±1	72.31	7.40	20.29	18.27
Labial palps	5weeks	10±1	69.64	10.50	19.86	29.33
Labial palps	5weeks	10±1	78.71	4.69	16.61	16.04
Labial palps	5weeks	10±1	74.21	7.09	18.70	20.56
Labial palps	5weeks	10±1	73.78	7.62	18.60	19.77

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	initial	15	89.23	2.03	8.74	9.96
Labial palps	initial	15	80.89	8.61	10.51	19.92
Labial palps	initial	15	83.30	6.15	10.54	17.07
Labial palps	initial	15	84.17	5.81	10.02	15.53
Labial palps	initial	15	87.36	3.88	8.76	13.11
Labial palps	initial	15	91.54	1.80	6.66	11.22
Labial palps	initial	15	90.89	2.03	7.08	12.92
Labial palps	initial	15	89.75	1.87	8.38	10.53
Labial palps	initial	15	89.61	3.54	6.85	11.45
Labial palps	initial	15	84.38	6.15	9.47	17.79
Labial palps	initial	15	80.98	4.39	14.64	11.18
Labial palps	initial	15	81.80	4.82	13.38	13.12
Labial palps	initial	15	85.69	4.15	10.16	10.12
Labial palps	initial	15	69.03	9.33	21.64	26.02
Labial palps	initial	15	76.33	7.50	16.17	14.51
Labial palps	3 weeks after	15	73.80	7.68	18.52	12.31
Labial palps	3 weeks after	15	65.65	9.18	25.17	14.44
Labial palps	3 weeks after	15	65.57	9.97	24.46	15.81
Labial palps	3 weeks after	15	58.92	9.20	31.88	15.76
Labial palps	3 weeks after	15	63.97	9.96	26.07	16.11
Labial palps	3 weeks after	15	74.58	4.11	21.31	8.18
Labial palps	3 weeks after	15	72.40	6.85	20.75	12.40
Labial palps	3 weeks after	15	70.52	10.44	19.03	22.15
Labial palps	3 weeks after	15	75.88	6.51	17.61	11.96
Labial palps	3 weeks after	15	71.37	10.81	17.82	20.28
Labial palps	3 weeks after	15	66.57	7.92	25.51	14.35
Labial palps	3 weeks after	15	68.72	14.54	16.74	23.26

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	3 weeks after	15	72.98	8.69	18.33	16.97
Labial palps	3 weeks after	15	75.20	10.44	14.36	20.27
Labial palps	before	15	77.81	6.95	15.24	15.92
Labial palps	before	15	91.33	2.00	6.67	8.01
Labial palps	before	15	84.34	3.74	11.91	9.71
Labial palps	before	15	74.23	6.86	18.91	16.96
Labial palps	before	15	76.88	7.42	15.69	17.44
Labial palps	before	15	83.16	4.66	12.18	13.28
Labial palps	before	15	70.23	6.88	22.90	16.71
Labial palps	before	15	75.40	4.57	20.03	11.79
Labial palps	before	15	73.21	7.85	18.94	16.76
Labial palps	before	15	81.80	5.79	12.41	13.89
Labial palps	before	15	74.89	9.08	16.03	20.60
Labial palps	before	15	75.19	6.76	18.05	13.90
Labial palps	before	15	76.49	5.49	18.02	10.57
Labial palps	before	15	73.58	6.43	19.99	11.76
Labial palps	before	15	74.59	7.45	17.96	13.49
Labial palps	1week	15±1	62.46	11.57	25.97	24.33
Labial palps	1week	15±1	67.41	6.36	26.23	17.75
Labial palps	1week	15±1	62.91	9.31	27.78	21.83
Labial palps	1week	15±1	74.43	12.63	12.94	22.52
Labial palps	1week	15±1	67.94	11.15	20.92	24.83
Labial palps	1week	15±1	62.26	14.07	23.67	25.21
Labial palps	1week	15±1	72.93	14.88	12.19	24.74
Labial palps	1week	15±1	72.43	14.85	12.72	26.77
Labial palps	2weeks	15±1	70.38	9.35	20.27	28.43
Labial palps	2weeks	15±1	78.21	4.60	17.19	14.07

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	2weeks	15±1	74.27	8.10	17.63	22.78
Labial palps	2weeks	15±1	78.73	3.22	18.05	10.41
Labial palps	2weeks	15±1	78.93	3.41	17.66	9.33
Labial palps	2weeks	15±1	67.22	7.54	25.24	23.59
Labial palps	2weeks	15±1	80.96	3.07	15.97	8.69
Labial palps	2weeks	15±1	81.04	3.52	15.45	13.22
Labial palps	2weeks	15±1	80.14	1.61	18.25	7.81
Labial palps	2weeks	15±1	80.74	3.45	15.81	7.51
Labial palps	2weeks	15±1	75.87	3.27	20.86	11.37
Labial palps	2weeks	15±1	76.57	5.13	18.29	12.91
Labial palps	5weeks	15±1	82.71	2.60	14.69	7.71
Labial palps	5weeks	15±1	82.78	2.05	15.17	5.26
Labial palps	5weeks	15±1	82.21	2.33	15.47	5.28
Labial palps	5weeks	15±1	80.07	3.28	16.65	9.81
Labial palps	5weeks	15±1	81.57	4.49	13.94	12.26
Labial palps	5weeks	15±1	84.82	2.23	12.95	9.12
Labial palps	5weeks	15±1	82.95	3.57	13.48	11.32
Labial palps	5weeks	15±1	76.19	3.18	20.64	11.43
Labial palps	5weeks	15±1	79.85	5.09	15.06	14.54
Labial palps	initial	20	89.23	2.03	8.74	9.96
Labial palps	initial	20	80.89	8.61	10.51	19.92
Labial palps	initial	20	83.30	6.15	10.54	17.07
Labial palps	initial	20	84.17	5.81	10.02	15.53
Labial palps	initial	20	87.36	3.88	8.76	13.11
Labial palps	initial	20	91.54	1.80	6.66	11.22
Labial palps	initial	20	90.89	2.03	7.08	12.92

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	initial	20	89.75	1.87	8.38	10.53
Labial palps	initial	20	89.61	3.54	6.85	11.45
Labial palps	initial	20	84.38	6.15	9.47	17.79
Labial palps	initial	20	80.98	4.39	14.64	11.18
Labial palps	initial	20	81.80	4.82	13.38	13.12
Labial palps	initial	20	85.69	4.15	10.16	10.12
Labial palps	initial	20	69.03	9.33	21.64	26.02
Labial palps	initial	20	76.33	7.50	16.17	14.51
Labial palps	3 weeks after	20	73.80	7.68	18.52	12.31
Labial palps	3 weeks after	20	65.65	9.18	25.17	14.44
Labial palps	3 weeks after	20	65.57	9.97	24.46	15.81
Labial palps	3 weeks after	20	58.92	9.20	31.88	15.76
Labial palps	3 weeks after	20	63.97	9.96	26.07	16.11
Labial palps	3 weeks after	20	74.58	4.11	21.31	8.18
Labial palps	3 weeks after	20	72.40	6.85	20.75	12.40
Labial palps	3 weeks after	20	70.52	10.44	19.03	22.15
Labial palps	3 weeks after	20	75.88	6.51	17.61	11.96
Labial palps	3 weeks after	20	71.37	10.81	17.82	20.28
Labial palps	3 weeks after	20	66.57	7.92	25.51	14.35
Labial palps	3 weeks after	20	68.72	14.54	16.74	23.26
Labial palps	3 weeks after	20	72.98	8.69	18.33	16.97
Labial palps	3 weeks after	20	75.20	10.44	14.36	20.27
Labial palps	before	20	86.36	2.84	10.80	7.85
Labial palps	before	20	83.54	3.75	12.71	9.14
Labial palps	before	20	75.85	10.11	14.04	18.57
Labial palps	before	20	80.63	3.29	16.08	10.06
Labial palps	before	20	84.25	6.51	9.24	17.56

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	before	20	80.14	3.56	16.30	10.85
Labial palps	before	20	74.97	6.37	18.66	16.57
Labial palps	before	20	69.53	9.93	20.54	20.55
Labial palps	before	20	74.72	4.07	21.22	11.63
Labial palps	before	20	70.01	6.10	23.89	15.68
Labial palps	before	20	72.71	6.07	21.22	17.97
Labial palps	before	20	65.87	7.31	26.82	18.67
Labial palps	before	20	70.23	7.43	22.33	16.31
Labial palps	before	20	79.31	3.85	16.84	9.23
Labial palps	before	20	70.33	7.00	22.66	16.23
Labial palps	1week	20±1	67.46	7.51	25.03	13.49
Labial palps	1week	20±1	62.47	12.55	24.99	24.88
Labial palps	1week	20±1	61.66	13.72	24.61	26.31
Labial palps	1week	20±1	68.29	13.39	18.33	29.52
Labial palps	1week	20±1	66.72	15.53	17.75	36.53
Labial palps	1week	20±1	63.63	10.83	25.53	24.17
Labial palps	1week	20±1	70.59	5.30	24.10	15.06
Labial palps	1week	20±1	64.94	9.59	25.47	26.94
Labial palps	1week	20±1	69.72	4.23	26.05	14.07
Labial palps	1week	20±1	65.73	10.99	23.28	22.68
Labial palps	1week	20±1	66.77	11.02	22.20	25.43
Labial palps	1week	20±1	64.49	10.78	24.72	24.19
Labial palps	2weeks	20±1	72.86	8.72	18.42	22.05
Labial palps	2weeks	20±1	73.52	6.23	20.24	13.44
Labial palps	2weeks	20±1	74.95	4.58	20.48	11.68
Labial palps	2weeks	20±1	77.40	2.72	19.88	10.72
Labial palps	2weeks	20±1	78.28	5.58	16.14	17.24

