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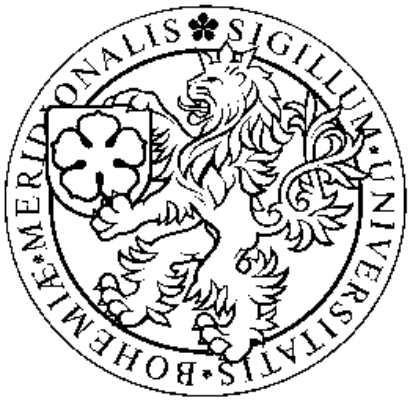

Bachellor thesis

Transcriptional analysis of genes coding for ion pumps during cold acclimation in drosophilid flies

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Bachellor thesis

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Annotation

The relative abundance of mRNA transcripts of genes coding for α subunit of Na^+K^+-ATPase and *Vha55* (B) subunit of H^+-ATPase were examined (using the qRT-PCR method) in third instar larvae of two fly species, *Drosophila melanogaster* and *Chymomyza costata*, that were reared at various acclimation conditions. No differences in mRNA levels were observed in *D. melanogaster* Malpighian tubules and central nervous system (CNS), when the flies were reared at 25°C or 15°C. *C. costata* revealed the up-regulation of both ion pump genes in the CNS at 15°C (in comparison to 25°C), and also after the transfer from 25°C to 15°C. This result indicates that compensation of ion pumps function took place at moderately low temperature (still permissive for continuation of activity, growth and development). In contrast, down-regulation of both ion pump genes was seen in the CNS of *C. costata* larvae at temperature of 5°C. It indicates that channel arrest strategy was adopted at very low temperatures (inducing dormancy).

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

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České Budějovice 2008 .....

Petra Šimůnková

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1. Introduction

1.1 Poikilotherms and the temperature changes

Poikilotherms have evolved effective acclimatory responses to cope with daily and seasonal temperature fluctuations. Insects can survive at a wide range of environmental temperature conditions. The physiological limits of insect cold survival go even beyond any imaginable extremities. Some overwintering insects can survive at temperatures below -50ºC in supercooled or frozen state (Lee, 1991). Hydrated mature larvae of a *Chymomyza costata* fly were successfully cryopreserved by gradually freezing to liquid nitrogen temperature of -196ºC (Moon et al., 1996) and anhydrobiotic insects recovered even after exposure to -270ºC (Watanabe, 2006).

This work was focused on biological membranes, because they are considered as primary target of chilling injury (Košťál et al, 2004; 2006), and the situation in insects may be compared to (more advanced) knowledge on fishes and other poikilotherms (Hazel, 1989; Cossins, 1994). The biological membrane is the only cell structural component noted for its qualitative alteration in response to cold (Hazel, 1989). Considering the adaptive meaning of acclimatory responses, two wide categories can be theoretically differentiated: compensation of physiological function (capacity adaptation), and preservation of biological structure (resistance adaptation) (Cossins and Bowler, 1987). Some insects living in temperate zones are fully active in buffered microhabitats during the cold season (Aitchinson, 1979). These species of insects probably need a specific level of physiological compensation (Koštál et al., 2004; 2006). Most species, however, spend winter in dormancy (Koštál et al., 2006), and those probably have to rely more on resistance mechanisms. Strong resistance mechanisms can be expected in those insects, which almost completely resign on any functioning during the cold season and undergo freezing or dehydration (Zachariassen et al., 2004).

1.2 Biological membranes

1.2.1 Temperature fluctuations and the changes in membrane structure and function

Biological membranes play crucial roles in cellular physiology. Low temperatures may badly bring down all the membrane functions. The basic features of lipidic bilayers; the *phase* state and the *fluidity*, are intensively sensitive to temperature. The idea of cold acclimation predicts that poikilotherm organism will respond to a change in environmental temperature by either

- modification of the membrane lipid composition (Cossins and Sinensky, 1984; Cossins, 1994; Hazel, 1989; 1990; 1995); or
- changing the amounts/activities of membrane proteins (or altering the expression of isozymes) (Juriaan et al., 2003).

