FACULTY OF SCIENCE

UNIVERSITY OF SOUTH BOHEMIA

ČESKÉ BUDĚJOVICE 2011

Master thesis

Transcriptional analysis of cell cycle regulators during the induction phase of diapause in Chymomyza costata

Petra Šimůnková

Supervisor: Doc. Ing. Vladimír Košťál, CSc.

Master thesis

Šimůnková P., 2011. Transcriptional analysis of cell cycle regulators during the induction phase of diapause in Chymomyza costata. 42 p., Faculty of Science, The University of South Bohemia, České Budějovice, Czech Republic.

Annotation

The changes of relative mRNA levels of seven different genes, coding for key cell cycle regulatory factors (Cyclins D and E, kinases Wee1 and Myt1, Phosphatase Cdc25 (String), Dacapo (p27), and Pcna) were performed using qRT-PCR method. Two reference genes (Rp49 and β-tubulin) served as a background. Significant transcriptional response to photoperiodic transfer were observed for two genes. While the relative levels of Dacapo mRNA increased during the rapid entry into G2 arrest, the Pcna expression was significantly downregulated during the beginning of G0/G1 arrest. Moderate transcriptional upregulations of the genes coding for two cell cycle inhibitory kinases, Wee1 and Myt1 accompanied the entry into diapause. The other genes were expressed equally in all photoperiodic conditions.

I declare here that I wrote this work on my own, with usage of advices from my supervisor and of literature cited below.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své diplomové práce, a to v nezkrácené podobě – v úpravě vzniklé vypuštěním význačných částí archivovaných Přírodovědeckou fakultou – elektronickou cestou veveřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. Zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

České Budějovice 2011 .....

Petra Šimůnková

ACKNOWLEDGEMENTS

My immense thanks go to my supervisor Vladimír Košťál, for his incessant assistance, nerve and optimistic state of mind during the whole work. I'm also very grateful to Michaela Borovanská for her willingness to give me valuable advice whenever I needed some.

TABLE OF CONTENTS

1. Introduction

1.1 Familiarization with the cell cycle

All organisms consist of cells that multiply through cell division. An adult human being has approximately 100 000 billion cells, all originating from a single cell, the fertilized egg cell. In adults there is also an enormous number of continuously dividing cells replacing those dying. Before a cell can divide it has to grow in size, duplicate its chromosomes and separate the chromosomes for exact distribution between the two daughter cells. These different processes are coordinated in the cell cycle.

It has been known for over one hundred years that cells multiply through division. It is however only during the last two decades that it has become possible to identify the molecular mechanisms that regulate the cell cycle and thereby cell division. These fundamental mechanisms are highly conserved through evolution and operate in the same manner in all eukaryotic organisms (Harris, 2000).

The cell cycle consists of several phases (see figure 1). In the first phase (G1) the cell grows and becomes larger. When it has reached a certain size it enters the next phase (S), in which DNA-synthesis takes place. The cell duplicates its hereditary material (DNA-replication) and a copy of each chromosome is formed. During the next phase (G2) the cell checks that DNA-replication is completed and prepares for cell division. The chromosomes are separated (mitosis, M) and the cell divides into two daughter cells. Through this mechanism the daughter cells receive identical chromosome set ups. After division, the cells are back in G1 and the cell cycle is completed.

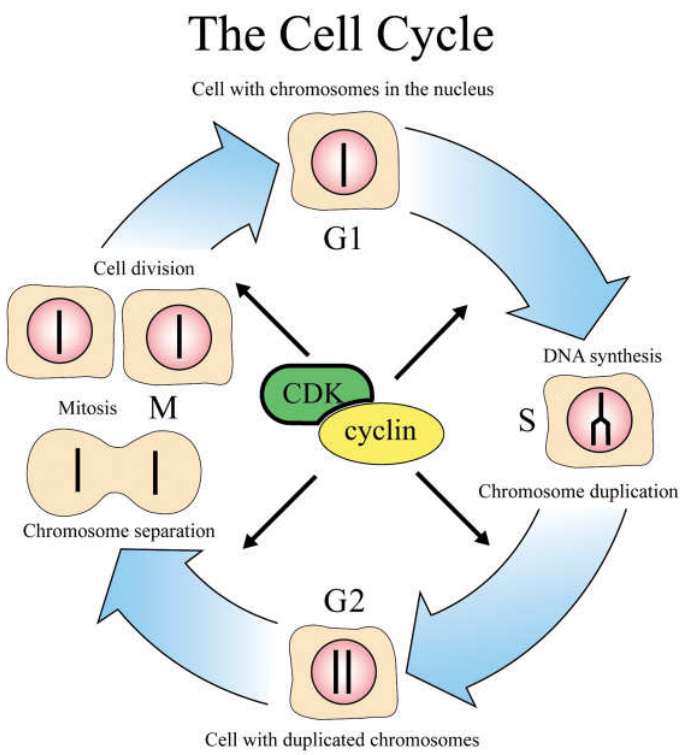


Fig.1. The cell cycle

The different phases of the cell cycle. In the first phase (G1) the cell grows. When it has reached a certain size it enters the phase of DNA-synthesis (S) where the chromosomes are duplicated. During the next phase (G2) the cell prepares itself for division. During mitosis (M) the chromosomes are separated and segregated to the daughter cells, which thereby get exactly the same chromosome set up. The cells are then back in G1 and the cell cycle is completed (Alberts et al., 2002).

The duration of the cell cycle varies between different cell types. In most mammalian cells it lasts between 10 and 30 hours. Cells in the first cell cycle phase (G1) do not always continue through the cycle. Instead they can exit from the cell cycle and enter a resting stage (G0).

1.2. The cell cycle regulation

The mechanism which controls the cell cycle is huge regulatory network that controls the arrangement and preciseness of cell cycle events.

The cardinal components of the cell cycle control system are **cyclin-dependent kinases (Cdks)**. As the cell cycle grow up through the cycle, huge changes in the enzymatic activities of these kinases lead to changes in the phosphorylation state and thus the state of activation, of proteins that control cell cycle processes. Concentration of Cdk proteins are constatnt troughout the cell cycle. Oscillations in their activity depend on corresponding oscillations in

levels of the regulatory subunits known as **cyclins**, which bind tightly to Cdks and stimulate their catalytic activity (Morgan, 2007).

1.2.1. The cyclin-dependent kinase (Cdks)

The cyclin-dependent kinases (Cdks) are family of serine/threonine protein kinases whose members are small proteins (~34-40 kDa) composed of little more than the catalytic core shared by all protein kinases (Morgan, 1997). All Cdks share the salience that their enzymatic activation requires the binding of a regulatory cyclin subunit. Generally, full activation also reguires phosphorylation of a threonine residue near kinase active site (Malumbres and Barbacid, 2009).

1.2.2. Cyclins

Cyclins are a family of proteins that control the progression of cells through the cell cycle by activating cyclin-dependent kinase (Cdk) enzymes (Galderisi et al., 2003). Cyclins are so named because their concentration varies in a cyclical fashion during the cell cycle. The oscillations of the cyclins, namely fluctuations in cyclin gene expression and destruction by proteolysis, induce oscillations in Cdk activity to drive the cell cycle. A cyclin forms a complex with Cdk, which begins to activate the Cdk, but the complete activation requires phosphorylation, as well. Cyclins themselves have no enzymatic activity and were discovered by R. Timothy Hunt in 1982 while studying the cell cycle of sea urchins.

Cyclins, when bound with the dependent kinases form the maturation-promoting factor. MPFs activate other proteins through phosphorylation (Masui, 2001). These phosphorylated proteins, in turn, are responsible for specific events during cycle division such as microtubule formation and chromatin remodeling. Cyclins can be divided into four classes based on their behavior in the cell cycle of vertebrate somatic cells and yeast cells: G1/S cyclins, S cyclins, M cyclins, G1 cyclins (Evans et al., 1983).

- **G1/S Cyclins** rise in late G1 and fall in early S phase. The Cdk- G1/S cyclin complex begins to induce the initial processes of DNA replication, primarily by arresting systems that prevent S phase Cdk activity in G1. The cyclins also promote other activities to progress the cell cycle, like centrosome duplication in vertebrates or

spindle pole body in yeast. The rise in presence of G1/S cyclins is paralleled by a rise in S cyclins.

- **S cyclins** bind to Cdk and the complex directly induces DNA replication. The levels of S cyclins remain high, not only throughout S phase, but through G2 and early mitosis as well to promote early events in mitosis.
- **M cyclin** concentrations rise as the cell begins to enter mitosis and the concentrations peak at metaphase. Cell changes in the cell cycle like the assembly of mitotic spindles and alignment of sister-chromatids along the spindles are induced by M cyclin- Cdk complexes. The destruction of M cyclins during anaphase causes the exit of mitosis and cytokinesis.
- **G1 cyclins** do not behave like the other cyclins, in that the concentrations increase gradually (with no oscillation), throughout the cell cycle based on cell growth and the external growth-regulatory signals. The presence of G cyclins coordinate cell growth with the entry to a new cell cycle.

1.2.3. Switches in cell cycle and some key players

A series of biochemical **switches** control transitions between and within the various phases of the cell cycle. This switches maintain the orderly progression of the cell cycle and act as **checkpoints** to ensure that each phase has been properly completed before progression to the next phase (Morgan, 2007). Cyclin dependent kinases, are a major control switches for the cell cycle. Such multi-component (involving multiple inter-linked proteins) switches have been shown to generate decisive, robust (and potentially irreversible) transitions and trigger stable oscillations (Santos, 2008).

1.2.3.1. The G1/S switch

The G1/S transition, more commonly known as the **Start checkpoint** or the restriction point regulates cell cycle commitment (Morgan, 2007) At this checkpoint, cells either arrest before DNA replication (due to limiting nutrients or a pheromone signal), prolong G1 (size control), or begin replication and progress through the rest of the cell cycle.