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	2weeks	20±1	76.99	4.01	19.00	12.74
Labial palps	2weeks	20±1	69.92	7.35	22.73	16.58
Labial palps	2weeks	20±1	76.69	5.25	18.06	13.72
Labial palps	2weeks	20±1	72.38	5.99	21.64	17.51
Labial palps	2weeks	20±1	76.28	6.77	16.95	16.45
Labial palps	2weeks	20±1	79.11	4.33	16.56	9.63
Labial palps	2weeks	20±1	77.64	3.05	19.31	9.34
Labial palps	5weeks	20±1	84.83	2.76	12.41	10.01
Labial palps	5weeks	20±1	79.64	3.52	16.84	10.53
Labial palps	5weeks	20±1	82.23	2.28	15.49	8.57
Labial palps	5weeks	20±1	73.27	5.73	21.00	19.50
Labial palps	5weeks	20±1	78.51	5.48	16.01	17.51
Labial palps	5weeks	20±1	75.79	4.79	19.42	17.89
Labial palps	5weeks	20±1	79.95	5.20	14.85	17.01
Labial palps	5weeks	20±1	77.41	5.51	17.08	14.50
Labial palps	5weeks	20±1	73.04	6.99	19.97	21.65
Labial palps	1week	10±5	76.54	4.34	19.11	13.26
Labial palps	1week	10±5	77.99	2.36	19.64	10.10
Labial palps	1week	10±5	74.34	5.75	19.91	14.16
Labial palps	1week	10±5	81.21	2.24	16.54	11.60
Labial palps	1week	10±5	73.59	5.03	21.36	15.61
Labial palps	1week	10±5	76.68	4.02	19.29	12.77
Labial palps	1week	10±5	77.55	2.51	19.94	9.42
Labial palps	1week	10±5	78.64	3.38	17.98	10.59
Labial palps	1week	10±5	78.32	3.83	17.84	13.14
Labial palps	1week	10±5	76.50	4.12	19.38	13.53

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	1week	10±5	75.22	2.89	21.88	9.81
Labial palps	1week	10±5	77.25	2.57	20.18	10.42
Labial palps	2weeks	10±5	71.33	7.31	21.35	17.98
Labial palps	2weeks	10±5	75.84	3.28	20.88	14.51
Labial palps	2weeks	10±5	83.33	1.60	15.06	5.57
Labial palps	2weeks	10±5	77.30	2.81	19.88	10.26
Labial palps	2weeks	10±5	75.43	3.11	21.45	12.01
Labial palps	2weeks	10±5	80.86	1.77	17.36	8.10
Labial palps	2weeks	10±5	73.33	6.51	20.15	17.30
Labial palps	2weeks	10±5	82.09	1.91	15.99	6.94
Labial palps	2weeks	10±5	82.59	3.24	14.17	7.62
Labial palps	2weeks	10±5	75.54	3.50	20.96	9.33
Labial palps	2weeks	10±5	76.78	3.53	19.69	11.95
Labial palps	2weeks	10±5	81.20	4.44	14.36	15.62
Labial palps	5weeks	10±5	70.38	8.81	20.80	22.03
Labial palps	5weeks	10±5	78.77	6.61	14.60	12.06
Labial palps	5weeks	10±5	74.39	6.93	18.68	11.85
Labial palps	5weeks	10±5	74.12	6.56	19.31	15.22
Labial palps	5weeks	10±5	75.13	5.14	19.72	11.42
Labial palps	5weeks	10±5	69.72	12.34	17.93	21.97
Labial palps	5weeks	10±5	79.63	2.11	18.25	7.66
Labial palps	5weeks	10±5	73.13	6.40	20.45	13.08
Labial palps	5weeks	10±5	69.43	10.81	19.75	25.51
Labial palps	5weeks	10±5	75.49	6.55	17.95	10.11
Labial palps	5weeks	10±5	75.48	4.13	20.38	11.62
Labial palps	5weeks	10±5	76.31	2.85	20.82	8.29

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	1week	15±5	62.84	10.55	26.61	23.79
Labial palps	1week	15±5	66.67	8.64	24.69	22.84
Labial palps	1week	15±5	66.52	7.92	25.56	19.81
Labial palps	1week	15±5	73.98	7.18	18.84	17.78
Labial palps	1week	15±5	66.15	9.52	24.33	21.31
Labial palps	1week	15±5	62.87	8.06	29.07	20.14
Labial palps	1week	15±5	67.49	13.82	18.69	27.21
Labial palps	1week	15±5	70.89	10.78	18.33	23.03
Labial palps	2weeks	15±5	77.96	7.78	14.26	20.22
Labial palps	2weeks	15±5	79.28	5.03	15.69	14.61
Labial palps	2weeks	15±5	77.79	2.74	19.48	11.54
Labial palps	2weeks	15±5	78.65	4.84	16.51	18.30
Labial palps	2weeks	15±5	79.66	3.33	17.01	11.82
Labial palps	2weeks	15±5	77.72	4.70	17.59	14.84
Labial palps	2weeks	15±5	80.96	2.35	16.69	6.35
Labial palps	2weeks	15±5	79.46	6.29	14.25	18.19
Labial palps	2weeks	15±5	79.50	3.54	16.97	11.49
Labial palps	2weeks	15±5	80.22	3.36	16.42	12.06
Labial palps	2weeks	15±5	76.48	3.67	19.85	12.72
Labial palps	5weeks	15±5	74.00	8.02	17.98	13.83
Labial palps	5weeks	15±5	83.36	4.65	11.99	8.62
Labial palps	5weeks	15±5	73.86	4.40	21.74	8.96
Labial palps	5weeks	15±5	78.48	3.93	17.59	9.24
Labial palps	5weeks	15±5	81.57	4.16	14.27	10.87
Labial palps	5weeks	15±5	74.24	5.64	20.12	11.22
Labial palps	5weeks	15±5	80.54	5.45	14.01	10.50
Labial palps	5weeks	15±5	78.67	3.75	17.57	8.37

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	5weeks	15±5	72.79	11.20	16.01	25.13
Labial palps	1week	15±10	65.19	11.08	23.73	27.77
Labial palps	1week	15±10	68.47	10.43	21.11	26.36
Labial palps	1week	15±10	63.97	9.26	26.77	24.26
Labial palps	1week	15±10	61.86	14.76	23.38	36.09
Labial palps	1week	15±10	68.92	7.02	24.07	16.97
Labial palps	1week	15±10	63.94	11.32	24.73	26.92
Labial palps	1week	15±10	66.16	11.82	22.02	24.72
Labial palps	1week	15±10	63.77	12.67	23.57	29.41
Labial palps	2weeks	15±10	75.53	10.88	13.58	23.24
Labial palps	2weeks	15±10	79.13	1.84	19.03	5.90
Labial palps	2weeks	15±10	77.42	5.56	17.02	20.23
Labial palps	2weeks	15±10	75.66	2.93	21.42	11.19
Labial palps	2weeks	15±10	79.28	3.80	16.93	13.09
Labial palps	2weeks	15±10	80.71	5.35	13.94	12.05
Labial palps	2weeks	15±10	78.54	6.16	15.30	19.10
Labial palps	2weeks	15±10	79.31	3.13	17.56	12.04
Labial palps	2weeks	15±10	78.08	5.99	15.93	14.78
Labial palps	2weeks	15±10	80.97	2.28	16.76	7.58
Labial palps	2weeks	15±10	80.45	4.29	15.25	10.00
Labial palps	2weeks	15±10	77.53	3.06	19.41	11.13
Labial palps	5weeks	15±10	81.14	4.31	14.55	10.17
Labial palps	5weeks	15±10	78.51	8.47	13.02	23.33
Labial palps	5weeks	15±10	79.51	5.37	15.11	12.81
Labial palps	5weeks	15±10	75.88	6.25	17.87	18.07
Labial palps	5weeks	15±10	77.13	6.00	16.88	14.53

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	5weeks	15±10	77.61	5.03	17.36	9.14
Labial palps	5weeks	15±10	84.29	4.46	11.24	10.35
Labial palps	5weeks	15±10	77.89	3.97	18.15	15.86
Labial palps	5weeks	15±10	80.04	4.48	15.47	11.26
Labial palps	1week	20±5	65.67	8.64	25.68	13.65
Labial palps	1week	20±5	66.30	8.67	25.03	14.84
Labial palps	1week	20±5	63.13	10.38	26.48	21.87
Labial palps	1week	20±5	67.60	8.23	24.17	17.70
Labial palps	1week	20±5	65.10	9.35	25.55	18.38
Labial palps	1week	20±5	62.17	10.13	27.70	20.13
Labial palps	1week	20±5	75.97	12.38	11.65	29.94
Labial palps	1week	20±5	79.62	9.13	11.25	14.19
Labial palps	2weeks	20±5	73.35	4.58	22.07	14.23
Labial palps	2weeks	20±5	75.33	4.60	20.06	12.13
Labial palps	2weeks	20±5	73.14	5.71	21.16	15.98
Labial palps	2weeks	20±5	72.03	9.32	18.65	23.11
Labial palps	2weeks	20±5	78.11	5.06	16.83	11.62
Labial palps	2weeks	20±5	76.77	7.27	15.96	17.37
Labial palps	2weeks	20±5	70.73	7.99	21.28	21.22
Labial palps	2weeks	20±5	74.52	6.81	18.67	12.82
Labial palps	2weeks	20±5	78.83	5.57	15.60	16.55
Labial palps	2weeks	20±5	74.44	6.25	19.31	15.81
Labial palps	2weeks	20±5	75.45	7.03	17.52	18.12
Labial palps	2weeks	20±5	75.12	4.92	19.96	13.73
Labial palps	5weeks	20±5	79.27	2.41	18.32	12.94
Labial palps	5weeks	20±5	79.13	3.65	17.22	11.33

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	5weeks	20±5	85.58	0.99	13.43	6.09
Labial palps	5weeks	20±5	73.23	8.82	17.94	16.87
Labial palps	5weeks	20±5	76.33	5.82	17.84	15.79
Labial palps	5weeks	20±5	76.89	5.57	17.54	15.23
Labial palps	5weeks	20±5	76.55	6.63	16.82	18.48
Labial palps	5weeks	20±5	79.93	3.89	16.17	11.60
Labial palps	5weeks	20±5	78.85	2.92	18.24	13.70
Labial palps	initial	27	75.16	11.14	13.70	16.23
Labial palps	initial	27	75.79	4.45	19.76	7.61
Labial palps	initial	27	76.32	9.06	14.62	10.06
Labial palps	initial	27	74.12	10.51	15.37	14.70
Labial palps	initial	27	81.59	4.81	13.61	6.54
Labial palps	initial	27	76.96	9.11	13.93	15.46
Labial palps	initial	27	75.32	9.31	15.37	12.29
Labial palps	initial	27	77.38	8.59	14.02	12.61
Labial palps	initial	27	75.56	8.47	15.97	11.91
Labial palps	initial	27	79.95	3.51	16.54	7.27
Labial palps	initial	27	80.87	4.58	14.55	7.76
Labial palps	initial	27	78.68	5.22	16.10	10.09
Labial palps	initial	27	78.68	6.36	14.96	10.16
Labial palps	3 weeks after	27	81.55	7.26	11.19	10.21
Labial palps	3 weeks after	27	80.96	6.12	12.92	7.17
Labial palps	3 weeks after	27	76.87	4.05	19.09	8.34
Labial palps	3 weeks after	27	72.96	10.66	16.38	12.96
Labial palps	3 weeks after	27	71.82	10.97	17.21	14.12
Labial palps	3 weeks after	27	70.68	6.69	22.63	10.92