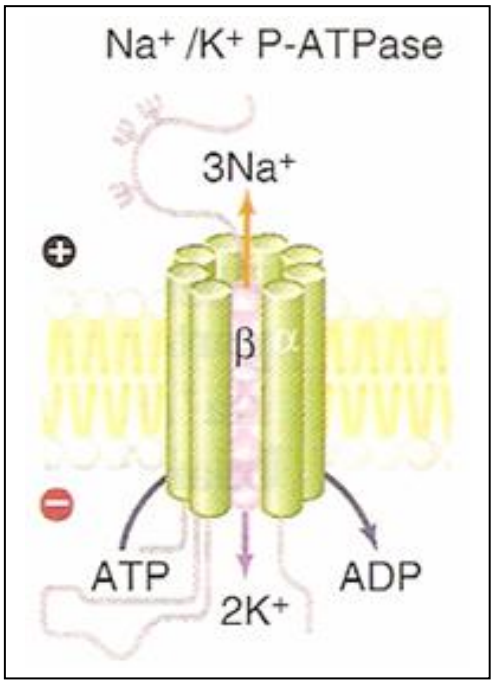
There are two directions how poikilotherms can change amount of their membrane proteins. One way is the **up-regulation** of membrane proteins (increase the number of proteins, in response to external variable), which means the compensation. The second way is to decrease the number of protein molecules, in response to an external variable, the **down-regulation**, which can lead, for instance, to the channel arrest (or inhibition of ion pump and channel function). The concept of channel arrest was put forward by Hochachka (1986).

1.2.2 Ion pumps

Transcription of two ion membrane pumps is compared in this thesis:

- **Na^+K^+-ATPase** (sodium/potassium pump). Na^+K^+-ATPase is ubiquitous to animal cells. It is required for maintenance of transmembrane electro-chemical potential differences (EPDs), that are essential for cell signaling and secondary transports. Na^+K^+-ATPase is especially abundant in nerve tissue, and is characteristically expressed among other cell types and regions (Cameron et al., 1993; Levenson, 1994; Shyjan and Levenson, 1989; Watts et al.,1991; Baumann and Takeyasu, 1993; Jungreis and Vaughan, 1997; Lebovitz et al., 1989).

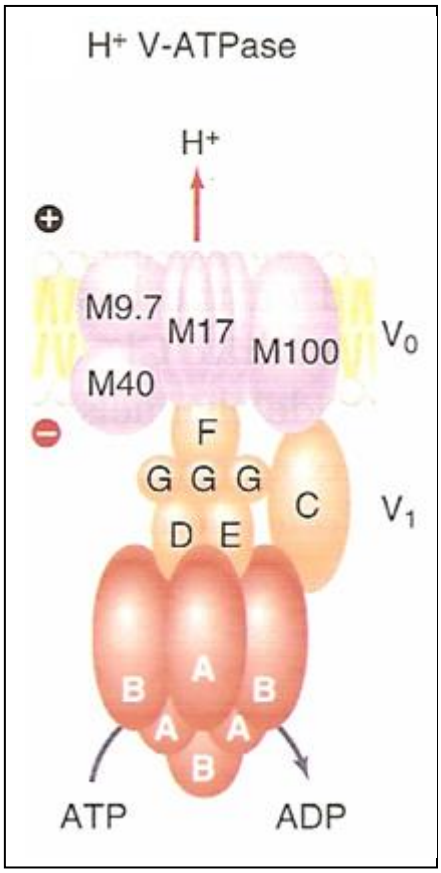
Fig. 1. A model of the Na^+K^+ - ATPase



The distribution of Na^+K^+-ATPase in insect tissues varies between species. For example, muscle and Malpighian tubules, but not the midgut, of *D. melanogaster* express the enzyme (Lebovitz et al., 1989). The abundance and presence of Na^+K^+-ATPase in the insect midgut varies taxonomically and with diet. All P-type Na^+K^+-ATPases have a simple structure. The single catalytic subunit (α-subunit) is approximately 100 kDa, and contains regions that are highly conserved; especially at the hydrophilic and cytoplasmic site of catalytic phosphorylation (Fagan and Saier, 1994; Green and Stokes, 1992). The catalytic subunit is membrane embedded with several transmembrane α-helices (Horisberger et al., 1991; Fitzgerald et al., 1996; Nicolson, 1993; Wieczorek, 1992).

- **H^+–ATPase (V–ATPase)**

Fig. 2. A model of the V-ATPase



V–ATPases are ubiquitous, vital proton pumps that play a multiple roles in higher organisms. In many epithelia they are the major energizers of cotransport processes and have been implicated in functions as diverse as fluid secretion and longevity. The first animal knockout of a V–ATPase was identified in *Drosophila*, and its recessive lethality demonstrated the essential nature of V-ATPases (Allan et al., 2005).