The G1/S cell cycle checkpoint controls the passage of eukaryotic cells from the first gap phase, G1, into the DNA synthesis phase, S. In this switch in mammalian cells, there are two cell cycle kinases that help to control the checkpoint: cell cycle kinases **CDK4/6-cyclin D** and

CDK2-cyclin E (Baserga, 1985). Together they phosphorylate the pocket pRB proteins. Phosphorylated pRB proteins dissociate from transcription regulators E2F. The E2F regulators than control the expression of huge numer of genes, including cyclins E and A, which are crucial for G1-S transition and S phase progression (Attwooll et al., 2004).

Another important players, that govern the G1/S-and S Cdk aktivity during entry into the cell cycle, is **Dacapo (p27)**; specific cdk-inhibitory protein (Sherr et al., 1999) and **Proliferating Cell Nuclear Antigen (Pcna)**. Pcna can bind Cyclin A-Cdk2 (S-Cdk) and thus produce an active complex promoting the progression through the S phase (Koundrioukoff et al., 2000).

1.2.3.2. The G2/M switch

This transition is commenced by E2F-mediated transcription of cyclin A, forming the cyclin A-Cdk2 complex. This is useful in regulating events in prophase. In order to proceed past prophase, the cyclin B-Cdk1 complex (first discovered as MPF or M-phase promoting factor) is activated by **Cdc 25 (String)**, a protein phosphatase (Edgar et al., 1994). As mitosis starts, the nuclear envelope disintegrates, chromosomes condense and become visibile, and the cells prepares for division. The Cyclin B-Cdk1 activation results in nuclear envelope breakdown, which is a characteristic of the initiation of mitosis. It is evident that the cyclin A and B complexes with Cdks help regulate mitotic events at the G2/M transition (Lehner et al., 1990).

As mentioned above, entry into mitosis is controlled by the Cyclin B-Cdk1 complex (first discovered as a M-phase promoting factor; Cdk1 is also known as Cdc2 in fission yeast and Cdc28 in budding yeast). This complex forms an element of an interesting regulatory circuit in which Cdk1 can phosphorylate and activate its activator, the phosphatase **String** (**Cdc25)** (positive feedback), and phosphorylate and inactivate its inactivators, the kinases **Wee1 and Myt1** (Ferrell, 2002; Russell et al., 1987).

1.2.3.3. The metaphase-to-anaphase transition

The metaphase-to-anaphase transition is the third major ceckpoint of the cell cycle. This checkpoint leads to sister chromatid segregation, completion of mitosis and cytokinesis. Progression through this checkpoint occurs when M-phase cyclin-Cdk complexes stimulate an

enzyme called the anaphase-promoting complex; which causes the proteolytic destruction of cyclins and of proteins that hold the sister chromatids together (Hartwell et al., 1989).

1.2.4. Higher regulation of cell cycle – upstream control

The cells of multicellular organism divide only when the organism as a whole needs new cells to build or maintain tissues (Alberts et al., 2002). In general terms, entry into a new cell cycle comes in animals only when the cell is exposed to the relevant **mitogens**. In general, mitogens are soluble peptides or small proteins secreted by neighboring cells, or they can be insoluble components of the extracellular matrix (Adhikary et al., 2005). Mitogens trigger signal transduction pathways in which mitogen-activated protein kinase is involved, leading to mitosis.

1.3. Cell cycle arrest

To allow regulation of cell cycle progression, the cell cycle control mechanism is supplemented by molecular braking mechanism that can be used, if necessary, to inhibit the Cdks and other regulators that drive progression through the three major checkpoints (Murray and Hunt, 1993). If environmental conditions are not appropriate for cell proliferation, inhibitory signals prevent activation of G1/S – and S-phase Cdks – thereby blocking progression through Start (Morgan, 1997). Similary, the failure to complete DNA replication blocks entry into mitosis by inhibiting M-phase cyclin-Cdk activation (Murray and Kirschner, 1989). Delays in spindle assembly inhibit the proteolytic machinery that drives the metaphase-to-anaphase transition. By these and numerous other mechanisms, the cell arrests cell cycle progression at an appropriate point when conditions are not ideal and continues it when they are.

1.4. Cell cycle arrest during diapause in insects

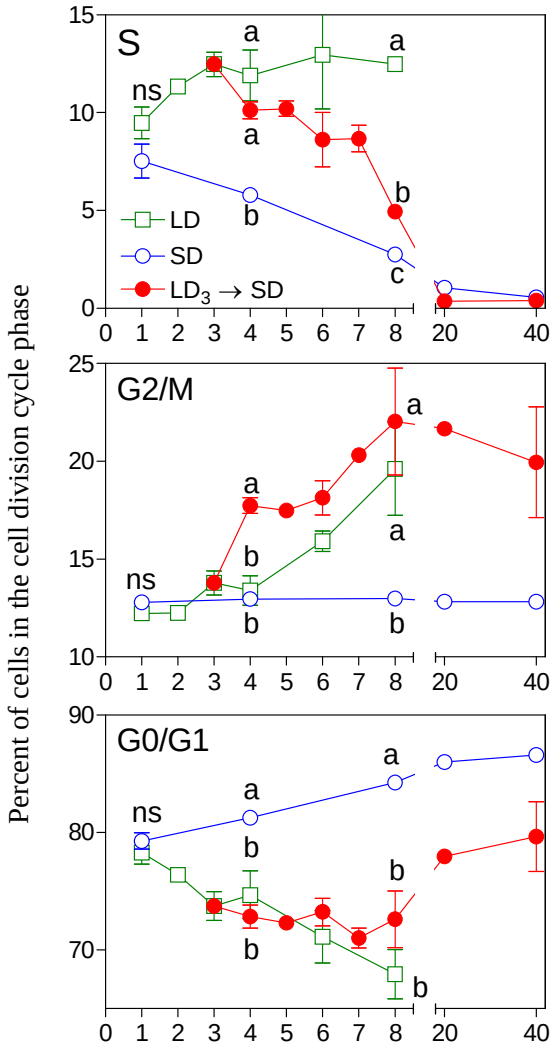
Diapause helps insects to pass through hostile periods and, as a result, to prosper from seasonally fluctuating resources, diversify in tropical habitats, and colonize temperate and polar regions. Diapause is a centrally-mediated seasonal response to token stimuli. The diapause results in induction of developmental arrest in advance of the emergence of the environmental stress. At the level of whole organism, the ontogeny is blocked in a diapause stage, which ranges from early embryo to adult (Lees, 1955; Danks, 1987; Denlinger, 2002; MacRae, 2005; Košťál, 2006). At the cellular level, cells in target organs in larval and pupal

stages or ovaries in adults arrest their differentiation and pregressio through the cell cycle (Tammariello and Derlinger, 1998). The cell cycle arrest is so the one of the characteristics of insect diapause.

However, there are only few information about the cell cycle arrest in insect diapause. The three of them should be mentioned here:

- In the silkworm, *Bombyx mori*, diapause occurs at a specific embryonic stage when 98% of cells are arested in G2 phase (Nakagaki et al., 1991).
- In contrast to *B. mori*, more that 97% of brain cells in diapausing larvae of the fleshfly, *Sarcophaga crassipalpis*, are arrested in G0/G1 phase (Tammariello and Denlinger, 1998). The regulatory events foregoing the cell cycle to stop remain practically unknown. Tammariello and Denlinger (1998) compared the expression of four regulatory genes in the brains of diapausing and nondiapausing pupae of *S. crassipalpis.* They found clear difference for one of this genes, Pcna (proliferating cell nuclear antigen). Very low levels of pcna expression were detected in diapausing brains using Nothern blot analysis and I*n situ* hybridization. Conversely, high levels of Pcna transcripts found in nondiapausing brains.
- V. Košťál (Košťál et al., 2009) used flow cytometry analysis to detect the phases at which the cells of brain tissues are arrested during larval diapause of *Chymomyza costata*.

Fig. 2. The flow cytometry results



Flow cytometry analysis of relative proportions of *Chymomyza costata* larval brain cells in various stages of the cell division cycle: S, G2/M, G0/G1. Each data point is a mean ± S.D. of two independent samples (n = 2; 4 x 10 000 nuclei in each sample). Each sample was prepared by pooling ten brains dissected from larvae of different age (x axis) and reared under three different photoperiodic regimes: LD, SD and Ld_3 – SD.

This experiment showed that:

- during the S phase of cell cycle the proportion of cells decreased notably in day 40 in diapausing larvae - SD -. When the larvae were transfered from LD_3 – SD conditions, the larvae changed their developmental destiny from direct development end entered the diapause state and the cell division cycle in their brain was gradualy arrested.
- whereas the number of cells decreased in S phase of cell cycle, the relative proportions of cells in G2/M and G0/G1 arrest increased.

2. Specific aims

- To clone selected transcriptionally regulated cell cycle regulators in *Chymomyza costata*.

  Selected regulators:
  - My1 and Wee1 kinases
  - String (Cdc25) phosphatase

- To optimize the qRT – PCR method (design primers, test optimal PCR conditions) for relative quantification of mRNA transcripts.
- Check the relative amounts of mRNA transcript levels of seven selected cell cycle regulators during the photoperiodically induced diapause in *Chymomyza costata*. Selected regulators:
  - Myt1 kinase
  - Wee1 kinase
  - String (Cdc25) phosphatase
  - Pcna (Proliferating Cell Nuclear Antigen)
  - Dacapo (p27)
  - Cyclin D
  - Cyclin E

3. Materials and methods

3.1. Insect and disections

A laboratory strain Sapporo of *Chymomyza costata* (Zetterstedt) (Diptera: Drosophilidae) was used in this study. *C.costata* is spread over cool-temperate, Holarctic area (Hackman et al., 1970). *C. costata* enters winter diapause as a mature 3rd instar larva; responding to long nightlength and / or low temperature (Riihimaa and Kimura, 1988; Yoshida and Kimura, 1995). During its embryonic and 1st larval instar, *C. costata* is sensitive to photoperiodic stimulus. This sensitivity increases gradually during the 2nd and early 3rd larval instars and reaches the maximum cca in 15-day old larvae (since egg laying), which corresponds to 3-d-old 3rd instar. Until this age, all larvae react on the transfer from long-day (LD) to short day (SD) conditions by altering their developmental destiny from direct development to diapause (Košťál et al., 2000a).