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	3 weeks after	27	77.76	7.93	14.30	13.76
Labial palps	3 weeks after	27	73.61	9.73	16.66	15.79
Labial palps	3 weeks after	27	73.60	8.46	17.94	14.09
Labial palps	3 weeks after	27	79.25	7.13	13.62	12.05
Labial palps	3 weeks after	27	78.20	7.17	14.63	13.73
Labial palps	3 weeks after	27	77.95	4.76	17.29	8.44
Labial palps	3 weeks after	27	75.33	9.64	15.03	16.06
Labial palps	3 weeks after	27	78.42	4.24	17.34	8.12
Labial palps	3 weeks after	27	78.66	9.73	11.62	17.64
Labial palps	before	27	84.87	4.95	10.18	14.66
Labial palps	before	27	81.90	5.12	12.98	17.83
Labial palps	before	27	77.99	9.58	12.43	22.56
Labial palps	before	27	82.33	6.34	11.33	16.45
Labial palps	before	27	82.04	9.25	8.70	20.78
Labial palps	before	27	83.48	2.92	13.60	12.49
Labial palps	before	27	75.46	6.39	18.15	16.05
Labial palps	before	27	75.73	5.90	18.37	16.90
Labial palps	before	27	72.28	8.76	18.96	19.49
Labial palps	before	27	68.93	12.33	18.74	27.04
Labial palps	before	27	79.42	4.18	16.40	14.91
Labial palps	before	27	76.08	7.25	16.67	18.27
Labial palps	before	27	76.87	10.19	12.94	19.08
Labial palps	before	27	71.37	11.33	17.30	23.27
Labial palps	before	27	80.26	8.61	11.13	20.09
Labial palps	1week	27	73.05	9.29	17.66	30.62
Labial palps	1week	27	67.82	9.49	22.68	26.33
Labial palps	1week	27	67.82	13.99	18.19	33.43

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	1week	27	69.63	12.95	17.42	34.74
Labial palps	1week	27	72.53	10.87	16.60	31.32
Labial palps	1week	27	70.40	15.57	14.03	36.39
Labial palps	1week	27	74.38	6.01	19.61	20.27
Labial palps	2weeks	27	83.39	2.58	14.03	6.02
Labial palps	2weeks	27	74.62	8.34	17.04	19.82
Labial palps	2weeks	27	79.19	5.71	15.10	16.74
Labial palps	2weeks	27	80.66	5.31	14.02	14.19
Labial palps	2weeks	27	75.79	6.60	17.61	15.07
Labial palps	2weeks	27	79.26	7.48	13.26	22.72
Labial palps	2weeks	27	77.06	5.75	17.19	14.65
Labial palps	2weeks	27	69.18	16.49	14.33	34.07
Labial palps	2weeks	27	82.25	4.18	13.57	15.50
Labial palps	2weeks	27	80.91	4.69	14.40	14.34
Labial palps	2weeks	27	84.04	5.63	10.33	18.23
Labial palps	2weeks	27	73.29	11.47	15.24	24.70
Labial palps	5weeks	27	79.60	6.37	14.04	19.32
Labial palps	5weeks	27	79.08	6.13	14.79	18.82
Labial palps	5weeks	27	85.99	3.77	10.24	11.05
Labial palps	5weeks	27	85.21	3.31	11.47	9.81
Labial palps	5weeks	27	80.75	5.80	13.45	17.31
Labial palps	5weeks	27	79.10	4.91	15.99	19.63
Labial palps	5weeks	27	83.67	3.96	12.38	14.68
Labial palps	5weeks	27	70.82	10.53	18.65	25.17
Labial palps	5weeks	27	78.86	5.94	15.20	19.94
Labial palps	5weeks	27	78.53	6.33	15.14	17.90

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	initial	GI	89.23	2.03	8.74	9.96
Labial palps	initial	GI	80.89	8.61	10.51	19.92
Labial palps	initial	GI	83.30	6.15	10.54	17.07
Labial palps	initial	GI	84.17	5.81	10.02	15.53
Labial palps	initial	GI	87.36	3.88	8.76	13.11
Labial palps	initial	GI	91.54	1.80	6.66	11.22
Labial palps	initial	GI	90.89	2.03	7.08	12.92
Labial palps	initial	GI	89.75	1.87	8.38	10.53
Labial palps	initial	GI	89.61	3.54	6.85	11.45
Labial palps	initial	GI	84.38	6.15	9.47	17.79
Labial palps	initial	GI	80.98	4.39	14.64	11.18
Labial palps	initial	GI	81.80	4.82	13.38	13.12
Labial palps	initial	GI	85.69	4.15	10.16	10.12
Labial palps	initial	GI	69.03	9.33	21.64	26.02
Labial palps	initial	GI	76.33	7.50	16.17	14.51
Labial palps	3 weeks after	GI	73.80	7.68	18.52	12.31
Labial palps	3 weeks after	GI	65.65	9.18	25.17	14.44
Labial palps	3 weeks after	GI	65.57	9.97	24.46	15.81
Labial palps	3 weeks after	GI	58.92	9.20	31.88	15.76
Labial palps	3 weeks after	GI	63.97	9.96	26.07	16.11
Labial palps	3 weeks after	GI	74.58	4.11	21.31	8.18
Labial palps	3 weeks after	GI	72.40	6.85	20.75	12.40
Labial palps	3 weeks after	GI	70.52	10.44	19.03	22.15
Labial palps	3 weeks after	GI	75.88	6.51	17.61	11.96
Labial palps	3 weeks after	GI	71.37	10.81	17.82	20.28
Labial palps	3 weeks after	GI	66.57	7.92	25.51	14.35
Labial palps	3 weeks after	GI	68.72	14.54	16.74	23.26

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	3 weeks after	GI	72.98	8.69	18.33	16.97
Labial palps	3 weeks after	GI	75.20	10.44	14.36	20.27
Labial palps	before	GI	71.34	11.13	17.52	20.04
Labial palps	before	GI	71.13	7.51	21.37	16.70
Labial palps	before	GI	80.33	5.15	14.52	14.35
Labial palps	before	GI	70.74	9.11	20.15	19.00
Labial palps	before	GI	71.89	8.36	19.74	17.26
Labial palps	before	GI	65.99	12.44	21.58	26.06
Labial palps	before	GI	69.32	5.58	25.10	11.24
Labial palps	before	GI	67.08	11.23	21.70	22.62
Labial palps	before	GI	64.63	7.10	28.27	12.46
Labial palps	before	GI	67.54	8.79	23.66	16.44
Labial palps	before	GI	73.21	5.67	21.12	11.77
Labial palps	before	GI	73.89	6.26	19.85	12.49
Labial palps	before	GI	72.10	8.60	19.30	17.61
Labial palps	before	GI	73.09	6.27	20.64	16.95
Labial palps	before	GI	66.11	10.07	23.82	19.17
Labial palps	1week	GI	67.25	7.04	25.72	17.20
Labial palps	1week	GI	69.39	7.32	23.29	17.42
Labial palps	1week	GI	63.21	9.98	26.82	26.01
Labial palps	1week	GI	66.84	7.53	25.63	20.33
Labial palps	1week	GI	64.95	9.05	26.01	23.12
Labial palps	1week	GI	64.69	9.23	26.08	20.76
Labial palps	1week	GI	81.26	2.40	16.34	6.59
Labial palps	1week	GI	73.10	7.85	19.05	15.60
Labial palps	1week	GI	77.87	8.06	14.07	15.20
Labial palps	1week	GI	68.56	5.59	25.85	18.11

Tissue	Time	Temperature	%G0/G1	%S	%G2/M	%PCNA
Labial palps	1week	GI	71.00	5.76	23.25	18.46
Labial palps	1week	GI	72.35	5.97	21.68	16.98
Labial palps	2weeks	GI	72.42	5.88	21.70	19.32
Labial palps	2weeks	GI	69.09	8.54	22.37	20.48
Labial palps	2weeks	GI	75.07	6.16	18.77	18.32
Labial palps	2weeks	GI	73.09	10.43	16.48	24.61
Labial palps	2weeks	GI	77.04	3.68	19.28	8.20
Labial palps	2weeks	GI	75.39	3.61	21.00	13.36
Labial palps	2weeks	GI	78.12	3.74	18.14	14.51
Labial palps	2weeks	GI	77.24	2.62	20.15	10.38
Labial palps	2weeks	GI	75.93	4.87	19.20	11.70
Labial palps	2weeks	GI	75.63	4.32	20.06	13.39
Labial palps	2weeks	GI	84.57	2.82	12.60	12.73
Labial palps	2weeks	GI	73.75	5.52	20.73	17.00
Labial palps	5weeks	GI	73.34	5.53	21.13	17.44
Labial palps	5weeks	GI	79.44	3.43	17.13	14.45
Labial palps	5weeks	GI	79.11	2.50	18.39	9.52
Labial palps	5weeks	GI	85.22	3.02	11.77	9.25
Labial palps	5weeks	GI	82.13	2.89	14.99	10.10
Labial palps	5weeks	GI	79.80	3.13	17.07	10.96
Labial palps	5weeks	GI	70.97	5.67	23.36	19.01
Labial palps	5weeks	GI	84.69	1.97	13.34	8.81
Labial palps	5weeks	GI	75.87	5.06	19.07	16.56

Table B.6. Data of heart nuclei for the analysis of temperature effects on nuclear RNA/DNA ratios at each phase of the cell cycle (Chapter 4 and 5).