In insect, V–ATPases are located in the apical membranes of nearly all epithelial tissues (such as salivary glands, midgut, and Malpighian tubules), where they energize secondary active transport processes across the epithelium (Wieczorek et al., 2003). Tissue specificity has also been demonstrated for V–ATPase isoforms for the a- and c- subunits in *Caenorhabditis elegans* (Oka and Futai,

2000; Oka et al., 2001; Oka et al., 1998). The Malpighian tubule is a classic insect transporting epithelium in which the plasma membrane role of V–ATPases in principal cells is well documented (Bertram and Wessing, 1994; Davies et al., 1996; Dow, 1999; Dow and Davies, 2003; O'Donnell et al., 1996; Sozen et al., 1997).

V–ATPases are large, multisubunit pumps that transport hydrogen ions in exchange for energy, in the form of ATP. They are present in the endomembranes of all cells and in the plasma membranes of many specialized eukaryotic cells (Nishi and Forgac, 2002). The V–ATPases of animals, plants, and fungi are structurally very similar and are composed of two functional domains, V_1 and V_0. The V_1 domain is located on the cytoplasmic side of the membrane, and is composed of eight different subunits (A-H) and is responsible for ATP hydrolysis. The V_0 domain is a membrane bound, proton-conducting complex, composed of at least four, possibly five, subunits (a-e). The V–ATPase multigene family is encoded by 33 genes, which encode 14 subunits of the V – ATPase in *Drosophila melanogaster* (Dow, 1999; Wang et al., 2004).

Our experiments were focused on the α-subunit of Na^+K^+-ATPase and *Vha55* (B) subunit of H^+-ATPase in two fly species, *Drosophila melanogaster* and *Chymomyza costata*. Sequences of *D. melanogaster* genes were obtained from NCBI GenBank databank. An α-subunit of Na^+K^+-ATPase of *C. costata* was previously cloned and sequenced by V. Kostal (unpublished results). *Vha55* (B) subunit of H^+-ATPase cloning and sequencing was the first part of this thesis.

1.3 Na^+K^+-ATPase in fishes – What is its role during cold acclimation?

The common carp *Cyprinus carpio* L. is a stenohaline freshwater fish. Its ability to adapt allowed this species to extend its habitat throughout the temperate zone of the northern hemisphere, in water temperatures ranging from 0 to 30°C (Billard, 2001). The optimal temperature for carp growth is around 25°C. Temperature strongly influences all biochemical processes and, obviously, when a set of crucial physiological processes is subjected to changes in water temperature, internal homeostasis may become at risk.

Isogenic carp *Cyprinus carpio* L., acclimated to water temperatures of 15, 22 and 29°C (Juriaan R. Metz et al., 2003), showed slightly, but significantly, different plasma osmolality, sodium, potassium and chloride concentrations. In this study appeared that at 15°C – acclimated fish, a lower apparent Na^+K^+-ATPase activity were compensated by strongly enhanced Na^+K^+-ATPase expression (determined biochemically and

immunohistochemically). In summary, at a lower ambient temperature, branchial Na^+K^+-ATPase expression is up-regulated to neutralize the inhibitory effect of low temperature on molecular activity of the ion pump (Juriaan, R. Metz et al., 2003).

In goldfish, the activities of membrane-associated enzymes of the mitochondrial respiratory chain (Caldwell 1969; Wodtke 1981; Hazel 1972) and enterocyte Na^+K^+-ATPase (Smith and Ellory 1971) increase with cold acclimation even though protein titers remain constant. In goldfish undergoing temperature acclimation, adaptive displacements in the threshold temperature for the onset of hyperexcitability and loss of equilibrium were strongly correlated with temporal changes in the fluidities of synaptosomal membranes (Cossins et al. 1977). Strong correlations have been established between membrane fluidity and the activity of several membrane-associated enzymes, particularly Na^+K^+-ATPase (Sinensky et al. 1979, Harris 1985). The LT_{50} (the temperature required to produce a 50% reduction in enzyme activity in a 15-min period) for of synaptosomal membrane was 2°C higher at 28°C – (47,7°C) than 6°C – acclimated (44,9°C) goldfish (Cossins et al. 1981).