C. costata larvae were rised up on an artificial diet of Lakovaara (1969), composed of agar, malted barley, corn flour and yeasts under a constant temperature of 18 °C. Two different photoperiodic regimes were used in this study: a long-day regime (LD) (16 h light : 8h dark), at which larvae continue direct development (pupariate); and a short-day regime (SD) (12 h light : 12 h dark) that induces diapause in all larvae (Košťál et al., 2000a). Beside, the diapause was induced in 3-day-old 3rd instar larvae by transfering them from LD to SD conditions.

Central nervous system (CNS) was dissected from larvae in a cold insect saline solution. Tissues from 10 larvae were gathered to obtain one sample. The CNS samples for a qRT-PCR analysis were prepared in biological triplicates and stored at -80°C in the RNA Blue solution (TopBio, Prague, Czech Republic).

3.2. Cloning

All 3 genes (Myt1, Wee1 and Cdc25) were cloned using the same procedure (mentioned below).

3.2.1. Total-RNA isolation (RNA Blue, Top-Bio) of CNS (brain tissue) samples

The routine:

- Add 40 µL of TE buffer, mix, spin tissues down at 5 000g/5 sec;
  remove carefully as much as possible of RNAlater
- Add 200 µL of RNA Blue
- Homogenize thoroughly using pellet pestle battery driven homogenizer, add 300µL RNA Blue (total volume is 500 µL), mix, spin down
- Keep at r.t. (room temperature) for 5 min, mix occasionally
- Add 100 µL of chloroform, shake vigorously for 15 sec, Vortex
- Keep at r.t. for 5 min
- 15 000g/10 min/4°C → 3 phases: take ca. 250 (or less) µL of upper aqueous phase, (discard the rest)
- During previous step, prepare tubes with 250 µL of isopropanol (ice), and add 250 µL of aqua-phase to it; finally add 1 µL of RNA-free glycogen solution (20 µg/mL)
- Allow precipitate for 30 min at 4°C
- 22 000g/20 min/4°C, remove liquid
- Wash pellet with 500 µL of 75 % EtOH in DEPC water (-20°C), mix by inverting 22 000g/5 min/4°C, remove liquid - (large pipette → spin down → small pipette)
- Dry (3 - 6 min on air)
- Dissolve in 5 µL DEPC water (keep on ice, mix by taping, spin down), no measuring of concentration, take all for cDNA synthesis

3.2.2. cDNA synthesis (Reverse Transcription System, Promega)

The routine:

- Set 70°C & 42°C on Epe tube incubators
- Heat a 5 µg aliquot of tRNA in a small Epe tube at 70°C for 5 min, place on ice, spin down
- Assemble a 20 µL reactions in the order listed (prepare Master Mix - MM without RNA template):

	1x
$MgCl_2$	4
10x buffer	2
dNTP mix	2
RNasin	0.5
AMV RT	0.8
Oligo $(dT)_{15}$	1
tRNA template	5
DEPC water	4.7
total volume	20.0 µL

- Add à 15 µL of MM into Epe tubes with denatured RNA templatse, Vortex, spin down (avoid formation of air bubles)
- Incubate the reactions at 42°C for 45 min (RT)
- Heat at 95°C for 5 min
- Cool down on ice for 5 min
- The resulting first-strand cDNA synthesis reactions should be diluted to:
  no dilution for cloning purposes
  100 µL (5x) with sterile water (add 80 µL) for normal PCR
  500 µL (25x) with sterile water (add 480 µL) for quantitative PCR on light cycler

3.2.3. Design of primers

For amplification by PCR method, degenerated oligonucleotide primers were used. These primers were designed using CODEHOP freeware on the basis of known sequences from other insects as published in NCBI GenBank database.

3.2.4. PCR and agarose electrophoresis

The routine:

- Assemble a 20 µL reactions in the order listed:

		4x Master Mix
Water		112
10x buffer		20
dNTP mix		16
Forward Primer		20
Reverse Primer		20
cDNA Template		12
ExTaq (HS ExTaq)		1,6
DEPC water		103.4
total volume	20.0 µL	200 µL (4 x 50 µL MM)

PCR conditions were: 95°C/3 min – initial impulse to denature strands

94°C/30 s – denaturation in progress
50°C/30 s – primers annealing
72°C/1 min – new strand synthesis

35 x

72°C/5 min – polymerization complete
4°C/5 min – cooling

- Prepare 2% agarose gel:

Agarose	0,5 g
50x TAE (Tris, acetic acid, EDTA)	0,5 ml
Water	24,5 ml

- Place an agarose gel into buffer-filled box
- Apply a *DNA ladder* for sizing into the first lane of the gel
- Apply other lanes with PCR products
- Run electrophoresis for 30min
- Submerge gel into ethidium bromide for 15-20min
- Examine gel under UV light
- Cut the the product from an agarose gel under UV

3.2.5. PCR product extraction from agarose gel (QIAquick Gel Extr. Kit)

The routine:

- Set incubator to 50°C, excise the DNA fragment (max. 300 mg of the gel)
- Add 3x vol. of Buffer QG - 750
- Incubate at 50°C for 10 min, Vortex every 2-3 min, dissolve the gel
  after dissolving, the mixture should remain yellow
  if not (if turned orange or violet) add 10 μL of sodium acetate (3M, pH 5.2)
- Add 1x vol. of isopropanol, mix, do not spin down - 250
- Apply to the spin column and > 18 0000 g/1 min/r.t.
- Add 750 μL of Buffer PE and > 18 0000 g/1 min/r.t., discard flowthrough
- 18 0000 g/1 min/r.t. in an empty collection tube (remove residual ethanol)
- Place column in a new tube, add 30 μL of Buffer EB, let stand for 1 min at r.t.
- 18 0000 g/1 min/r.t. → DNA product is in 30 μL of EB buffer
- Pool DNA products from all bands (4 x 30 = 120 μL)
  Measure concentration in 10 μL

3.2.6. Ligation into vector (pGEM-T Easy, Promega)

A. Optimizing the DNA insert concentration

Electrophoresis gel extraction product (insert) was 0,9kb (900bp) long.

Required molar ratio of insert DNA / vector DNA is 8/1

(accepted ratios are between 8/1 – 1/8)

(optimum ratios are between 3/1 – 1/3)

Formula: $\frac{50 \times 0,9}{3} \times \frac{8}{3} = 120ng$

→ 120 ng of the insert DNA should be used for ligation reaction

dilute DNA product of PCR to: 120 ng / 1 µL

B. Ligation reaction

- Set up the reaction in sterile 0,5 mL Epe tube

pGEM vector	3µL	(50 ng of DNA)
2 x Ligation Buffer	15µL	(store in aliquots)
sterile w.	6 µL	
DNA insert	3 µL	(carrying optimal amount of DNA insert)
MIX, spin down		
T4 DNA ligase 3 µL	(3 U)	
	10 µL	

- Incubate overnight at 4°C

3.2.7. Transformation of competent cells (JM 109, Promega)

The routine:

- Set water bath to 42°C, equlibrate LB solution to r.t.
- Transfer full ligation reaction (10 µL) into a new 1,5 mL Epe tube
- Thaw cells from -80°C to ice temp. for approx. 5 min
- Transfer 50 µL of cells to ligation reaction, mix, keep on ice for 20 min
- Heat shock the cells at 42°C for 50 sec.
- Immediately to ice for 2 min
- Add 450 µL of LB solution
- Incubate for 1,5 h at 37°C with shaking (160 RPM)

3.2.8. Preparation of LB plates

The routine:

- Dissolve 1 tablet of LB broth in 50 mL of water, autoclave (or microvawe) and store at 4°C
- Make 1,2% solution of Agar in the LB solution, for instance: melt 1,44g of Agar in 120 mL of LB sol. → 6 dishes à 20 mL
- Cool down to ca. 60°C
- Pour into the dishes
- Allow solidify for 1 h
- Close dishes, wrap, store at 4°C for up to 1 mo
- Equilibrate dishes at r.t.
- Add to centre:

Ampicilin	40 µL	(stock 50 mg/mL w.)
IPTG	20	(0.1 M; 23.8 mg/mL w.)
X-gal	20	(20 mg/ mL DMF)

- Keep open at 37°C for approx. 30 min, then close

3.2.9. Plating and incubation

The routine:

- Add 20 – 100 µL of cell solution (in LB) to the centre (20 for fresh cells, 100 for old and repeatedly melted cells)
- Spread
- Cultivate at 37°C overnight

Three plates were set and left to incubate at 37°C overnight. White colonies were suspected of taking the plasmid.