Tissue	Time	Temperature	G0/G1	S	G2/M
Heart	3 weeks after	10	0.11	0.20	0.13
Heart	3 weeks after	10	0.09	0.15	0.10
Heart	3 weeks after	10	0.10	0.13	0.10
Heart	3 weeks after	10	0.09	0.11	0.07
Heart	3 weeks after	10	0.07	0.08	0.07
Heart	3 weeks after	10	0.09	0.25	0.03
Heart	3 weeks after	10	0.11	0.27	0.10
Heart	3 weeks after	10	0.09	0.16	0.10
Heart	3 weeks after	10	0.06	0.14	0.07
Heart	3 weeks after	10	0.06	0.14	0.07
Heart	3 weeks after	10	0.07	0.16	0.08
Heart	3 weeks after	10	0.06	0.13	0.06
Heart	3 weeks after	10	0.08	0.11	0.08
Heart	before	10	0.07	0.17	0.07
Heart	before	10	0.08	0.19	0.08
Heart	before	10	0.09	0.19	0.09
Heart	before	10	0.06	0.19	0.06
Heart	before	10	0.08	0.19	0.07
Heart	before	10	0.07	0.14	0.07
Heart	before	10	0.07	0.19	0.07
Heart	before	10	0.07	0.16	0.07
Heart	before	10	0.05	0.10	0.06
Heart	before	10	0.06	0.13	0.06
Heart	before	10	0.07	0.21	0.08
Heart	before	10	0.06	0.11	0.06

Tissue	Time	Temperature	G0/G1	S	G2/M
Heart	before	10	0.06	0.19	0.06
Heart	before	10	0.07	0.21	0.07
Heart	before	10	0.06	0.14	0.07
Heart	1week	10±1	0.26	0.77	0.40
Heart	1week	10±1	0.07	0.31	0.08
Heart	1week	10±1	0.08	0.33	0.10
Heart	1week	10±1	0.08	0.31	0.11
Heart	1week	10±1	0.14	0.40	0.21
Heart	1week	10±1	0.06	0.19	0.09
Heart	1week	10±1	0.12	0.49	0.15
Heart	1week	10±1	0.11	0.33	0.15
Heart	1week	10±1	0.14	0.54	0.21
Heart	1week	10±1	0.09	0.28	0.12
Heart	2weeks	10±1	0.05	0.16	0.07
Heart	2weeks	10±1	0.03	0.09	0.04
Heart	2weeks	10±1	0.15	0.55	0.23
Heart	2weeks	10±1	0.07	0.37	0.09
Heart	2weeks	10±1	0.08	0.66	0.10
Heart	2weeks	10±1	0.05	0.30	0.08
Heart	2weeks	10±1	0.04	0.12	0.05
Heart	2weeks	10±1	0.05	0.12	0.06
Heart	2weeks	10±1	0.05	0.27	0.07
Heart	2weeks	10±1	0.05	0.16	0.09
Heart	2weeks	10±1	0.05	0.20	0.07
Heart	2weeks	10±1	0.04	0.12	0.05
Heart	5weeks	10±1	0.14	0.64	0.15
Heart	5weeks	10±1	0.13	0.46	0.18

Tissue	Time	Temperature	G0/G1	S	G2/M
Heart	5weeks	10±1	0.19	0.69	0.27
Heart	5weeks	10±1	0.22	0.81	0.33
Heart	5weeks	10±1	0.19	1.02	0.33
Heart	5weeks	10±1	0.14	0.59	0.23
Heart	5weeks	10±1	0.15	0.49	0.20
Heart	5weeks	10±1	0.21	0.81	0.32
Heart	5weeks	10±1	0.11	0.53	0.15
Heart	5weeks	10±1	0.13	0.41	0.17
Heart	3 weeks after	15	0.11	0.20	0.13
Heart	3 weeks after	15	0.09	0.15	0.10
Heart	3 weeks after	15	0.10	0.13	0.10
Heart	3 weeks after	15	0.09	0.11	0.07
Heart	3 weeks after	15	0.07	0.08	0.07
Heart	3 weeks after	15	0.09	0.25	0.03
Heart	3 weeks after	15	0.11	0.27	0.10
Heart	3 weeks after	15	0.09	0.16	0.10
Heart	3 weeks after	15	0.06	0.14	0.07
Heart	3 weeks after	15	0.06	0.14	0.07
Heart	3 weeks after	15	0.07	0.16	0.08
Heart	3 weeks after	15	0.06	0.13	0.06
Heart	3 weeks after	15	0.08	0.11	0.08
Heart	before	15	0.20	0.34	0.19
Heart	before	15	0.37	0.70	0.49
Heart	before	15	0.18	0.35	0.17
Heart	before	15	0.11	0.40	0.12
Heart	before	15	0.14	0.41	0.16

Tissue	Time	Temperature	G0/G1	S	G2/M
Heart	before	15	0.17	0.65	0.19
Heart	before	15	0.17	0.28	0.16
Heart	before	15	0.19	0.23	0.17
Heart	before	15	0.17	0.27	0.15
Heart	before	15	0.26	0.42	0.25
Heart	before	15	0.15	0.30	0.15
Heart	before	15	0.15	0.31	0.13
Heart	before	15	0.11	0.26	0.11
Heart	before	15	0.09	0.18	0.08
Heart	before	15	0.08	0.14	0.07
Heart	1week	15±1	0.34	0.59	0.35
Heart	1week	15±1	0.34	0.79	0.37
Heart	1week	15±1	0.23	0.57	0.27
Heart	1week	15±1	0.74	1.68	0.88
Heart	1week	15±1	0.23	0.57	0.22
Heart	1week	15±1	0.38	0.86	0.37
Heart	1week	15±1	0.44	0.58	0.44
Heart	2weeks	15±1	0.50	0.98	0.67
Heart	2weeks	15±1	0.35	0.80	0.40
Heart	2weeks	15±1	0.37	1.02	0.43
Heart	2weeks	15±1	0.12	0.23	0.14
Heart	2weeks	15±1	0.19	0.36	0.25
Heart	2weeks	15±1	0.16	0.35	0.22
Heart	2weeks	15±1	0.18	0.83	0.30
Heart	2weeks	15±1	0.44	0.93	0.59
Heart	2weeks	15±1	0.34	1.44	0.72
Heart	2weeks	15±1	0.19	0.78	0.27

Tissue	Time	Temperature	G0/G1	S	G2/M
Heart	2weeks	15±1	0.16	0.47	0.23
Heart	5weeks	15±1	0.11	0.25	0.12
Heart	5weeks	15±1	0.09	0.22	0.10
Heart	5weeks	15±1	0.12	0.29	0.14
Heart	5weeks	15±1	0.13	0.17	0.17
Heart	5weeks	15±1	0.10	0.22	0.12
Heart	5weeks	15±1	0.11	0.27	0.14
Heart	5weeks	15±1	0.02	0.03	0.02
Heart	5weeks	15±1	0.15	0.34	0.15
Heart	5weeks	15±1	0.11	0.31	0.12
Heart	5weeks	15±1	0.15	0.34	0.15
Heart	3 weeks after	20	0.11	0.20	0.13
Heart	3 weeks after	20	0.09	0.15	0.10
Heart	3 weeks after	20	0.10	0.13	0.10
Heart	3 weeks after	20	0.09	0.11	0.07
Heart	3 weeks after	20	0.07	0.08	0.07
Heart	3 weeks after	20	0.09	0.25	0.03
Heart	3 weeks after	20	0.11	0.27	0.10
Heart	3 weeks after	20	0.09	0.16	0.10
Heart	3 weeks after	20	0.06	0.14	0.07
Heart	3 weeks after	20	0.06	0.14	0.07
Heart	3 weeks after	20	0.07	0.16	0.08
Heart	3 weeks after	20	0.06	0.13	0.06
Heart	3 weeks after	20	0.08	0.11	0.08
Heart	before	20	0.09	0.17	0.09
Heart	before	20	0.13	0.41	0.14

Tissue	Time	Temperature	G0/G1	S	G2/M
Heart	before	20	0.13	0.20	0.14
Heart	before	20	0.13	0.29	0.14
Heart	before	20	0.16	0.35	0.17
Heart	before	20	0.24	0.43	0.27
Heart	before	20	0.11	0.18	0.11
Heart	before	20	0.16	0.27	0.16
Heart	before	20	0.11	0.21	0.11
Heart	before	20	0.15	0.20	0.13
Heart	before	20	0.17	0.27	0.16
Heart	before	20	0.16	0.20	0.14
Heart	before	20	0.09	0.17	0.09
Heart	before	20	0.07	0.15	0.07
Heart	before	20	0.10	0.18	0.09
Heart	1week	20±1	0.21	0.37	0.20
Heart	1week	20±1	0.21	0.39	0.30
Heart	1week	20±1	0.21	0.42	0.20
Heart	1week	20±1	0.24	0.68	0.21
Heart	1week	20±1	0.25	0.51	0.21
Heart	1week	20±1	0.35	0.63	0.29
Heart	1week	20±1	0.41	0.96	0.45
Heart	1week	20±1	0.23	0.58	0.23
Heart	1week	20±1	0.29	0.57	0.28
Heart	1week	20±1	0.30	0.71	0.27
Heart	1week	20±1	0.40	0.75	0.41
Heart	1week	20±1	0.45	0.94	0.52
Heart	2weeks	20±1	0.12	0.30	0.14
Heart	2weeks	20±1	0.08	0.35	0.11

Tissue	Time	Temperature	G0/G1	S	G2/M
Heart	2weeks	20±1	0.22	0.63	0.28
Heart	2weeks	20±1	0.31	0.98	0.46
Heart	2weeks	20±1	0.16	0.72	0.21
Heart	2weeks	20±1	0.33	1.47	0.51
Heart	2weeks	20±1	0.21	0.39	0.31
Heart	2weeks	20±1	0.20	0.38	0.23
Heart	2weeks	20±1	0.42	0.89	0.64
Heart	2weeks	20±1	0.19	0.30	0.26
Heart	2weeks	20±1	0.16	0.29	0.19
Heart	2weeks	20±1	0.17	0.42	0.21
Heart	5weeks	20±1	0.10	0.20	0.12
Heart	5weeks	20±1	0.17	0.46	0.17
Heart	5weeks	20±1	0.09	0.14	0.10
Heart	5weeks	20±1	0.08	0.19	0.10
Heart	5weeks	20±1	0.16	0.29	0.18
Heart	5weeks	20±1	0.11	0.24	0.14
Heart	5weeks	20±1	0.10	0.22	0.10
Heart	5weeks	20±1	0.13	0.29	0.15
Heart	5weeks	20±1	0.15	0.32	0.18
Heart	1week	10±5	0.10	0.41	0.15
Heart	1week	10±5	0.07	0.26	0.10
Heart	1week	10±5	0.06	0.42	0.10
Heart	1week	10±5	0.09	0.55	0.13
Heart	1week	10±5	0.10	0.72	0.17
Heart	1week	10±5	0.09	0.31	0.11
Heart	1week	10±5	0.12	0.51	0.18