1.4 Na^+K^+-ATPase in insect – What is its role during cold acclimation?

It was shown in earlier studies, that cold acclimated insects maintain the transmembrane electrochemical potential differences (EPDs) at low (even sub-zero) temperatures, which is essential for maintaining regulated cell metabolism, cell signaling, secondary transports and other vital processes (Košťál et al., 2004; 2006). Non-cold acclimated insects, in contrast, can not maintain EPDs, can not survive at low temperatures and die. How acclimated insects arrange, that they survive at low temperatures? There are two theoretical possibilities: (a) cold acclimated insect can up-regulate the amount of membrane pumps. This possibility, however, requires constant access to energy (ATP) (capacity adaptation or compensation, likely to occur at low temperatures, but still permissive for growth and development); (b) the other possible way is to down-regulate the amount of membrane pumps and channels, which may lead to channel arrest and saving energy (resistance adaptation, likely to occur at temperatures not permissive for growth and development). In this work the up/down-regulation was tested at the level of transcription of the genes coding for catalytic subunits of membrane pumps. The ion pumps are relatively well known, including their functions and gene sequences. Also the down/up-regulation can be tested at the level of membrane channels, but this is much more complicated, because there are many different channels and this subject is not fully understood in insect.

1.5 Working hypotheses

Question: Is there any acclimation response to low temperature at a level of transcription of the genes coding for ion pumps in two fly species, *D. melanogaster* and *C. costata*?

Potential answers:

- The first alternative: there is no transcriptional response.
- The second alternative: transcriptional upregulation of ion pump genes; it means the compensation response under low temperatures. This can be generally expected from the ectotherms, which are continuing their activity, growth and development, and so they have to compensate their basal functions under low temperatures.
- The third alternative: transcriptional downregulation of ion pump genes, which may lead to channel arrest. Activity of pumps decreases in order to save the energy. Similar response can generally be expected in dormant species, which cease activity, growth and development (hibernate).

1.6 Specific aims

- To clone the *vha55* gene of the H^+ - ATPase subunit of *C. costata.*
- To optimize the qRT – PCR method (design primers, test optimal PCR conditions) for relative quantification of mRNA transcripts.
- To answer the question on if and how the abundances of mRNA transcripts of the genes coding for Na^+K^+-ATPase and V–ATPase change during the cold acclimation of two fly species *D. melanogaster* and *C. costata*.

2. Materials and methods

2.1 Experimental insect

Chymomyza costata:

- holarctic, cold temperate distribution
- high capacity for cold acclimation
- high level of cold and freeze tolerance

Drosophila melanogaster:

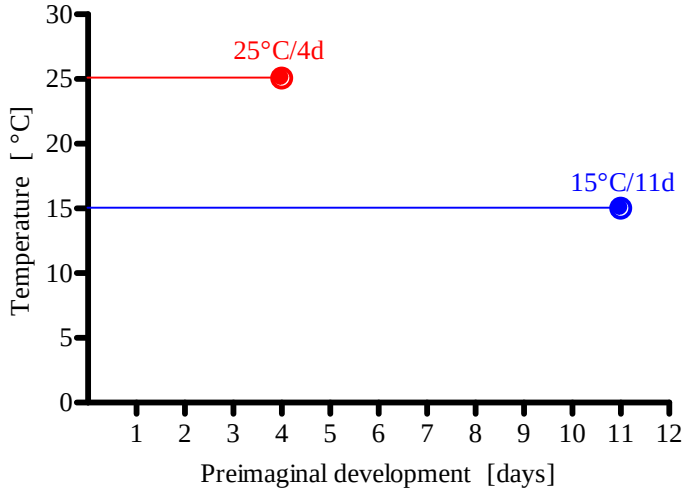
- tropic and subtropic distribution
- low capcity for cold acclimation
- low level of cold tolerance, no freeze tolerance

C. costata flies were raised on artificial diet, composed of agar, malted barley, cornflour and yeasts. Adult flies oviposited on a small piece of apple in the diet. After oviposition, a piece of apple was transferred into a new glass tube with fresh diet, where larval development was enabled.

D. melanogaster flies were raised on artificial diet composed of cornflour, agar, sugar and yeast. Adult flies oviposited directly into the diet.

2.2 Aclimation and experimental variants

Fig. 3. Schematic depiction of the acclimation protocol of the adults *Drosophila melanogaster*.