3.2.10. PCR control and cultivation of selected clones

The routine:

- Add Apmicilin to LB solution: (32) clones → 16(32) x 0.2 = 3.2(6.4) mL of LB solution with 3.2(6.4) µL Amp. sol. Pipette à 200 µL of LB+Amp. into the wells of sterile Elisa plate
- Prepare PCR (rTaq) master mix and PCR tubes:

	25xMM
10x Buffer	25 µL
d NTP	20 µL
M13 fw	1,25 µL
M13 rev	1,25 µL
water	201,25 µL
rTaq	1,25
	250 µL

- Sample white colony with a sterile wooden stick
  submerge the stick into the Elisa well, rotate
  submerge the stick into the PCR tube, rotate thoroughly
- Cultivate clones in Elisa plate wells at 37°C
- Run PCR (1h 55 min)
- Electrophoresis → select clones according the product size (1% agarose gel)
- Prepare Terrific broth (8 ml for each clone: 6 clones, 48 mL; 12 clones 96 mL)

	400 µL
Terr. medium	18.8 g
glycerol	3.2 mL

autoclave, store at 4°C
add Apmicilin before use: 100 µL Amp. stock/ 100 mL of Terrific broth

- Add into sterile 15 mL tubes (Corning):
  8 mL Terrific broth + Amp.
  80 µL of pre-cultivated clone from Elisa plate well
- Cultivate at 37°C overnight (shaking at 160 RPM)

3.2.11. Isolation of plasmid DNA (QIAPrep Miniprep, Qiagen)

The routine:

- 3 000 RPM (920 g)/5 min/ r.t. → collect cells
- Resuspend cells in 250 µL of Buffer P1, Vortex, transfer to new 1,5 mL tubes
- Add 350 µL of Buffer P2, gently mix, inverting, max. 5 min
- Add 350 µL of Buffer N3, immediatelly and vigorously mix
- 18 000 g/10 min/r.t., take supernatant carefully and apply to the column

- 18 000 g/60 sec, discard flow-through
- Add 500 μL of Buffer PB, 18 000 g/60 sec, discard flow-through
- Add 750 μL of Buffer PE, 18 000 g/60 sec, discard flow-through
- 18 000 g/60 sec, empty collection tube (remove traces of ethanol)
- Place column in a new 1,5 tube, add 50 μL of Buffer EB to the center let stand for 1 min
- 18 000 g/90 sec → plasmid DNA in 50 μL of the EB buffer
- Take 5 μL for concentration measurement

3.2.12. Preparation of sequencing reaction (BigDye Terminator, Applied Biosystems)

The routine:

One reaction requires 0.3 – 1 μg of plasmid DNA

- Prepare PCR reaction

	11x
water	77 μL
plasmid DNA	x
5 x buffer	22 μL
M13 primer	11 forward μL
BigDye mix	44 μL
total	20 μL

- Run PCR (file SEQ) (2h 40 min)

3.2.13. Purification of PCR product

The routine:

- Transfer PCR product to 1,5 mL tube, add 80 μL of 80% isopropanol
- Precipitate for 15 min at r.t. in the darkness
- 22 000 g/30 min/4°C → pellet is almost INVISIBLE, discard liquid
- Add 200 μL of 80% isopropanol, 22 000 g/30 min/4°C, discard liquid
- Dry for 10 min at r.t., store at 4°C in darkness → pass to Sequencer lab.

3.2.14. DNA sequencing

In this work, the ABI Prism 377 DNA Sequencer was used to obtain the final sequence of *all 3 genes* of *C. costata*. This procedure was made by A. Trojanová.

3.3. mRNA transcripts' quantification

Totally 10 CNS of *C.costata* were pooled in each sample. Larvae were reared under three different photoperiodic regimes and at constant temperature of 18°C:

- a long-day (LD) regime (16 h light : 8 h dark); all larvae continue direct development (pupariate)
- a short-day (SD) regime (12 h light : 12 h dark); induction of diapause in all larvae
- a LD-SD transfer; larvae transferred in 3-day-old 3rd instar – induction of diapause

The total RNA was extracted from tissue samples using RNA Blue kit (TopBio, Prague, Czech Republic). And each sample was stored at -80°C for further analysis.

3.3.1. Isolation of total RNA (RNA Blue, Top-Bio s.r.o.)

The routine: was described above

3.3.2. Synthesis of cDNA (reverse transcription) (RT Promega)

The routine: was described above

- Resulting cDNA was diluted 25x (480µL of sterile water was added to Epes with 20 µL cDNA-synthesi reaction)
- 4 aliquots were made from stock solution

3.3.3. qPCR (Light-cycler Corbett Res.)

- Relative abundance of mRNA transcripts for target genes were measured by quantitative Real Time PCR (qRT-PCR) technique using a Rotor Gene RG 3000 PCR light cycler (Corbett Research, Sydney, Australia) and a Hot Start version of TaKaRa ExTaq polymerase (Takara, Shiga, Japan)
- Treshold cycle (C_T), i.e. the cycle at which there is a significant detectable increase in fluorescence signal was determined for each sample

The routine (rotor with 36 holes = 4x9 samples):

- Assemble Master Mix 40

sterile water	388,8 µL
dNTP mix	64
10x buffer	80
Syber Green (1:1000)	32
ExTaq HS	3,4
Total	568
+ primers	2 x 8 + 8
+cDNA	40 x 5

- Mix Ex Taq with the pipette tip before adding
- Mix well the Master Mix after its assembled
- Melt cDNA samples that will be measured
- Divide Master Mix to two aliquots a 284 µL
- Add 8 + 8 µL of the upper and lower primers (20 pmol/1µL), mix, keep on ice protected from direct light
- Prepare 36 PCR tubes (0,2 mL)
- Pipette a 5 µL of cDNA into each PCR tube
- Add a 15 µL of the Master Mix into each PCR tube
- Close lids
- Keep on ice and protected from direct light before starting PCR run

3.4. Analysis of results

The relative quantification of a target gene to a reference gene wa sdone according to Pfaffl (2001).

The C_T values were further recalculated to the relative changes of total amount in 3- -days old LD-larvae as 100%, and using the real amplification efficiency of 1,85-fold per one PCR cycle.

3.4.1. Reference mRNA transcripts

During the 3rd larval instar the CNS tissue undergo a rapid growth and development. This is the limitation for choosing the suitable reference gene for qRT-PCR analysis. Two

reference standards; *Ribosomal protein 49* (Rp49, coding for a structural constituent of ribosome involved in nucleic acid binding) and *β-tubulin* (coding for a structural constituent of a cell cystoskeleton) were choosen in this work.

Structural homologs of the Rp49 and β-tubulin genes in *C.costata* were cloned and partially sequenced earlier (Stehlík et al., 2008; Shimada, unpublished data).

3.4.2. Statistical analysis

In this work all data were statistically anylyzed by two-way ANOVA followed by Bonferroni post-hoc test (Prism5, GraphPad Software, San Diego, CA, USA). The details of statistical analysis are presented in the Suplementary online material to the paper Koštál et al. (2009) (doi: 10.1016/j.ibmb.2009.10.004).

4. Results

4.1. Results of cloning and sequencing

4.1.1. Results of PCR using degenerated oligonucleotide primers

Products of approximately 1000 bp (Wee), 350 bp (Myt), and 600 bp String were obtained, cut out and extracted from gel. The products were then ligated into the vector, in which the products were multiplied and then isolated from plasmids. Finally, the products were prepared for sequencing and sequenced.

4.1.2. Results of BLAST analysis for Wee, Myt and String

The "nucleotide blast" (NCBI BLAST) method was used to compare the obtained sequences of *C. costata* to their nearest homologues:

- **Wee1** of fruit fly *Drosophila melanogaster* was found as a nearest structural homologue of *C. costata* Wee (homology 91%). The second nearest sequence was mitosis inhibitor kinase of *Aedes aeypti* (homology 77%).

Fig.3. Alignement of amino acid sequences of *D.melanogaster* **Wee1** (NCBI GenBank) and its *C. costata* structural homolog (obtained in this study)

> ref|NP_477035.1| wee CG4488-PA [Drosophila melanogaster]
sp|P54350|WEE1_DROME Wee1-like protein kinase (Dwee1)
gb|AAF52453.2| CG4488-PA [Drosophila melanogaster]
gb|AAM50802.1| LD27552p [Drosophila melanogaster]
Length=609

GENE ID: 33965 wee | wee [Drosophila melanogaster] (Over 10 PubMed links)

Score = 615 bits (1585), Expect = 2e-174, Method: Compositional matrix adjust.
Identities = 297/346 (85%), Positives = 317/346 (91%), Gaps = 9/346 (2%)

```
Query  1    DTANVNPFTPDSLLAHNKKRCRTQFGRENLN--TNSVQKYLLTDRSDEDGAWLPEDSSAD  58
            DTANVNPFTPDSL+AHNKKRCRTQFGRENLN  N++QKYLL+D  D+D     + + D
Sbjct  157  DTANVNPFTPDSLMAHNKKRCRTQFGRENLNLNVNAMQKYLLSDACDDDVT----EEAGD  212

Query  59   SMREIHQ-APKRLALHDTNISRFKREFMQVSVIGVGEFGVVFQCVNRLDGCIYAIKKSKK  117
            SMREIHQ APKRLALHDTNISRFKREFMQV+VIGVGEFGVVFQCVNRLDGCIYAIKKSKK
Sbjct  213  SMREIHQQAPKRLALHDTNISRFKREFMQVNVIGVGEFGVVFQCVNRLDGCIYAIKKSKK  272

Query  118  PVAGSSFEKRALNEVWAHAVLGKHDNVVRYYSAWAEDDHMLIQNEYCDGGSLHARIKDQC  177
            PVAGSSFEKRALNEVWAHAVLGKHDNVVRYYSAWAEDDHMLIQNE+CDGGSLHARI+D C
Sbjct  273  PVAGSSFEKRALNEVWAHAVLGKHDNVVRYYSAWAEDDHMLIQNEFCDGGSLHARIQDHC  332
```

Query 178 LSEAELKIFLMHVIEGLRYIHSNDLVHMDLKPENIFSTMNP-VTRLTEVQ-QQCRDEDGM 235
L EAELKI LMHVIEGLRYIHSNDLVHMDLKPENIFSTMNP +L EVQ QQ +D+DGM
Sbjct 333 LGEAELKIVLMHVIEGLRYIHSNDLVHMDLKPENIFSTMNPNAHKLVEVQPQQTKDDDGM 392

Query 236 DSVYEELRKSENLVTYKIGDLGHVTSVKEPHVEEGDCRYLPKEILQEDYSNLFKADIFSL 295
DSVYEELR SENLVTYKIGDLGHVTSVKEP+VEEGDCRYLPKEIL EDYSNLFKADIFSL
Sbjct 393 DSVYEELRHSENLVTYKIGDLGHVTSVKEPYVEEGDCRYLPKEILHEDYSNLFKADIFSL 452

Query 296 GITLYEVAGGGPLPKNGPEWHKLRNGEVPCIPTLSKDFNELIALMM 341
GITL+E AGGGPLPKNGPEWH LR+G+VP +P+LS+DFNELIA MM
Sbjct 453 GITLFEAAGGGPLPKNGPEWHNLRDGKVPILPSLSRDFNELIAQMM 498

- **Myt1** of fruit fly *Drosophila melanogaster* was found as a nearest structural homologue of *C. costata* Wee (homology 96%). The second nearest sequence was mitosis inhibitor kinase of *Culex pipiens* (homology 82%).