Tissue	Time	Temperature	G0/G1	S	G2/M
Heart	1week	10±5	0.14	0.97	0.24
Heart	1week	10±5	0.09	0.37	0.15
Heart	1week	10±5	0.12	0.65	0.17
Heart	1week	10±5	0.11	0.31	0.15
Heart	1week	10±5	0.09	0.25	0.12
Heart	2weeks	10±5	0.11	0.46	0.17
Heart	2weeks	10±5	0.04	0.12	0.06
Heart	2weeks	10±5	0.05	0.13	0.07
Heart	2weeks	10±5	0.03	0.08	0.04
Heart	2weeks	10±5	0.05	0.16	0.07
Heart	2weeks	10±5	0.04	0.09	0.05
Heart	2weeks	10±5	0.03	0.10	0.05
Heart	2weeks	10±5	0.03	0.11	0.05
Heart	2weeks	10±5	0.04	0.07	0.05
Heart	2weeks	10±5	0.04	0.13	0.06
Heart	2weeks	10±5	0.02	0.03	0.02
Heart	2weeks	10±5	0.04	0.12	0.05
Heart	5weeks	10±5	0.23	0.60	0.31
Heart	5weeks	10±5	0.14	0.42	0.20
Heart	5weeks	10±5	0.20	0.68	0.29
Heart	5weeks	10±5	0.23	0.87	0.30
Heart	5weeks	10±5	0.24	0.92	0.50
Heart	5weeks	10±5	0.06	0.27	0.11
Heart	5weeks	10±5	0.04	0.19	0.06
Heart	5weeks	10±5	0.12	0.38	0.18
Heart	5weeks	10±5	0.13	0.38	0.16
Heart	5weeks	10±5	0.15	0.49	0.20

Tissue	Time	Temperature	G0/G1	S	G2/M
Heart	5weeks	10±5	0.09	0.26	0.12
Heart	1week	15±5	0.22	0.35	0.20
Heart	1week	15±5	0.26	0.39	0.20
Heart	1week	15±5	0.31	0.46	0.27
Heart	1week	15±5	0.18	0.30	0.18
Heart	1week	15±5	0.25	0.40	0.24
Heart	2weeks	15±5	0.23	0.60	0.32
Heart	2weeks	15±5	0.26	0.74	0.40
Heart	2weeks	15±5	0.21	0.76	0.30
Heart	2weeks	15±5	0.18	0.37	0.23
Heart	2weeks	15±5	0.13	0.24	0.17
Heart	2weeks	15±5	0.26	0.60	0.37
Heart	2weeks	15±5	0.15	0.44	0.21
Heart	2weeks	15±5	0.13	0.30	0.17
Heart	2weeks	15±5	0.17	0.41	0.23
Heart	2weeks	15±5	0.19	0.43	0.28
Heart	2weeks	15±5	0.27	0.50	0.20
Heart	2weeks	15±5	0.27	0.48	0.29
Heart	5weeks	15±5	0.11	0.34	0.14
Heart	5weeks	15±5	0.09	0.14	0.10
Heart	5weeks	15±5	0.15	0.54	0.17
Heart	5weeks	15±5	0.11	0.29	0.13
Heart	5weeks	15±5	0.08	0.19	0.10
Heart	5weeks	15±5	0.10	0.26	0.12
Heart	5weeks	15±5	0.17	0.59	0.18
Heart	5weeks	15±5	0.11	0.21	0.12

Tissue	Time	Temperature	G0/G1	S	G2/M
Heart	1week	15±10	0.20	0.44	0.21
Heart	1week	15±10	0.30	0.70	0.37
Heart	1week	15±10	0.27	0.51	0.22
Heart	1week	15±10	0.44	1.05	0.55
Heart	1week	15±10	0.23	0.61	0.22
Heart	1week	15±10	0.66	1.35	0.70
Heart	2weeks	15±10	0.20	0.67	0.36
Heart	2weeks	15±10	0.07	0.17	0.09
Heart	2weeks	15±10	0.35	0.89	0.47
Heart	2weeks	15±10	0.18	0.59	0.36
Heart	2weeks	15±10	0.18	0.74	0.26
Heart	2weeks	15±10	0.08	0.14	0.09
Heart	2weeks	15±10	0.11	0.29	0.13
Heart	2weeks	15±10	0.13	0.33	0.16
Heart	2weeks	15±10	0.17	0.51	0.23
Heart	2weeks	15±10	0.17	0.33	0.24
Heart	2weeks	15±10	0.20	0.78	0.30
Heart	2weeks	15±10	0.14	0.33	0.17
Heart	5weeks	15±10	0.16	0.63	0.21
Heart	5weeks	15±10	0.10	0.16	0.12
Heart	5weeks	15±10	0.12	0.33	0.14
Heart	5weeks	15±10	0.13	0.23	0.18
Heart	5weeks	15±10	0.11	0.21	0.16
Heart	5weeks	15±10	0.11	0.25	0.16
Heart	5weeks	15±10	0.07	0.18	0.11
Heart	5weeks	15±10	0.10	0.30	0.12
Heart	5weeks	15±10	0.08	0.26	0.10

Tissue	Time	Temperature	G0/G1	S	G2/M
Heart	5weeks	15±10	0.11	0.32	0.14
Heart	5weeks	15±10	0.17	0.80	0.20
Heart	1week	20±5	0.16	0.29	0.20
Heart	1week	20±5	0.22	0.35	0.19
Heart	1week	20±5	0.44	1.06	0.43
Heart	1week	20±5	0.37	1.21	0.34
Heart	1week	20±5	0.26	0.38	0.22
Heart	2weeks	20±5	0.13	0.31	0.16
Heart	2weeks	20±5	0.15	0.76	0.19
Heart	2weeks	20±5	0.15	0.36	0.19
Heart	2weeks	20±5	0.22	0.57	0.30
Heart	2weeks	20±5	0.08	0.13	0.10
Heart	2weeks	20±5	0.14	0.32	0.19
Heart	2weeks	20±5	0.07	0.18	0.08
Heart	2weeks	20±5	0.07	0.20	0.09
Heart	2weeks	20±5	0.13	0.36	0.15
Heart	2weeks	20±5	0.16	0.49	0.18
Heart	2weeks	20±5	0.22	0.48	0.24
Heart	5weeks	20±5	0.14	0.32	0.15
Heart	5weeks	20±5	0.11	0.44	0.12
Heart	5weeks	20±5	0.11	0.29	0.12
Heart	5weeks	20±5	0.11	0.22	0.14
Heart	5weeks	20±5	0.09	0.22	0.11
Heart	5weeks	20±5	0.09	0.26	0.11
Heart	5weeks	20±5	0.14	0.28	0.15

Tissue	Time	Temperature	G0/G1	S	G2/M
Heart	5weeks	20±5	0.12	0.31	0.14
Heart	5weeks	20±5	0.08	0.21	0.09
Heart	3 weeks after	27	0.11	0.20	0.13
Heart	3 weeks after	27	0.09	0.15	0.10
Heart	3 weeks after	27	0.10	0.13	0.10
Heart	3 weeks after	27	0.09	0.11	0.07
Heart	3 weeks after	27	0.07	0.08	0.07
Heart	3 weeks after	27	0.09	0.25	0.03
Heart	3 weeks after	27	0.11	0.27	0.10
Heart	3 weeks after	27	0.09	0.16	0.10
Heart	3 weeks after	27	0.06	0.14	0.07
Heart	3 weeks after	27	0.06	0.14	0.07
Heart	3 weeks after	27	0.07	0.16	0.08
Heart	3 weeks after	27	0.06	0.13	0.06
Heart	3 weeks after	27	0.08	0.11	0.08
Heart	before	27	0.23	0.58	0.23
Heart	before	27	0.18	0.40	0.17
Heart	before	27	0.19	0.27	0.17
Heart	before	27	0.28	0.40	0.30
Heart	before	27	0.38	0.89	0.47
Heart	before	27	0.15	0.29	0.16
Heart	before	27	0.15	0.35	0.13
Heart	before	27	0.16	0.24	0.14
Heart	before	27	0.11	0.19	0.10
Heart	before	27	0.14	0.23	0.12
Heart	before	27	0.16	0.26	0.15

Tissue	Time	Temperature	G0/G1	S	G2/M
Heart	before	27	0.14	0.20	0.12
Heart	before	27	0.12	0.23	0.10
Heart	before	27	0.16	0.30	0.12
Heart	before	27	0.08	0.14	0.07
Heart	1week	27	0.15	0.18	0.14
Heart	1week	27	0.13	0.20	0.12
Heart	1week	27	0.22	0.34	0.22
Heart	1week	27	0.24	0.36	0.23
Heart	1week	27	0.26	0.48	0.27
Heart	1week	27	0.25	0.47	0.28
Heart	2weeks	27	0.08	0.23	0.10
Heart	2weeks	27	0.13	0.80	0.20
Heart	2weeks	27	0.12	0.51	0.17
Heart	2weeks	27	0.10	0.37	0.13
Heart	2weeks	27	0.19	0.54	0.31
Heart	2weeks	27	0.15	0.68	0.22
Heart	2weeks	27	0.11	0.39	0.14
Heart	2weeks	27	0.30	1.67	0.55
Heart	2weeks	27	0.07	0.28	0.09
Heart	2weeks	27	0.16	0.52	0.20
Heart	2weeks	27	0.14	0.47	0.18
Heart	2weeks	27	0.17	0.71	0.25
Heart	5weeks	27	0.10	0.47	0.12
Heart	5weeks	27	0.10	0.38	0.12
Heart	5weeks	27	0.10	0.41	0.12
Heart	5weeks	27	0.10	0.45	0.15
Heart	5weeks	27	0.16	0.67	0.23

Tissue	Time	Temperature	G0/G1	S	G2/M
Heart	5weeks	27	0.07	0.26	0.10
Heart	5weeks	27	0.13	0.61	0.20
Heart	5weeks	27	0.15	0.33	0.17
Heart	5weeks	27	0.10	0.36	0.12
Heart	3 weeks after	GI	0.11	0.20	0.13
Heart	3 weeks after	GI	0.09	0.15	0.10
Heart	3 weeks after	GI	0.10	0.13	0.10
Heart	3 weeks after	GI	0.09	0.11	0.07
Heart	3 weeks after	GI	0.07	0.08	0.07
Heart	3 weeks after	GI	0.09	0.25	0.03
Heart	3 weeks after	GI	0.11	0.27	0.10
Heart	3 weeks after	GI	0.09	0.16	0.10
Heart	3 weeks after	GI	0.06	0.14	0.07
Heart	3 weeks after	GI	0.06	0.14	0.07
Heart	3 weeks after	GI	0.07	0.16	0.08
Heart	3 weeks after	GI	0.06	0.13	0.06
Heart	3 weeks after	GI	0.08	0.11	0.08
Heart	before	GI	0.20	0.29	0.14
Heart	before	GI	0.12	0.17	0.10
Heart	before	GI	0.08	0.14	0.07
Heart	before	GI	0.09	0.20	0.08
Heart	before	GI	0.09	0.15	0.07
Heart	before	GI	0.08	0.15	0.07
Heart	before	GI	0.08	0.15	0.07
Heart	before	GI	0.09	0.16	0.07
Heart	before	GI	0.07	0.17	0.06