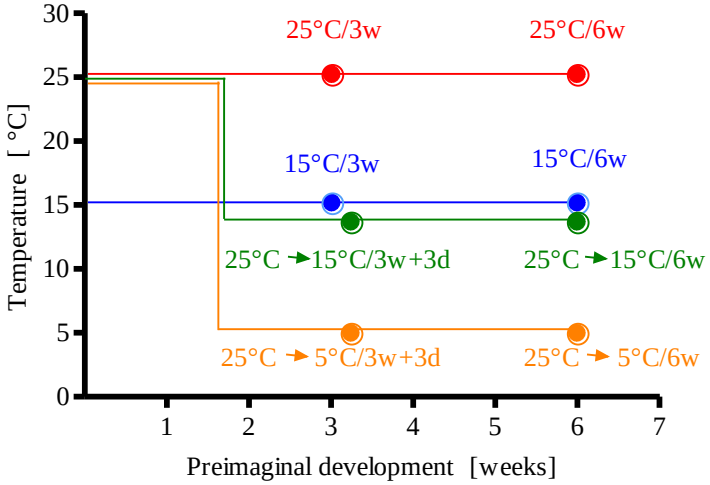


***D. melanogaster* experimental variants**

Larvae were raised at 25°C for 4 days (25°C/4d), or at 15 °C for 11 days (15°C/11d) to achieve similar physiological ages in both variants. See text for more details.

Larval development requires only 4 days at 25°C but 11 days at 15°C. Mature larvae (just before they would start wandering behaviour) were dissected. Two tissues were dissected, central nervous tissue (CNS, "brain") and Malpighi tubules, in Ringer solution on ice and then transferred into RNase free microtubes with 40µL of RNAlater. After dissections, the samples were stored at – 20°C and saved for future analysis.

Fig. 4. Schematic depiction of the acclimation protocol of the larvae of *Chymomyza costata*



***C. costata* experimental variants**

All larvae were raised with photoperiod of 12 hours light / 12 hours dark, which means that all larvae were destined to (3w) or already in (6w) the diapause state. See text for more details.

- 25°C/3w and 25°C/6w: larvae were raised at 25°C for 3 or 6 w.
- 15°C/3w and 15°C/6w: larvae were raised at 15°C for 3 or 6 w.
- 25°C→15°C/3w+3d or 25°C→15°C/6w: larvae were raised at 25°C for 3w, then transferred to 15°C and sample was taken after another 3 days or 3w, respectively.
- 25°C→5°C/3w+3d or 25°C→5°C/6w: larvae were raised at 25°C for 3w, then transferred to 5°C and sample was taken after another 3 days or 3w, respectively.

2.3 Dissections

Two larval tissue samples were collected; central nervous system (CNS) or "**brain**" and **Mapighi tubules**. Each sample consisted of the tissues dissected from 15 larvae. Each sample had three replications. In summary, 45 larvae were thus dissected per each sample. With 2 tissues, 45 larvae dissected, and 10 different sampling variants (experimental acclimation variants in both fly species) – more than 450 dissections were performed in this experiment. Larvae of *D. melanogaster* were able to continue their development at both temperatures. This

was verified by observing successful pupariation and adult emergence. No signs of dormancy were registered.

All samples were dissected in Ringer solution on ice than transferred into RNA tubes with 40 µl of RNA Later and put into – 20°C and stored for further analysis.

2.4 Cloning

- ***Chymomyza costata***

The α subunit of Na^+K^+ ATPase was previously cloned by V. Košťál .
The α subunit of Vha55 I cloned myself.

2.4.1 Isolation of total RNA (RNA Blue, Top-Bio)

RNA extraction – What is it?

RNA extraction is the purification of RNA from biological samples. This procedure is complicated by the ubiquitous presence of ribonuclease enzymes in cells and tissues, which can rapidly degrade RNA. Several methods are used to isolate RNA from samples, the most common of these is Guanidinium thiocyanate-phenol-chloroform extraction.

Careful and precise working is crucial to succeed in this part of work mainly because of possible sample contamination by RNAses.

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

2.4.2 Synthesis of cDNA (Reverse Transcription System, Promega)

cDNA – What is it?

copy DNA (**cDNA**) is DNA synthesized from a mature mRNA template in a reaction catalysed by the enzyme reverse transcriptase.

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

2.4.3 Design of primers

Primers – What are they?

A **primer** is a strand of nucleic acid that serves as a starting point for DNA replication. They are required because the enzymes that catalyze replication, DNA polymerases, can only add new nucleotides to an existing strand of DNA. The polymerase starts replication at the 3'-end of the primer, and copies the opposite strand.

Uses of synthetic primers.

In PCR (see below), primers are used to determine the DNA fragment to be amplified by the PCR process. The length of primers is usually not more than 30 nucleotides, and they match exactly the beginning and the end of the DNA fragment to be amplified. They direct replication towards each other - the extension of one primer by polymerase then becomes the template for the other, leading to an exponential increase in the target segment.