Fig.4. Alignement of amino acid sequences of *D.melanogaster* **Myt1** (NCBI GenBank) and its *C. costata* structural homolog (obtained in this study)

> gb|AAF32288.1| Myt1 kinase-like protein [Drosophila melanogaster]
Length=534

Score = 222 bits (566), Expect = 6e-57
Identities = 106/118 (89%), Positives = 114/118 (96%), Gaps = 1/118 (0%)
Frame = +1

Query 1 EVRRYEEFSGHENCIRFIRAWEQYDRLFMQMELCRENLEQYLFRCRIIPEERIWHILLDL 180
EVRRYEEFSGHENCIRFIRAWEQYDRL+MQMELCRE+LEQYL RC+ IPEERIWHILLDL
Sbjct 149 EVRRYEEFSGHENCIRFIRAWEQYDRLYMQMELCRESLEQYLLRCQRIPEERIWHILLDL 208

Query 181 LRGLQSLHDRNLIHLDIKLDNVLI-DDDDTCKLADFGLVIDVDKANNDHATEGDSRYM 351
LRGL+SLHDRNLIHLDIKLDNVLI +DD+TCKLADFGLVIDVD+AN+ HATEGDSRYM
Sbjct 209 LRGLKSLHDRNLIHLDIKLDNVLIGEDDETCKLADFGLVIDVDRANSHHATEGDSRYM 266

- **String (Cdc25)** of fruit fly *Drosophila melanogaster* was found as a nearest structural homologue of *C. costata* Wee (homology 77%). The second nearest sequence was m-phase inducer phosphatase (Cdc25) od *Aedes aegypti*.

Fig.5. Alignement of amino acid sequences of *D.melanogaster* **String (cdc25)** and its *C.costata* structural homolog (obtained in this study)

> ref|NP_524547.1| string [Drosophila melanogaster]
sp|P20483.2|MPIP_DROME RecName: Full=M-phase inducer phosphatase; AltName: Full=Cdc25-like protein; AltName: Full=Protein string
emb|CAA40732.1| CDC25 [Drosophila melanogaster]
gb|AAF56885.1| string [Drosophila melanogaster]
gb|AAL39849.1| LD47579p [Drosophila melanogaster]
gb|ACL90619.1| stg-PA [synthetic construct]
Length=479

GENE ID: 43466 stg | string [Drosophila melanogaster] (Over 100 PubMed links)

```
 Score =  251 bits (640),  Expect = 5e-65
 Identities = 139/201 (70%), Positives = 154/201 (77%), Gaps = 19/201 (9%)
 Frame = +1

Query  1    YMELFEMETLAGQQQHSFGFPSGLNSLISGQIKVSSPAPLMPMNQSKTPETLTIMRRPPV  180
            YMELFEME+ +  QQ + GFPSGLNSLISGQIK   PA       +K+P  L+ MRRP V
Sbjct  128  YMELFEMESQS--QQTALGFPSGLNSLISGQIK-EQPA-------AKSPAGLS-MRRPSV  176

Query  181  RRCLSMTESNQQMQQSPVPKTPEQLLKETARDCFKRPEPPASTNCSPVHSKRHRFT--EK  354
            RRCLSMTESN     +P PKTPE     TARDCFKRPEPPAS NCSP+ SKRHR    EK
Sbjct  177  RRCLSMTESNTNSTTTPPPKTPE-----TARDCFKRPEPPASANCSPIQSKRHRCAAVEK  231

Query  355  ENCPAPQTVPAASM-HPPALRKCISLNDAEIASALARSENHNEPELIGDFSKAYCLPLIE  531
            ENCPAP  +   ++ HPP LRKC+SLNDAEI SALARSEN NEPELIGDFSKAY LPL+E
Sbjct  232  ENCPAPSPLSQVTISHPPPLRKCMSLNDAEIMSALARSENRNEPELIGDFSKAYALPLME  291

Query  532  GRHRDLKSISSNTVSRLLRGE  594
            GRHRDLKSIS TV+RLL+GE
Sbjct  292  GRHRDLKSISSETVARLLKGE  312
```

4.1.3. Designing of gene-specific primers

Based on BLAST results, gene-specific upper and lower primers for *C. costata* Wee1, Myt1 and String (Cdc25) were designed with the help of DNAStar software. These primers were used for further transcript quantifications (qRT-PCR).

- **Primer structure:**

Wee1 kinase upper primer:	5’ TGA CCG CAG CGA CGA GGA TGG 3’
Wee1 kinase lower primer:	5’ CAC GTT TAA AGC GGC TGA TGT TGG TA 3’
Myt1 kinase upper primer:	5’ ACT GCG TGG TCT CCA ATC GCT 3’
Myt 1 kinase lower primer:	5’ ATC GGC CAG TTT GCA AGT ATC ATC 3’
String (Cdc25) upper primer:	5’ CAT TTG GCT TTC CTA GTG GTC TCA 3’
String (Cdc25) lower primer:	5’ GTT TCC GGT GTT TTG CTT TGG TTC 3’

4.2. Transcript quantification results

4.2.1. Reference gene Rp49 and β-tubulin

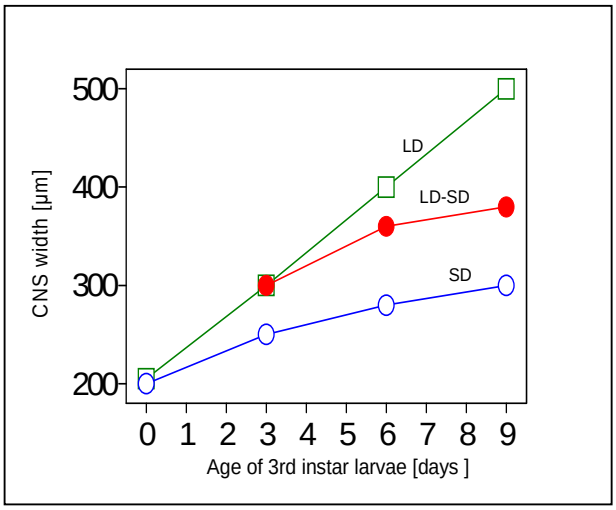


Fig.6.
Growth of CNS during ontogeny of the 3rd larvae instar of *C.costata* under different photoperiodic conditions (LD – 16 h light : 8 h dark; SD – 12 h: 12h; LD-SD tranfer on day 3). All larvae were raised undet constant temperature of 18°C. Data for this part were taken from earlier study (Košťál et al., 2000a). See the text for more explanation.

The size of CNS was almost identical in the 3rd instar larvae that were reared under LD and SD conditions. However, in the 8 day, there were differences between developing LD-larvae and diapause-destined SD and LD-SD larvae. This indicated that as the cells number increased, the absolute amount of mRNA transcripts of reference genes will change as well.

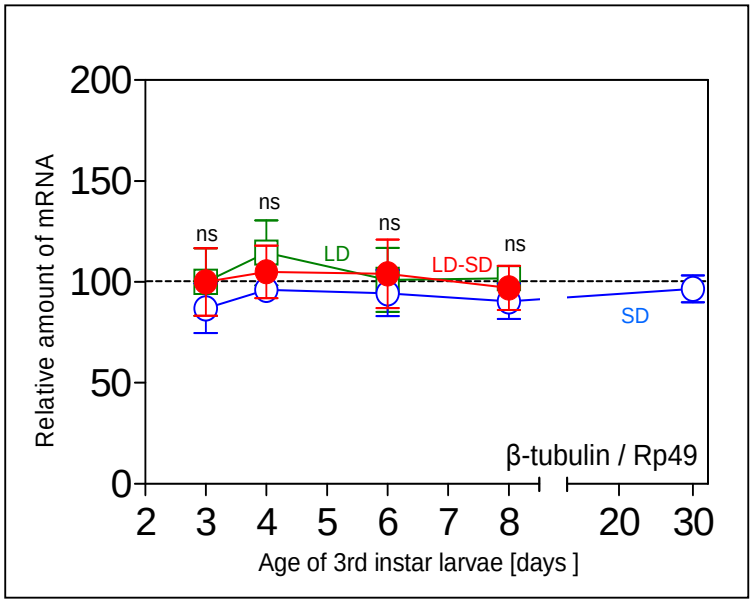


Fig.7.
qRT-PCR analysis of critical cycle (C_T) of the Rp49 amplicon. Each data point is a mean ± S.D. (n = 8). See the text for more explanation.

The absolute amount of Rp49 mRNA in samples is shown in this figure.

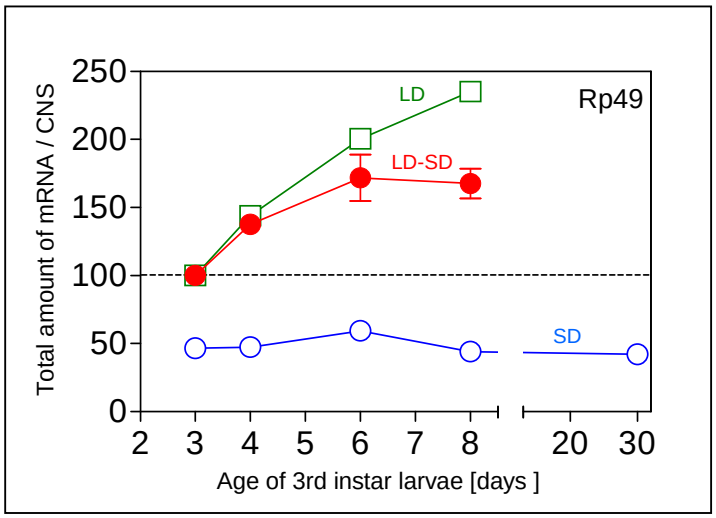


Fig.8.
The recalculation of C_T values to the relative changes in total amount of Rp49 mRNA per CNS. Each data point Each data point is a mean ± S.D. (n = 8). See the text for more explanation.