Tissue	Time	Temperature	G0/G1	S	G2/M
Heart	before	GI	0.07	0.13	0.07
Heart	before	GI	0.08	0.14	0.07
Heart	before	GI	0.07	0.15	0.10
Heart	before	GI	0.06	0.19	0.05
Heart	before	GI	0.06	0.19	0.05
Heart	1week	GI	0.12	0.17	0.13
Heart	1week	GI	0.19	0.49	0.19
Heart	1week	GI	0.22	0.46	0.20
Heart	1week	GI	0.40	0.61	0.36
Heart	1week	GI	0.27	0.35	0.25
Heart	2weeks	GI	0.20	0.65	0.24
Heart	2weeks	GI	0.17	0.53	0.26
Heart	2weeks	GI	0.09	0.20	0.10
Heart	2weeks	GI	0.06	0.20	0.07
Heart	2weeks	GI	0.12	0.35	0.15
Heart	2weeks	GI	0.11	0.25	0.13
Heart	2weeks	GI	0.08	0.25	0.11
Heart	2weeks	GI	0.07	0.28	0.10
Heart	2weeks	GI	0.07	0.16	0.09
Heart	2weeks	GI	0.11	0.43	0.17
Heart	2weeks	GI	0.08	0.31	0.09
Heart	2weeks	GI	0.08	0.26	0.09
Heart	5weeks	GI	0.12	0.39	0.16
Heart	5weeks	GI	0.08	0.29	0.10
Heart	5weeks	GI	0.05	0.19	0.05
Heart	5weeks	GI	0.09	0.28	0.12
Heart	5weeks	GI	0.08	0.24	0.10

Tissue	Time	Temperature	G0/G1	S	G2/M
Heart	5weeks	GI	0.27	0.79	0.37
Heart	5weeks	GI	0.17	0.35	0.28
Heart	5weeks	GI	0.09	0.25	0.12
Heart	5weeks	GI	0.13	0.45	0.18

Table B.7. Data of labial palp nuclei for the analysis of temperature effects on nuclear RNA/DNA ratios at each phase of the cell cycle (Chapter 4 and 5).

Tissue	Time	Temperature	G0/G1	S	G2/M
Labial palps	3 weeks after	10	0.12	0.22	0.11
Labial palps	3 weeks after	10	0.10	0.21	0.09
Labial palps	3 weeks after	10	0.10	0.21	0.10
Labial palps	3 weeks after	10	0.13	0.13	0.13
Labial palps	3 weeks after	10	0.12	0.20	0.11
Labial palps	3 weeks after	10	0.12	0.21	0.10
Labial palps	3 weeks after	10	0.20	0.35	0.20
Labial palps	3 weeks after	10	0.13	0.30	0.15
Labial palps	3 weeks after	10	0.06	0.11	0.06
Labial palps	3 weeks after	10	0.10	0.12	0.08
Labial palps	3 weeks after	10	0.09	0.13	0.07
Labial palps	3 weeks after	10	0.07	0.12	0.07
Labial palps	3 weeks after	10	0.07	0.10	0.06
Labial palps	3 weeks after	10	0.08	0.09	0.07
Labial palps	before	10	0.08	0.15	0.08
Labial palps	before	10	0.08	0.20	0.08
Labial palps	before	10	0.07	0.26	0.07
Labial palps	before	10	0.08	0.15	0.08
Labial palps	before	10	0.07	0.22	0.07

Tissue	Time	Temperature	G0/G1	S	G2/M
Labial palps	before	10	0.06	0.20	0.06
Labial palps	before	10	0.09	0.38	0.09
Labial palps	before	10	0.06	0.32	0.07
Labial palps	before	10	0.10	0.32	0.10
Labial palps	before	10	0.10	0.35	0.10
Labial palps	before	10	0.08	0.25	0.09
Labial palps	before	10	0.07	0.22	0.08
Labial palps	before	10	0.06	0.21	0.07
Labial palps	before	10	0.07	0.27	0.08
Labial palps	before	10	0.05	0.12	0.05
Labial palps	1week	10±1	0.14	0.68	0.19
Labial palps	1week	10±1	0.16	0.49	0.20
Labial palps	1week	10±1	0.14	0.69	0.17
Labial palps	1week	10±1	0.10	0.16	0.11
Labial palps	1week	10±1	0.13	0.50	0.15
Labial palps	1week	10±1	0.08	0.51	0.11
Labial palps	1week	10±1	0.10	0.31	0.12
Labial palps	1week	10±1	0.11	0.43	0.12
Labial palps	1week	10±1	0.19	0.99	0.21
Labial palps	1week	10±1	0.12	0.55	0.14
Labial palps	1week	10±1	0.10	0.29	0.11
Labial palps	1week	10±1	0.16	0.83	0.19
Labial palps	2weeks	10±1	0.07	0.26	0.08
Labial palps	2weeks	10±1	0.07	0.41	0.08
Labial palps	2weeks	10±1	0.10	0.27	0.11
Labial palps	2weeks	10±1	0.05	0.15	0.05
Labial palps	2weeks	10±1	0.06	0.18	0.07

Tissue	Time	Temperature	G0/G1	S	G2/M
Labial palps	2weeks	10±1	0.08	0.17	0.08
Labial palps	2weeks	10±1	0.06	0.10	0.06
Labial palps	2weeks	10±1	0.07	0.29	0.08
Labial palps	2weeks	10±1	0.07	0.12	0.07
Labial palps	2weeks	10±1	0.07	0.29	0.07
Labial palps	2weeks	10±1	0.07	0.21	0.08
Labial palps	2weeks	10±1	0.05	0.08	0.06
Labial palps	5weeks	10±1	0.34	0.58	0.40
Labial palps	5weeks	10±1	0.25	1.48	0.31
Labial palps	5weeks	10±1	0.29	1.29	0.33
Labial palps	5weeks	10±1	0.28	0.50	0.32
Labial palps	5weeks	10±1	0.25	1.19	0.29
Labial palps	5weeks	10±1	0.31	0.52	0.34
Labial palps	5weeks	10±1	0.29	0.76	0.27
Labial palps	5weeks	10±1	0.26	0.74	0.39
Labial palps	5weeks	10±1	0.20	0.59	0.27
Labial palps	5weeks	10±1	0.28	0.66	0.30
Labial palps	3 weeks after	15	0.12	0.22	0.11
Labial palps	3 weeks after	15	0.10	0.21	0.09
Labial palps	3 weeks after	15	0.10	0.21	0.10
Labial palps	3 weeks after	15	0.13	0.13	0.13
Labial palps	3 weeks after	15	0.12	0.20	0.11
Labial palps	3 weeks after	15	0.12	0.21	0.10
Labial palps	3 weeks after	15	0.20	0.35	0.20
Labial palps	3 weeks after	15	0.13	0.30	0.15
Labial palps	3 weeks after	15	0.06	0.11	0.06

Tissue	Time	Temperature	G0/G1	S	G2/M
Labial palps	3 weeks after	15	0.10	0.12	0.08
Labial palps	3 weeks after	15	0.09	0.13	0.07
Labial palps	3 weeks after	15	0.07	0.12	0.07
Labial palps	3 weeks after	15	0.07	0.10	0.06
Labial palps	3 weeks after	15	0.08	0.09	0.07
Labial palps	before	15	0.20	0.48	0.23
Labial palps	before	15	0.25	0.55	0.30
Labial palps	before	15	0.20	0.42	0.26
Labial palps	before	15	0.12	0.30	0.14
Labial palps	before	15	0.20	0.34	0.20
Labial palps	before	15	0.22	0.42	0.23
Labial palps	before	15	0.18	0.44	0.17
Labial palps	before	15	0.14	0.42	0.12
Labial palps	before	15	0.11	0.23	0.11
Labial palps	before	15	0.20	0.52	0.21
Labial palps	before	15	0.17	0.56	0.18
Labial palps	before	15	0.16	0.48	0.15
Labial palps	before	15	0.07	0.21	0.07
Labial palps	before	15	0.07	0.18	0.07
Labial palps	before	15	0.07	0.32	0.08
Labial palps	1week	15±1	0.30	0.70	0.28
Labial palps	1week	15±1	0.30	0.66	0.29
Labial palps	1week	15±1	0.28	0.58	0.27
Labial palps	1week	15±1	0.27	0.41	0.19
Labial palps	1week	15±1	0.19	0.37	0.17
Labial palps	1week	15±1	0.46	0.90	0.46
Labial palps	2weeks	15±1	0.20	1.02	0.33

Tissue	Time	Temperature	G0/G1	S	G2/M
Labial palps	2weeks	15±1	0.23	1.25	0.24
Labial palps	2weeks	15±1	0.28	1.07	0.28
Labial palps	2weeks	15±1	0.13	0.46	0.15
Labial palps	2weeks	15±1	0.20	0.81	0.24
Labial palps	2weeks	15±1	0.23	0.65	0.24
Labial palps	2weeks	15±1	0.12	0.57	0.15
Labial palps	2weeks	15±1	0.15	0.61	0.21
Labial palps	2weeks	15±1	0.14	0.47	0.16
Labial palps	2weeks	15±1	0.25	0.59	0.28
Labial palps	2weeks	15±1	0.12	0.56	0.14
Labial palps	2weeks	15±1	0.15	0.72	0.19
Labial palps	5weeks	15±1	0.15	0.29	0.17
Labial palps	5weeks	15±1	0.13	0.22	0.13
Labial palps	5weeks	15±1	0.12	0.21	0.13
Labial palps	5weeks	15±1	0.09	0.28	0.10
Labial palps	5weeks	15±1	0.09	0.27	0.10
Labial palps	5weeks	15±1	0.10	0.24	0.11
Labial palps	5weeks	15±1	0.09	0.17	0.10
Labial palps	5weeks	15±1	0.06	0.31	0.07
Labial palps	5weeks	15±1	0.10	0.33	0.10
Labial palps	3 weeks after	20	0.12	0.22	0.11
Labial palps	3 weeks after	20	0.10	0.21	0.09
Labial palps	3 weeks after	20	0.10	0.21	0.10
Labial palps	3 weeks after	20	0.13	0.13	0.13
Labial palps	3 weeks after	20	0.12	0.20	0.11
Labial palps	3 weeks after	20	0.12	0.21	0.10