In this part of work, **F1 (Forward) x R1 (Reverse)** ***Vha55*** primers sepcific for *D. melanogaster Vha55 sequence* were used as they appeared to prime the synthesis of expected product band also when *C. costata* cDNA was used as PCR template. The primers were designed with help of **DNA Star.**

The **structure** of primers used for cloning:

D. melanogaster Vha55 **F1**: CGC CGG TCT GCC CCA CAC C - 3’

R1: GGA GGC CGG GAT ACG CTT GAG - 3’

2.4.4 PCR and electrophoresis (el-fo)

PCR – polymerase chain reaction – What is it?

The **polymerase chain reaction (PCR)** is a method widely used in molecular biology. It originates its name from one of its crucial components, a *DNA polymerase* used to amplify a piece of DNA by in vitro enzymatic replication. As PCR progresses the DNA is itself used as template for replication. Than the chain reaction is originated and the DNA template is exponentially amplified. With PCR it is possible to amplify a single or few copies of a piece of DNA, generating millions or more copies of the DNA piece.

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: constant : 95°C/5 min – initial impulse to denature strands

95°C/30 s – denaturation in progress
54°C/30 s – primers annealing
72°C/1 min – new strand synthesis
} 35s

72°C/2 min – polymerization complete
4°C/5 min – cooling

Electrophoresis (el-fo) – What is it?

Electrophoresis is the most known electrokinetic phenomena, discovered by Reuss in 1809. Generally, electrophoresis is the motion of dispersed particles relative to a fluid under the influence of an electric field.

Gel electrophoresis is an application of electrophoresis in molecular biology. Biological macromolecules are loaded on a gel and separated on the basis of their electrophoretic mobility. The gel greatly slow up the mobility of all present molecules.

- 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

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

Gel extraction – What is it?

Gel extraction or **gel isolation** is a technique used to isolate a desired fragment of intact DNA from an agarose gel. After extraction, fragments of interest can be mixed, precipitated, and enzymatically ligated together. This process, usually performed on plasmids, is basis for rudimentary genetic engineering.

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

2.4.6 Ligation into vector (pGEM Teasy)

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

DNA ligase – What is it?

DNA ligases are used with restriction enzymes to insert DNA fragments, often genes, into plasmids. The mechanism of DNA ligase is to form covalent phosphodiester bonds between 3' hydroxyl ends of one nucleotide with the 5' phosphate end of another. ATP is required for the ligase 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

2.4.7 Transformation of competent cells (JM 109, Promega)

Transformation – What is it?

Following ligation, the ligation product (plasmid) is transformed into bacteria for proliferation. The bacteria is then sowed on selective agar to select for bacteria that contains plasmid. Individual colonies are picked and tested for the desired insert.

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)

2.4.8 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

2.4.9 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.

2.4.10 PCR control and cultivation of selected clones

Which clone is a good one – How to pick out ?

As the mentioned-above procedures are of low efficiency, there is a need to identify the cells that contain the desired insert at the adequate orientation and isolate these from those not successfully transformed. Cloning vectors include frequently antibiotic resistance markers that allow to grow only cells in which the vector took root. Additionally, the cloning vectors may contain colour selection markers which provide blue/white screening. However, these selection steps do not absolutely guarantee that the DNA insert is present in the cells. Further investigation of the resulting colonies is required to confirm that cloning was successful. This may be achieved by PCR.

Generally 7 colonies were picked from 3 plates as follows; from first plate 4 colonies and from second plate 3 colonies, all unambiguously white which indicated the products reception into plasmid.

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)

After visualization electrophoresis products with UV light, 1170 kb product was found.

2.4.11 Isolation of plasmid DNA (QIAPrep Miniprep)

Minprep – What is it?

A **Miniprep** is a procedure to extract plasmid DNA from bacteria. It is based on the method of alkaline lysis. When bacteria are lysed under alkaline conditions, DNA and proteins are precipitated. After the addition of acetate including neutralization buffer the large and less supercoiled chromosomal DNA and proteins are precipitated, but small bacterial DNA plasmids can renature and stay in solution. Minipreps are used in the process of molecular cloning to analyze bacterial clones.

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

2.4.12 Preparation of sequencing reaction (BigDye Terminator)

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)

2.4.13 Purification of PCR product

- 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.

2.4.14 DNA sequencing

Sequencing – What is it?

The **DNA sequencing** contains biochemical methods for determining the order of the order of the nucleotide bases.

In this work the ABI Prism 377 DNA Sequencer was used to obtain the final sequence of *Vha55* gene of *C. costata*. This procedure was made by A. Trojanová.

2.4.15 Blasting

Blasting – What is it?