The C_T values were recalculated to the relative changes of total amount of Rp49 mRNa per tissue, the amount in 3 days old LD larvae was considered as 100%. All results were calculated considering 1,85-fold change (per one PCR cycle) as a real amplification efficiency of qPCR reactions.

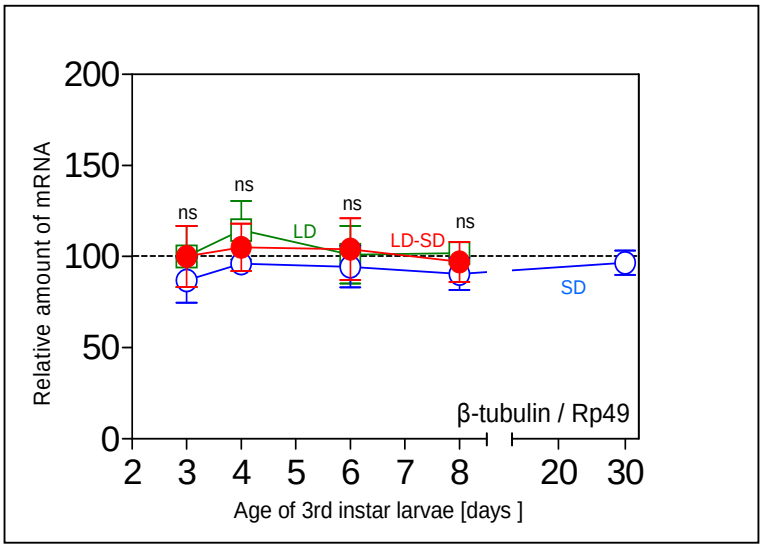


Fig. 9.
Relative amounts of β-tubulin mRNA transcripts standardized to the levels of Rp49 mRNA. Each data point is a mean ± S.D. result of qRt-PCR analysis of 3 independent CNS samples (n = 3). See the text for more explanation.

The levels of β-tubulin mRNA remained constant relative to levels of Rp49 mRNA. The trends in transcriptional changes of Rp49 and β-tubulin appeared equal during growth and development of the CNS tissue under different photoperiodic conditions. Depending on this results, it was decided to standardize the expression of all terget genes to the levels of

a RP49 only. In two cases, pcna and dacapo genes, results were confirmed by an independent qRT-PCR analysis against a β-tubulin background.

4.2.2 Expression of cell cycle regulatory genes with small changes

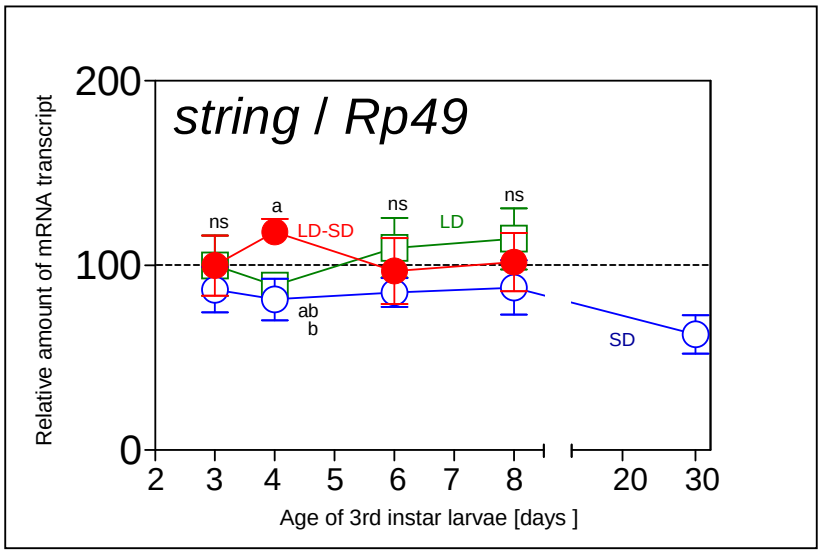


Fig.10.
qRT-PCR analysis of the relative mRNA transcriptsof String (Cdc25) in *C.costata* CNS under three photoperiodic regimes. Each data point is a mean ± S.D. relult of qRT-PCR analysis of 3 independent CNS samples (n = 3). Each sample was prepared by pooling 10 CNS. See the text for more explanation.

No, or only small (up-to 1,5-fold) photoperiod-related changes in the relative level of mRNA transcript of String (Cdc25) gene in *C.costata* CNS tissue were found.

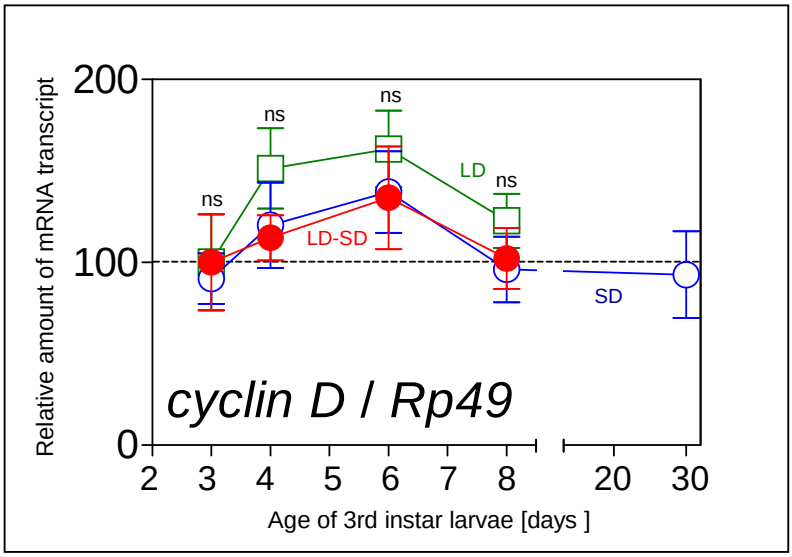


Fig.11.
qRT-PCR analysis of the relative mRNA transcriptsof **Cyclin D** in *C.costata* CNS under three photoperiodic regimes. Each data point is a mean ± S.D. relult of qRT-PCR analysis of 3 independent CNS samples (n = 3). Each sample was prepared by pooling 10 CNS. See the text for more explanation.

The ANOVA analysis found a transient increase of Cyclin D mRNA abundance during the mid - 3rd instar *C.costata* larvae regardless of photoperiodic conditions.

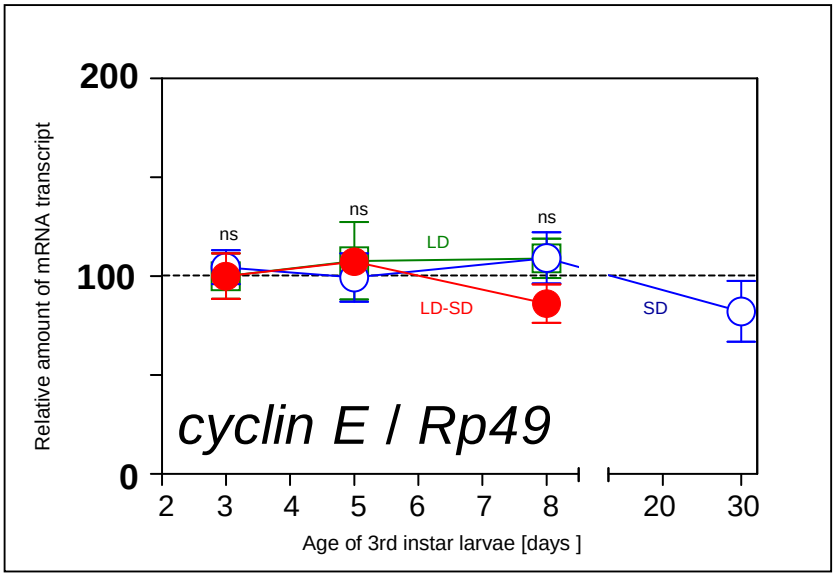


Fig.12.
qRT-PCR analysis of the relative mRNA transcriptsof **Cyclin E** in *C.costata* CNS under three photoperiodic regimes. Each data point is a mean ± S.D. relult of qRT-PCR analysis of 3 independent CNS samples (n = 3). Each sample was prepared by pooling 10 CNS. See the text for more explanation.

No photoperiod-related changes in the relative level of mRNA transcript of Cyclin E gene in *C.costata* CNS tissue were found.

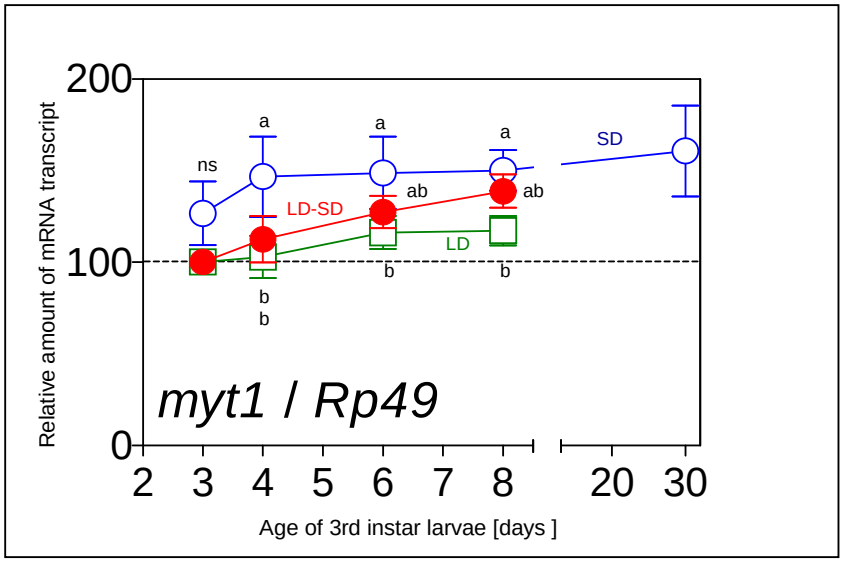


Fig.13.
qRT-PCR analysis of the relative mRNA transcriptsof **Myt 1** in *C.costata* CNS under three photoperiodic regimes. Each data point is a mean ± S.D. relult of qRT-PCR analysis of 3 independent CNS samples (n = 3). Each sample was prepared by pooling 10 CNS. See the text for more explanation.