Tissue	Time	Temperature	G0/G1	S	G2/M
Labial palps	3 weeks after	20	0.20	0.35	0.20
Labial palps	3 weeks after	20	0.13	0.30	0.15
Labial palps	3 weeks after	20	0.06	0.11	0.06
Labial palps	3 weeks after	20	0.10	0.12	0.08
Labial palps	3 weeks after	20	0.09	0.13	0.07
Labial palps	3 weeks after	20	0.07	0.12	0.07
Labial palps	3 weeks after	20	0.07	0.10	0.06
Labial palps	3 weeks after	20	0.08	0.09	0.07
Labial palps	before	20	0.24	0.50	0.29
Labial palps	before	20	0.27	0.67	0.31
Labial palps	before	20	0.25	0.33	0.26
Labial palps	before	20	0.22	0.58	0.22
Labial palps	before	20	0.25	0.44	0.30
Labial palps	before	20	0.19	0.51	0.18
Labial palps	before	20	0.29	0.54	0.26
Labial palps	before	20	0.37	1.06	0.51
Labial palps	before	20	0.22	0.56	0.23
Labial palps	before	20	0.17	0.37	0.19
Labial palps	before	20	0.37	0.63	0.38
Labial palps	before	20	0.38	0.59	0.32
Labial palps	before	20	0.16	0.37	0.14
Labial palps	before	20	0.09	0.24	0.09
Labial palps	before	20	0.08	0.13	0.07
Labial palps	1week	20±1	0.17	0.25	0.12
Labial palps	1week	20±1	0.21	0.47	0.20
Labial palps	1week	20±1	0.22	0.57	0.20
Labial palps	1week	20±1	0.23	0.80	0.21

Tissue	Time	Temperature	G0/G1	S	G2/M
Labial palps	1week	20±1	0.29	0.85	0.26
Labial palps	1week	20±1	0.16	0.57	0.13
Labial palps	1week	20±1	0.17	0.30	0.15
Labial palps	1week	20±1	0.19	0.51	0.17
Labial palps	1week	20±1	0.24	0.42	0.21
Labial palps	1week	20±1	0.31	0.60	0.28
Labial palps	1week	20±1	0.34	0.64	0.27
Labial palps	1week	20±1	0.30	0.53	0.28
Labial palps	2weeks	20±1	0.37	2.31	0.47
Labial palps	2weeks	20±1	0.26	0.64	0.28
Labial palps	2weeks	20±1	0.21	0.62	0.26
Labial palps	2weeks	20±1	0.20	0.31	0.23
Labial palps	2weeks	20±1	0.24	0.91	0.31
Labial palps	2weeks	20±1	0.19	0.51	0.21
Labial palps	2weeks	20±1	0.20	0.44	0.23
Labial palps	2weeks	20±1	0.17	0.37	0.19
Labial palps	2weeks	20±1	0.21	0.39	0.23
Labial palps	2weeks	20±1	0.17	0.42	0.18
Labial palps	2weeks	20±1	0.18	0.50	0.21
Labial palps	2weeks	20±1	0.19	0.63	0.24
Labial palps	5weeks	20±1	0.16	0.41	0.19
Labial palps	5weeks	20±1	0.10	0.23	0.11
Labial palps	5weeks	20±1	0.18	0.39	0.20
Labial palps	5weeks	20±1	0.08	0.21	0.09
Labial palps	5weeks	20±1	0.08	0.19	0.09
Labial palps	5weeks	20±1	0.11	0.24	0.12
Labial palps	5weeks	20±1	0.14	0.46	0.16

Tissue	Time	Temperature	G0/G1	S	G2/M
Labial palps	5weeks	20±1	0.09	0.15	0.09
Labial palps	5weeks	20±1	0.14	0.40	0.14
Labial palps	1week	10±5	0.11	0.33	0.14
Labial palps	1week	10±5	0.11	0.57	0.13
Labial palps	1week	10±5	0.09	0.43	0.11
Labial palps	1week	10±5	0.10	0.46	0.13
Labial palps	1week	10±5	0.12	0.62	0.15
Labial palps	1week	10±5	0.09	0.49	0.10
Labial palps	1week	10±5	0.11	0.39	0.12
Labial palps	1week	10±5	0.12	0.65	0.16
Labial palps	1week	10±5	0.09	0.65	0.12
Labial palps	1week	10±5	0.12	0.62	0.14
Labial palps	1week	10±5	0.09	0.31	0.10
Labial palps	1week	10±5	0.13	0.52	0.16
Labial palps	2weeks	10±5	0.06	0.05	0.07
Labial palps	2weeks	10±5	0.09	0.07	0.10
Labial palps	2weeks	10±5	0.09	0.22	0.10
Labial palps	2weeks	10±5	0.05	0.14	0.06
Labial palps	2weeks	10±5	0.08	0.27	0.08
Labial palps	2weeks	10±5	0.07	0.33	0.08
Labial palps	2weeks	10±5	0.06	0.26	0.07
Labial palps	2weeks	10±5	0.08	0.22	0.08
Labial palps	2weeks	10±5	0.10	0.46	0.12
Labial palps	2weeks	10±5	0.06	0.41	0.07
Labial palps	2weeks	10±5	0.06	0.18	0.07
Labial palps	2weeks	10±5	0.12	0.49	0.16

Tissue	Time	Temperature	G0/G1	S	G2/M
Labial palps	5weeks	10±5	0.50	1.06	0.56
Labial palps	5weeks	10±5	0.50	1.13	0.59
Labial palps	5weeks	10±5	0.42	0.84	0.45
Labial palps	5weeks	10±5	0.51	1.50	0.49
Labial palps	5weeks	10±5	0.33	0.71	0.33
Labial palps	5weeks	10±5	0.40	1.35	0.60
Labial palps	5weeks	10±5	0.12	0.46	0.15
Labial palps	5weeks	10±5	0.08	0.40	0.10
Labial palps	5weeks	10±5	0.14	0.62	0.15
Labial palps	5weeks	10±5	0.15	0.35	0.16
Labial palps	5weeks	10±5	0.06	0.31	0.07
Labial palps	5weeks	10±5	0.10	0.41	0.10
Labial plaps	1week	15±5	0.21	0.27	0.17
Labial plaps	1week	15±5	0.20	0.27	0.18
Labial plaps	1week	15±5	0.17	0.23	0.14
Labial plaps	1week	15±5	0.27	0.44	0.27
Labial plaps	1week	15±5	0.17	0.28	0.14
Labial plaps	1week	15±5	0.33	0.60	0.23
Labial plaps	1week	15±5	0.15	0.18	0.11
Labial plaps	1week	15±5	0.22	0.35	0.19
Labial plaps	1week	15±5	0.26	0.30	0.27
Labial plaps	1week	15±5	0.23	0.26	0.22
Labial plaps	1week	15±5	0.17	0.18	0.16
Labial plaps	1week	15±5	0.22	0.23	0.20
Labial plaps	2weeks	15±5	0.39	2.28	0.53
Labial plaps	2weeks	15±5	0.21	0.61	0.25

Tissue	Time	Temperature	G0/G1	S	G2/M
Labial plaps	2weeks	15±5	0.13	0.48	0.14
Labial plaps	2weeks	15±5	0.17	0.75	0.19
Labial plaps	2weeks	15±5	0.18	0.58	0.23
Labial plaps	2weeks	15±5	0.14	0.53	0.16
Labial plaps	2weeks	15±5	0.18	0.30	0.20
Labial plaps	2weeks	15±5	0.19	0.50	0.22
Labial plaps	2weeks	15±5	0.22	0.65	0.26
Labial plaps	2weeks	15±5	0.13	0.38	0.15
Labial plaps	5weeks	15±5	0.14	0.43	0.17
Labial plaps	5weeks	15±5	0.15	0.36	0.20
Labial plaps	5weeks	15±5	0.10	0.18	0.10
Labial plaps	5weeks	15±5	0.12	0.27	0.24
Labial plaps	5weeks	15±5	0.12	0.18	0.12
Labial plaps	5weeks	15±5	0.13	0.48	0.16
Labial plaps	5weeks	15±5	0.08	0.23	0.09
Labial plaps	5weeks	15±5	0.07	0.27	0.09
Labial plaps	5weeks	15±5	0.11	0.35	0.11
Labial plaps	5weeks	15±5	0.14	0.25	0.13
Labial palps	1week	15±10	0.19	0.38	0.17
Labial palps	1week	15±10	0.19	0.34	0.16
Labial palps	1week	15±10	0.20	0.32	0.17
Labial palps	1week	15±10	0.22	0.35	0.18
Labial palps	1week	15±10	0.15	0.33	0.13
Labial palps	1week	15±10	0.15	0.24	0.13
Labial palps	1week	15±10	0.18	0.23	0.15
Labial palps	1week	15±10	0.14	0.26	0.12

Tissue	Time	Temperature	G0/G1	S	G2/M
Labial palps	1week	15±10	0.15	0.26	0.12
Labial palps	1week	15±10	0.11	0.18	0.10
Labial palps	1week	15±10	0.08	0.13	0.07
Labial palps	2weeks	15±10	0.47	0.90	0.50
Labial palps	2weeks	15±10	0.20	0.57	0.22
Labial palps	2weeks	15±10	0.13	0.39	0.16
Labial palps	2weeks	15±10	0.16	0.62	0.19
Labial palps	2weeks	15±10	0.18	0.34	0.22
Labial palps	2weeks	15±10	0.21	0.45	0.27
Labial palps	2weeks	15±10	0.16	0.39	0.19
Labial palps	2weeks	15±10	0.15	0.38	0.19
Labial palps	2weeks	15±10	0.16	0.40	0.17
Labial palps	2weeks	15±10	0.18	0.40	0.26
Labial palps	2weeks	15±10	0.13	0.33	0.15
Labial palps	5weeks	15±10	0.12	0.30	0.13
Labial palps	5weeks	15±10	0.20	0.36	0.24
Labial palps	5weeks	15±10	0.15	0.36	0.17
Labial palps	5weeks	15±10	0.11	0.26	0.13
Labial palps	5weeks	15±10	0.08	0.30	0.10
Labial palps	5weeks	15±10	0.16	0.33	0.16
Labial palps	5weeks	15±10	0.07	0.20	0.08
Labial palps	5weeks	15±10	0.08	0.21	0.08
Labial palps	5weeks	15±10	0.33	0.50	0.40
Labial palps	1week	20±5	0.27	0.65	0.29
Labial palps	1week	20±5	0.22	0.50	0.22
Labial palps	1week	20±5	0.28	0.52	0.28