The **B**asic **L**ocal **A**lignment **S**earch **T**ool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST requires two sequences as input: a query sequence (also called the target sequence) and a sequence database. BLAST will find subsequences in the query that are similar to subsequences in

2.5 Transcript quantification

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

The routine: was described above

- 6 samples were usually processed
- (2 tissues: Brain, Malpighi – each contained 15 tissue samples. Each Brain and Malpighi had 3 repetition. Totally: 2 tissues x 3 repetition = 6 samples to process).

2.5.2 Synthesis of cDNA (reverse transcription) (RT Promega)

The routine: was described above

- After "cooling down" final point, 480μL of sterile water was added to Epes with samples so the cDNA stock solution was made.
- 4 aliquots were made from stock solution

2.5.3. qPCR (Light-cycler Corbett Res.)

qRT-PCR – What is it?

Real-time PCR allow accurate quantification of starting amounts of DNA, cDNA, and RNA targets. Fluorescence is measured during each cycle, which greatly increases the dynamic range of the reaction, since the amount of fluorescence is proportional to the amount of PCR product. PCR products can be detected using either fluorescent dyes that bind to the double-stranded DNA.

SYBR® Green I binds all double-stranded DNA molecules, emitting a fluorescent signal of a defined wavelength on binding. The excitation and emission maxima of SYBR Green I are at 494 nm and 521 nm, respectively, and are compatible for use with any real-time cycler. Detection takes place in the extension step of real-time PCR. Signal intensity increases with increasing cycle number due to the accumulation of PCR product.

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

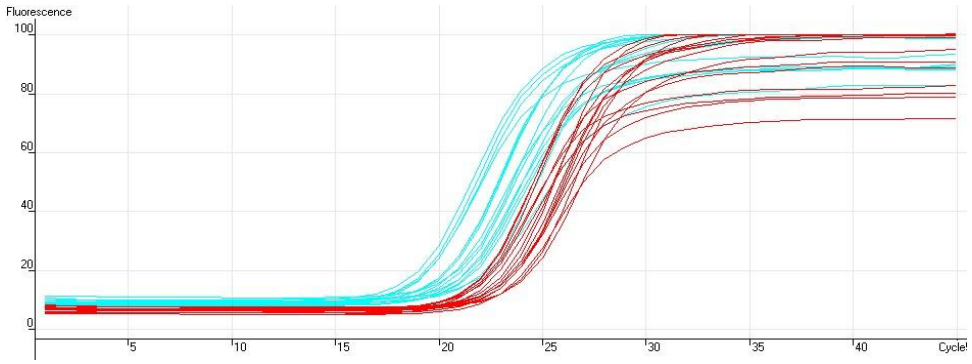
The mRNA transcript amounts were compared with Rp49 gene (ribosomal, house-keeping gene, showing constant transcript amount in cells, earlier cloned by V. Košťál). Rp49 was used as a constant background.

2.5.3 qPCR methodology

Tab.1. Example of the Excell sheet, which was used to process qPCR results (this example shows results for 9 different samples of in CNS of *C. costata* tested for relative abundance of Na^+K^+- ATPase transcripts). The threshold cycle (C_T) values (the cycle at which there is a significant detectable increase in fluorescence signal) are shown in columns 1 and 2 (doublet reaction). Real fficiency of transcript multiplication (theoretical maximum = 2 times in one PCR cycle). Means (av) and standard deviations (StDev) of the C_T doublet are also shown.