The levels of Myt1 mRNA were a slightly but significantly higher (up-to 1,4-fold) in the SD-larvae than in the LD-larvae. The levels increased also after the LD – SD transfer.

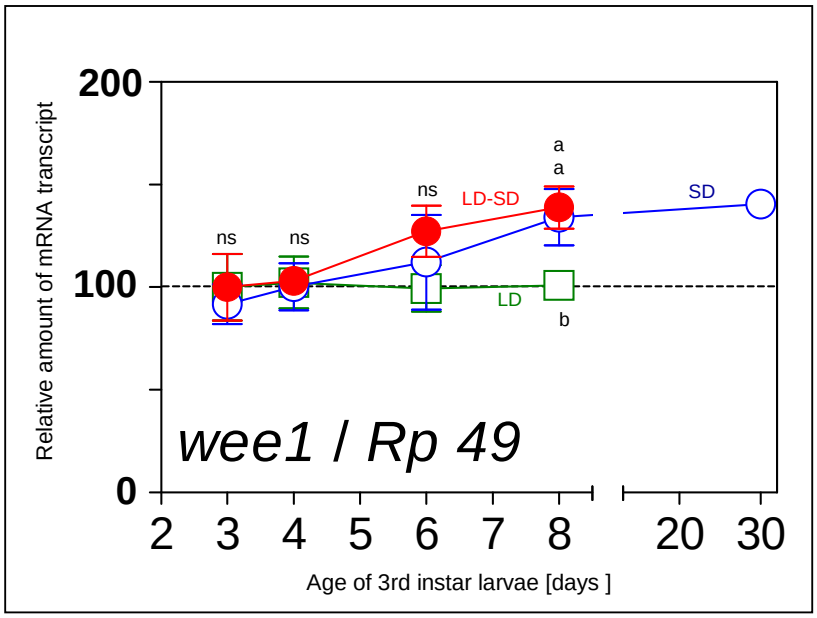


Fig.14.
qRT-PCR analysis of the relative mRNA transcriptsof **Wee1** in *C.costata* CNS under three photoperiodic regimes. Each data point is a mean ± S.D. relult of qRT-PCR analysis of 3 independent CNS samples (n = 3). Each sample was prepared by pooling 10 CNS. See the text for more explanation.

While the relative levels of Wee1 mRNA were maintained stable in LD-larvae, they gradually increased (up to 1,4-fold) between days 3 and 8 in SD-larvae and also after the LD-SD transfer.

4.2.3. Pcna - the cell cycle stimulant

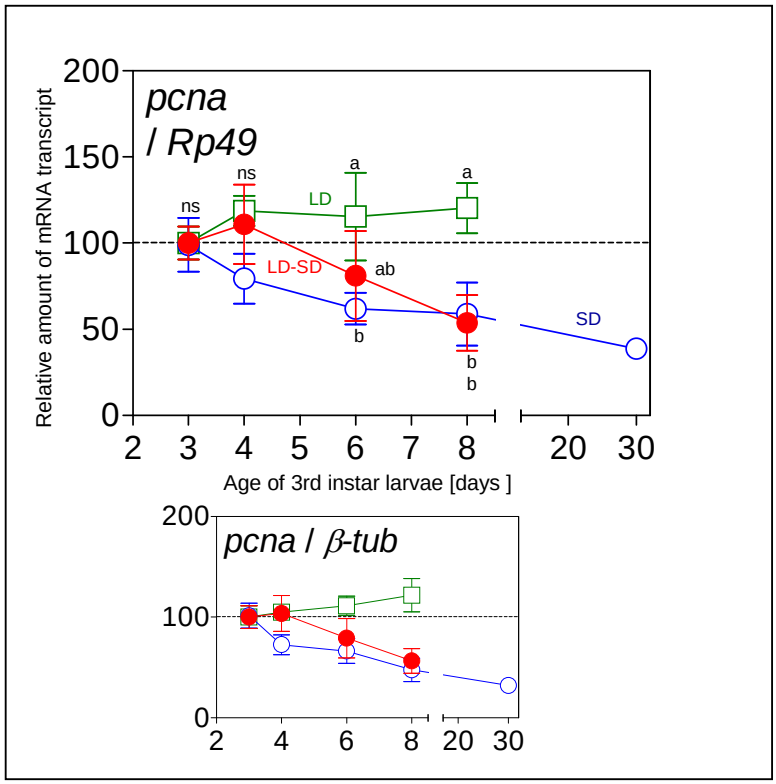


Fig.15.
qRT-PCR analysis of the relative mRNA transcriptsof **Pcna** in *C.costata* CNS under three photoperiodic regimes. And an independent qRT-PCR analyses using β – tubulin as a reference gene. Each data point is a mean ± S.D. relult of qRT-PCR analysis of 3 independent CNS samples (n = 3). Each sample was prepared by pooling 10 CNS. See the text for more explanation.

Clear photoperiod-related patterns of Pcna transcripts were found in the larval CNS. In the LD-larvae the relative levels of Pcna remained relatively constant. In contrast, it decreased significantly (1,7-fold) in 8 days old larvae that were reared under SD photoperiodic conditions. Further decrease (2,5-fold) was evident in 30 days old SD larvae.

A similar decreasing pattern could be seen after the transfer from LD to SD photoperiodic conditions (LD – SD transfer). Five days after the transfer. there was a 1,9-fold difference between the LD and LD – SD photoperiodic conditions.

The relative expression patterns of Pcna gene were equally described by an independent qRT-PCR analysis using β-tubuline as a second reference gene.

4.2.4. Dacapo - the cell cycle inhibitor

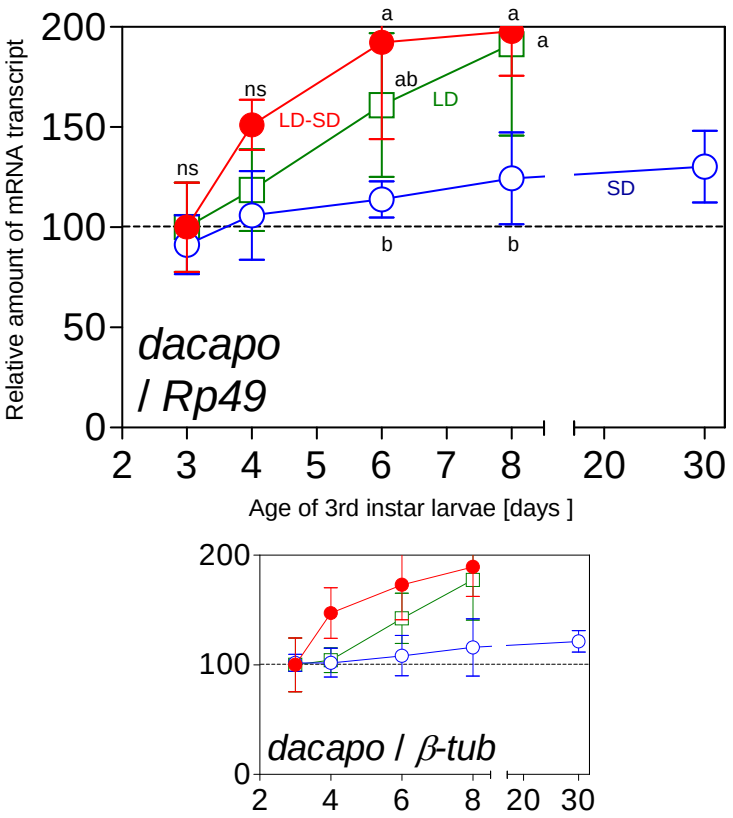


Fig.16. qRT-PCR analysis of the relative mRNA transcriptsof **Dacapo** in *C.costata* CNS under three photoperiodic regimes. And an independent qRT-PCR analyses using β – tubulin as a reference gene. Each data point is a mean ± S.D. relult of qRT-PCR analysis of 3 independent CNS samples (n = 3). Each sample was prepared by pooling 10 CNS. See the text for more explanation.

Clear photoperiod-related patterns of Dacapo transcripts were found in the larval CNS. The relative levels of dacapo transcripts increased under all photoperiodic regimes. The increase rate in SD-larvae was slow (1,4-fold change between days 3 and 30). More rapid

change (1,9-fold change between days 3 and 8) was observed in LD-larvae. The levels of Dacapo mRNA responded remarkably rapidly to the LD – SD transfer (1,5-fold increase within the first day after transfer) and finally reached a 2-fold difference (5 days after the transfer).

The relative expression patterns of Dacapo gene were equally described by independent qRT-PCR using β-tubuline as a second reference gene.

5. Discussion

The structural homologs of the genes coding for the cell cycle regulators: Myt1 kinase, Wee1 kinase and String (Cdc25) phosphatase, were sucsesfully cloned and partially sequenced in *C. costata*. The relative amounts of mRNA transcripts of these three genes plus four other (previously sequenced) cell cycle regulators: Cyclins D end E, Dacapo (p27) and Pcna were measured in the CNS of *C. costata* during the induction of larval diapause.
Only the results concerning the transcript quantification will be discussed here.

5.1. Cyclins D and E

Cyclins D and E give one of the major links between mitogens and the cell cycle control system. Cyclins D and E are needed for the entrance into S phase and initiation of the cell cycle. Their levels are transcriptionally controlled and oscilate during the cell cycle (Jackson et al., 1995; Edgar and Lehner, 1996). It was expected that the transcription of Cyclins D and E will be down-regulated during the entry into diapause. In contrary to this premise, it was found that the relative levels of Cyclins D and E mRNAs were only a little influenced by photoperiodic regime. This similar stability of Cyclin E was reported earlier by Tammariello and Derlinger (1998) in the brain of diapausing *S. crassipalpis*. In the fruitfly, the levels of Cyclin E need to oscillate in order to ensure the multiple rounds of endoreduplication cycles in larval tissues. When Cyclin E was experimentally overexpressed, the cell cycle was blocked (Follette et al., 1998).