Tissue	Time	Temperature	G0/G1	S	G2/M
Labial palps	1week	20±5	0.21	0.53	0.21
Labial palps	1week	20±5	0.23	0.67	0.23
Labial palps	1week	20±5	0.21	0.34	0.17
Labial palps	1week	20±5	0.26	0.35	0.24
Labial palps	2weeks	20±5	0.24	0.95	0.26
Labial palps	2weeks	20±5	0.18	0.60	0.20
Labial palps	2weeks	20±5	0.13	0.43	0.13
Labial palps	2weeks	20±5	0.34	1.09	0.46
Labial palps	2weeks	20±5	0.16	0.30	0.19
Labial palps	2weeks	20±5	0.17	0.36	0.21
Labial palps	2weeks	20±5	0.17	1.04	0.18
Labial palps	2weeks	20±5	0.12	0.35	0.13
Labial palps	2weeks	20±5	0.19	0.61	0.26
Labial palps	2weeks	20±5	0.29	0.98	0.33
Labial palps	2weeks	20±5	0.15	0.44	0.22
Labial palps	2weeks	20±5	0.20	0.66	0.23
Labial palps	5weeks	20±5	0.11	0.19	0.13
Labial palps	5weeks	20±5	0.09	0.21	0.10
Labial palps	5weeks	20±5	0.10	0.18	0.11
Labial palps	5weeks	20±5	0.13	0.27	0.12
Labial palps	5weeks	20±5	0.09	0.24	0.10
Labial palps	5weeks	20±5	0.14	0.21	0.10
Labial palps	5weeks	20±5	0.09	0.40	0.09
Labial palps	5weeks	20±5	0.05	0.15	0.05
Labial palps	5weeks	20±5	0.06	0.16	0.06

Tissue	Time	Temperature	G0/G1	S	G2/M
Labial palps	3 weeks after	27	0.12	0.22	0.11
Labial palps	3 weeks after	27	0.10	0.21	0.09
Labial palps	3 weeks after	27	0.10	0.21	0.10
Labial palps	3 weeks after	27	0.13	0.13	0.13
Labial palps	3 weeks after	27	0.12	0.20	0.11
Labial palps	3 weeks after	27	0.12	0.21	0.10
Labial palps	3 weeks after	27	0.20	0.35	0.20
Labial palps	3 weeks after	27	0.13	0.30	0.15
Labial palps	3 weeks after	27	0.06	0.11	0.06
Labial palps	3 weeks after	27	0.10	0.12	0.08
Labial palps	3 weeks after	27	0.09	0.13	0.07
Labial palps	3 weeks after	27	0.07	0.12	0.07
Labial palps	3 weeks after	27	0.07	0.10	0.06
Labial palps	3 weeks after	27	0.08	0.09	0.07
Labial palps	before	27	0.25	0.55	0.27
Labial palps	before	27	0.19	0.50	0.22
Labial palps	before	27	0.24	0.49	0.27
Labial palps	before	27	0.25	0.58	0.26
Labial palps	before	27	0.26	0.36	0.30
Labial palps	before	27	0.22	0.35	0.21
Labial palps	before	27	0.12	0.39	0.11
Labial palps	before	27	0.21	0.51	0.21
Labial palps	before	27	0.28	0.55	0.24
Labial palps	before	27	0.13	0.21	0.12
Labial palps	before	27	0.15	0.24	0.13
Labial palps	before	27	0.15	0.26	0.13
Labial palps	before	27	0.08	0.24	0.08

Tissue	Time	Temperature	G0/G1	S	G2/M
Labial palps	before	27	0.11	0.28	0.10
Labial palps	before	27	0.06	0.14	0.06
Labial palps	1week	27	0.13	0.19	0.12
Labial palps	1week	27	0.15	0.21	0.14
Labial palps	1week	27	0.14	0.19	0.12
Labial palps	1week	27	0.16	0.27	0.13
Labial palps	1week	27	0.20	0.35	0.17
Labial palps	1week	27	0.18	0.32	0.15
Labial palps	1week	27	0.10	0.18	0.09
Labial palps	2weeks	27	0.29	0.76	0.29
Labial palps	2weeks	27	0.26	0.95	0.30
Labial palps	2weeks	27	0.19	0.26	0.21
Labial palps	2weeks	27	0.18	0.59	0.18
Labial palps	2weeks	27	0.16	0.28	0.16
Labial palps	2weeks	27	0.19	0.51	0.20
Labial palps	2weeks	27	0.14	0.37	0.15
Labial palps	2weeks	27	0.23	0.61	0.26
Labial palps	2weeks	27	0.13	0.62	0.17
Labial palps	2weeks	27	0.11	0.39	0.13
Labial palps	2weeks	27	0.16	1.00	0.18
Labial palps	5weeks	27	0.14	0.50	0.16
Labial palps	5weeks	27	0.12	0.37	0.13
Labial palps	5weeks	27	0.13	0.55	0.20
Labial palps	5weeks	27	0.13	0.41	0.19
Labial palps	5weeks	27	0.07	0.22	0.09
Labial palps	5weeks	27	0.08	0.48	0.12
Labial palps	5weeks	27	0.13	0.24	0.17

Tissue	Time	Temperature	G0/G1	S	G2/M
Labial palps	5weeks	27	0.07	0.31	0.08
Labial palps	5weeks	27	0.10	0.32	0.13
Labial palps	5weeks	27	0.10	0.39	0.12
Labial palps	3 weeks after	GI	0.12	0.22	0.11
Labial palps	3 weeks after	GI	0.10	0.21	0.09
Labial palps	3 weeks after	GI	0.10	0.21	0.10
Labial palps	3 weeks after	GI	0.13	0.13	0.13
Labial palps	3 weeks after	GI	0.12	0.20	0.11
Labial palps	3 weeks after	GI	0.12	0.21	0.10
Labial palps	3 weeks after	GI	0.20	0.35	0.20
Labial palps	3 weeks after	GI	0.13	0.30	0.15
Labial palps	3 weeks after	GI	0.06	0.11	0.06
Labial palps	3 weeks after	GI	0.10	0.12	0.08
Labial palps	3 weeks after	GI	0.09	0.13	0.07
Labial palps	3 weeks after	GI	0.07	0.12	0.07
Labial palps	3 weeks after	GI	0.07	0.10	0.06
Labial palps	3 weeks after	GI	0.08	0.09	0.07
Labial palps	before	GI	0.08	0.18	0.07
Labial palps	before	GI	0.08	0.15	0.07
Labial palps	before	GI	0.05	0.11	0.05
Labial palps	before	GI	0.10	0.17	0.09
Labial palps	before	GI	0.10	0.18	0.09
Labial palps	before	GI	0.09	0.23	0.07
Labial palps	before	GI	0.05	0.11	0.05
Labial palps	before	GI	0.08	0.17	0.07
Labial palps	before	GI	0.07	0.11	0.07

Tissue	Time	Temperature	G0/G1	S	G2/M
Labial palps	before	GI	0.07	0.14	0.07
Labial palps	before	GI	0.07	0.15	0.07
Labial palps	before	GI	0.07	0.18	0.06
Labial palps	before	GI	0.07	0.18	0.06
Labial palps	before	GI	0.08	0.20	0.07
Labial palps	before	GI	0.08	0.16	0.07
Labial palps	1week	GI	0.20	0.35	0.21
Labial palps	1week	GI	0.17	0.40	0.15
Labial palps	1week	GI	0.23	0.29	0.19
Labial palps	1week	GI	0.19	0.34	0.16
Labial palps	1week	GI	0.20	0.28	0.17
Labial palps	1week	GI	0.20	0.30	0.16
Labial palps	1week	GI	0.16	0.21	0.15
Labial palps	1week	GI	0.18	0.29	0.14
Labial palps	1week	GI	0.19	0.27	0.17
Labial palps	1week	GI	0.39	0.40	0.25
Labial palps	1week	GI	0.24	0.29	0.17
Labial palps	1week	GI	0.22	0.31	0.16
Labial palps	2weeks	GI	0.18	0.52	0.20
Labial palps	2weeks	GI	0.25	0.83	0.36
Labial palps	2weeks	GI	0.16	0.51	0.19
Labial palps	2weeks	GI	0.19	0.72	0.21
Labial palps	2weeks	GI	0.13	0.41	0.15
Labial palps	2weeks	GI	0.17	0.58	0.20
Labial palps	2weeks	GI	0.06	0.27	0.09
Labial palps	2weeks	GI	0.10	0.49	0.12
Labial palps	2weeks	GI	0.08	0.27	0.11

Tissue	Time	Temperature	G0/G1	S	G2/M
Labial palps	2weeks	GI	0.06	0.35	0.07
Labial palps	2weeks	GI	0.09	0.15	0.12
Labial palps	2weeks	GI	0.09	0.27	0.11
Labial palps	5weeks	GI	0.14	0.48	0.15
Labial palps	5weeks	GI	0.10	0.29	0.14
Labial palps	5weeks	GI	0.07	0.28	0.09
Labial palps	5weeks	GI	0.09	0.24	0.11
Labial palps	5weeks	GI	0.02	0.04	0.02
Labial palps	5weeks	GI	0.13	0.28	0.14
Labial palps	5weeks	GI	0.10	0.32	0.12
Labial palps	5weeks	GI	0.10	0.28	0.11
Labial palps	5weeks	GI	0.06	0.12	0.08

Table B.8. Immunohistochemical data of labial palp nuclei labeling indices for high percent of PCNA concentrations (Chapter 2).

Tissue Number	Negative-PCNA	Positive-PCNA	Labeling Index (LI)
15±10-4-2	998	2	0.20
15±5-7-2	999	1	0.10
15±1-8-1	985	15	1.52
GI-6-1	992	8	0.81
INT-14	997	3	0.30
27-9-1	998	2	0.20
10±1-9-1	996	4	0.40
15±1-11-1	974	26	2.67
20±1-8-1	991	9	0.91
10±1-1-2	1000	0	0.00

Table B.9. Immunohistochemical data of labial palp nuclei labeling indices for low percent of PCNA concentrations (Chapter 2).

Tissue Number	Negative-PCNA	Positive-PCNA	Labeling Index (LI)
GI-11-4	983	17	1.73
20±1-3-4	998	2	0.20
15±5-11-4	997	3	0.30
10±5-7-4	999	1	0.10
27-6-4	984	16	1.63
20±5-3-4	999	1	0.10
15±10-2-3	993	7	0.70
10±5-3-3	971	29	2.99
15±1-3-4	1000	0	0.00
15±1-2-4	998	2	0.20

VITA

Fernando Jimenez was born in San Jose, Costa Rica. He attended Louisiana State University, Baton Rouge, Louisiana, where he received his Bachelor of Science in biochemistry in 2001. There after he worked for Pennington Biomedical Research Center as a research assistant until 2002. In August 2002 he began his master's program in fisheries at Louisiana State University, Baton Rouge. While a graduate student at LSU, Fernando worked with Dr. Terrence R. Tiersch as a graduate assistant, during which he attended various national and international conferences. He was awarded the best abstract at the 26th annual meeting of the American Fisheries Society (AFS) at Baton Rouge. His research focused on developing an assay for the detection of proliferating cells in eastern oysters by flow cytometry. Fernando is currently a candidate for the degree of Master of Science in fisheries on May 20, 2005.