5.2. Kinases Wee1, Myt1 and phosphatase String (Cdc25)

The Myt1 and Wee1 kinases and the phosphatase String play significant roles in the G2-M transition. The protein kinases Wee1 and Myt1 play their role in the cell cycle via inhibitory phosphorylation. The phosphatase String, on the other hand, removes this inhibitory phosphates.

Very small differences were found in the relative levels of String mRNA transcripts in response to photoperiod. The changes in mRNA levels of Myt1 and Wee1 genes were also relatively small but they were in the expected direction: the transcriptional levels of these inhibitory kinases increased during the transition into the diapause state. Still, this results

indicate that the transcriptional control over the activity of Myt1 and Wee1 kinases may play some role in the photoperiodically induced G2 arrest in *C. costata* larvae.

5.3. Pcna

The Proliferating Cell Nuclear Antigen is a necessary factor for cell cycle progression through the S phase (Maga and Hubscher, 2003). Pcna encircles DNA double helix and slides freely along its axis (Krishna et al., 1994). The primary function of Pcna is to increase the processivity of a δ subunit of DNA polymerase (Kelman and O' Donnell, 1995). Pcna can bind numerous other proteins and it is thus called: ”a dancer with many partners“ (Maga and Hübscher, 2003). Pcna is one of the proteins which are transcriptionally uregulated by E2F released from the binding pocket of pRB upon mitogen stimulation.

The relative levels of Pcna mRNA transcripts showed significantly decreasing trend in the CNS of diapausing *C. costata* larvae whereas they remained constant, or increased slightly in the larvae that continued their development. Similar results were previously described by Tammariello and Denlinger (1998). They found low levels of Pcna transcripts in the brains of diapausing pupae od *S. crassipalpis.*

5.4 Inhibitor Dacapo (p27)

The activities of Cyclin E-Cdk2 (G1/S-Cdk) and Cyclin A-Cdk2 (S-Cdk) complexes during the entry into the cell cycle and its progression. The activities of these cyclins are controlled by a specific inhibitory protein Dacapo in *D. melanogaster* (homolog of mammalian p27) (Sherr et al., 1999). Overexpression of Dacapo may represent a forcible mechanism for inhibition of the G2-M transition (Liu et al., 2002).

Very rapid upregulation od Dacapo expression was observed in the CNS of *C. costata* larvae in response to LD – SD transfer. The level of mRNA transcripts rose 1,5-fold within a day and doubled within 5 days after the transfer. The relative levels of Dacapo mRNA increased also in the brains of LD-larvae, which corresponds to the increasing proportion of G2-arrested cells. This results indicares that Dacapo is a potential regulator of diapause related cell cycle arrest in *C. costata*.

6. Conclusions

- The strucrural homologs of Wee1, Myt1 and String (Cdc25) were cloned and sequenced in *C. costata* and their sequences are deposited in NCBI GenBank under accession numbers: wee1, GQ329610; myt1, GQ329612; string, GQ329612.
- The levels of mRNA transcripts of **Cyclins D and E** showed no significant changes during the photoperiodic induction of larval diapause in *C. costata*.
- The transcriptional control over activity of Myt1 and Wee1 kinases may play some role in the G2 arrest following photoperiodic induction of diapause in *C. Costata*.
- The role for **Pcna,** as for potentially important player during the regulation of diapause-related cell cycle arrest in insects, was confirmed in *C. costata* in addition to earlier results obtained in different fly species, *Sarcophaga crassipalpis*.
- **Dacapo** was found as another potential regulator of diapause-related cell cycle arrest in *C. costata*.

7. References

Adhikary, S., Eilers, M., 2005. Transcriptional regulation and transformation by Myc proteins. Nat. Rev. Mol. Cell Biol. 6, 635-645.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2002. Molecular Biology of the Cell 4th ed. (Garland Science, New York).

Attwooll, C., Drncni, E.L., Helin, k., 2004. The E2F family: specific functions and overlapping interests. EMBO J. 23, 4709-4716.

Baserga, R., 1985. The biology of Cell Reproduction. Harvard University Press, Cambridge, MA.

Danks, H.V., 1987. Insect dormancy: An ecological perspective. Biological Survey of Canada (terrestrial Arthropods), Monograph series no. 1, Ottawa.

Denlinger, D.L., 2002. regulation of diapause. Annu. Rev. Entomol. 47, 93-122.

Edgar, B.A., Lehman, D.A., O' Farrell, P.H., 1994. Transcriptonal regulation of *string* (cdc25): a link between developmental programming and the cell cycle. Development 120, 3131-3143.

Edgar, B.A., Lehner, C.F., 1996. Developmental control of cell cycle regulators: a fly‘s perspective. Science 274, 1646-1651.

Evans, T. et al., 1983. yclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell 33, 389-396.

Ferrell, J.E. Jr, 2002. Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and biostability. Curr. Opin. Cell Biol. 14, 140-148.

Follette, P.J., Duronio, R.J., O' Farrel, P.H., 1998. Fluctuations in cyclin E levels are required for multiple rounds of endocycle S phase in *Drosophila*. Curr. Biol. 8, 235-238.

Galderisi, U., Jori, F. P., Giordano, A., 2003. Cell cycle regulation and neural differentiation. Oncogene 22 (33), 5208-19.

Hackman, W., Lakovaara, S., Saura, A., Sorsa, M., Vepsalainen, K., 1970. On the biology and karyology of *Chymomyza costata*Zetterstedt, with reference to the taxonomy and distribution of various species of *Chymomyza* (Dipt., Drosophilidae). Ann. Ent. Fenn 36, 1-9.

Harris, H., 2000. The birth of the Cell. (Yale University Press, New Haven).

Hartwell, L.H., Weinert, T.A., 1989. Checkpoints: controls that ensure the order of cell cycle events. Science 246, 629-634.

Chiu, J.F., Mack, A.F., Fernald, R.D., 1995. Daily rhytm of cell proliferation in the teleost retina. Brain res. 673, 119-125.

Jackson, P.K., Chevalier, S., Philippe, M., Kirschner, M.V., 1995. Early events in DNA replication require cyclin and are blocked by p21 ^{CIP1}. J. Cell Biol. 130, 755-769.

Košťál, V., 2006. Eco-physiological phases of insect diapause. J. Insect Physiol. 52, 113-127.

Košťál, V., Shimada, K., Hayakawa, Y., 2000a. Induction and development of winter larval diapause in a drosophilid fly, *Chymomyza costata*. J. Insect Physiol. 46, 417-428.

Košťál, V., Šimůnková, P., Kobelková, A., Shimada, K., 2009. Cell cycle arrest as a hallmark of insect diapause: Changes in gene transcription during diapause induction in the drosophilid fly, *Chymomyza costata*. Insect Biochem. Mol. Biol. 39, 875-883.

Koundrioukoff, S., Jansson, Z.O., Hasan, S., de Jong, R.N., van der Vliet, P.C., Hottiger, M.O., Hubscher, U., 2000. A direct interaction between proliferating cell nuclear antigen (PCNA) and Cdk2 targets PCNA interacting proteins for phosphorylation. J. Biol. Chem. 275, 22882-22887.

Lees, A.D., 1955. The Physiology of Diapause in Arthropods. Cambridge University Press, Cambridge, p. 151.

Lehner, C.F., O' Farrell, P.H., 1990. The roles of *Drosophila* Cyclins A and B in mitotic control. Cell 61, 535-547.

Liu, T.H., Li, L., Vaessin, H., 2002. Transcription of the *Drosophila* CKI gene *dacapo* is regulated by a modular array of cis-regulatory sequences. Mech. Dev. 112, 25-36.

MacRae, T.H., 2005. Diapause: diverse states of developmental arrest and metabolic arrest. J. Biol. Res. 3, 3-14.

Maga, G., Hubscher, U., 2003. Proliferating cell nuclear antigen (PCNA): a dancer with many partners. J. Cell Sci. 116, 3051-3060.

Malumbres, M., Barbacid, M., 2009. Cell cycle, CDKs and cancer: a changing paradigm. Nat. Rev. Cancer 9, 153-166.

Masui, Y., 2001. From oocyte maturation to the in vitro cell cycle: the history of discoveries of Maturation-Promoting Factor (MPF) and Cytostatic Factor (CFS). Differentiation 69, 1-17.

Morgan, D.O., 1997. Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu. Rev. Cell Dev. Biol. 13, 261-291.

Morgan, D.O., 2007. The Cell Cycle. Oxford University Press, Oxford.

Murray, A.W. and Hunt, T., 1993. The Cell Cycle: An introduction.(Freeman, New York).

Murray, A.W. and Kirschner, M.W., 1989. Cyclin synthesis drives the early embrionic cell cycle. Nature 339, 275-280.

Riihimaa, A.J., Kimura, M.T., 1988. A mutant strain of *Chymomyza costata*(Diptera: Drosophilidae) insensitive to diapause-inducing action of photoperiod. Physiol. Entomol. 13, 441-445.

Russell, P., Nurse, P., 1987. Negative regulation of mitosis by wee1^{+}, a gene encoding a protein dinase homolog. Cell 49, 559-567.

Santos, S.D.M.; Ferrell, J.E., 2008. On the cell cycle and its switches. *Nature* 454 (7202):288.

Sherr, C.J., Roberts, J.M., 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Devel.. 13, 1501-1512.

Stehlík, J., Závodská, R., Shimada, K., Šauman, I., Košťál, V., 2008. Photoperiodic induction of diapause requires regulated transcription of *timeless* in the larval brain of *Chymomyza costata*. J.Biol.Rhytms 23, 129-139.

Tammariello, S.P., Derlinger, D.L., 1998. G0/G1 cell cycle arrest in the brain of S*arcophaga crassipalpis* during pupal diapause and expression pattern of the cell cycle regulator, proliferating cell nuclear antigen. Insect Biochem. Mol. Biol. 28, 83-89.

Yoshida, T., Kimura. M.T., 1995. The photoperiodic clock in *Chymomyza costata*. J. Insect Physiol. 41, 217-222.