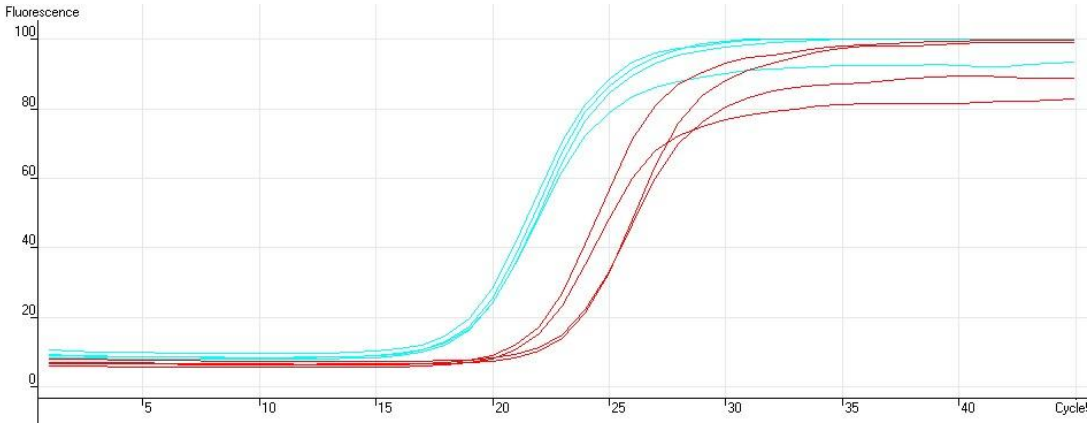
	test gene: NaK ATPase					control gene: Rp49				
	primers: DmNaKF1 vs.DmNaKR1					primers: CcRPF1 vs. CcRPR1				
sample	**1**	**2**	**REAL eff**	**av**	**StDev**	**1**	**2**	**REAL eff**	**av**	**StDev**
A2.B4	19,66	19,71	**1,85**	**19,685**	**0,035**	17,09	17,23	**1,83**	**17,16**	**0,099**
A2.B5	18,42	18,35	**1,85**	**18,385**	**0,049**	15,92	16,09	**1,83**	**16,005**	**0,12**
A2.B6	18,26	18,26	**1,85**	**18,26**	**0**	15,98	15,92	**1,83**	**15,95**	**0,042**
B1.3d.B1	20,02	19,88	**1,85**	**19,95**	**0,099**	18,39	18,35	**1,83**	**18,37**	**0,028**
B1.3d.B2	18,79	18,68	**1,85**	**18,735**	**0,078**	17,29	17,32	**1,83**	**17,305**	**0,021**
B1.3d.B3	19,43	19,34	**1,85**	**19,385**	**0,064**	17,81	17,74	**1,83**	**17,775**	**0,049**
B2.3d.B1	19,59	19,58	**1,85**	**19,585**	**0,007**	18,18	18,09	**1,83**	**18,135**	**0,064**
B2.3d.B2	20,75	20,45	**1,85**	**20,6**	**0,212**	18,95	19,07	**1,83**	**19,01**	**0,085**
B2.3d.B3	18,70	18,55	**1,85**	**18,625**	**0,106**	17,32	17,53	**1,83**	**17,425**	**0,148**

Fig. 5. Sample of the qRT-PCr methodology. The record of data reading during single cycles by Light-Cycler machine.



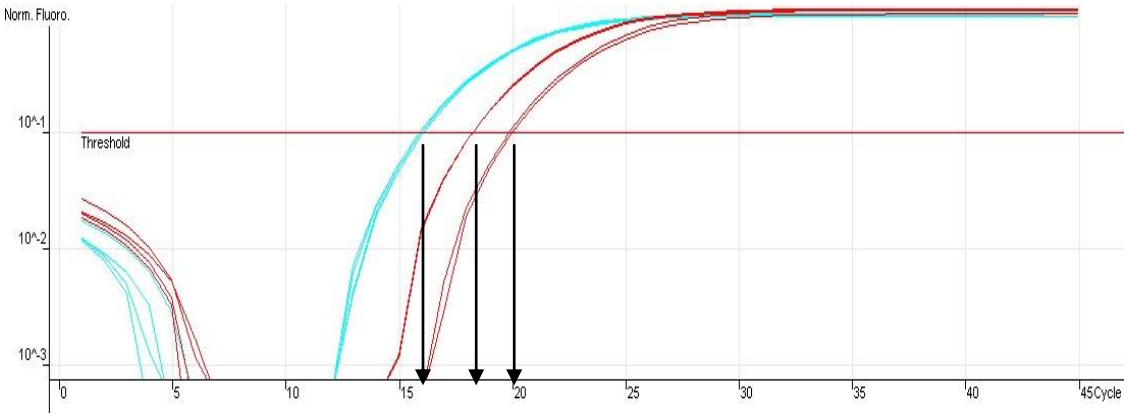
Nine samples (presented in Fig. 4) were compared in a single run. Each sample was run as a doublet of the target gene and doublet of Rp49 reactions. In summary, 18 reactions for Rp49 (blue) and 18 reactions for Na^+K^+-ATPase (red) are shown in figure.

Fig. 6. Sample of the qRT-PCr methodology. The record od data during single cycles by Light-Cycler machine.



Two (of nine presented in Fig. 4) selected cDNA samples are displayed. Each sample was run as a doublet of the target gene and doublet of Rp49 reactions. In summary, 4 reactions for Rp49 (blue) and 4 reactions for Na^+K^+-ATPase (red) are shown.

Fig. 7. Sample of the qRT-PCr methodology. RotorGene 6 anylysis of samles.



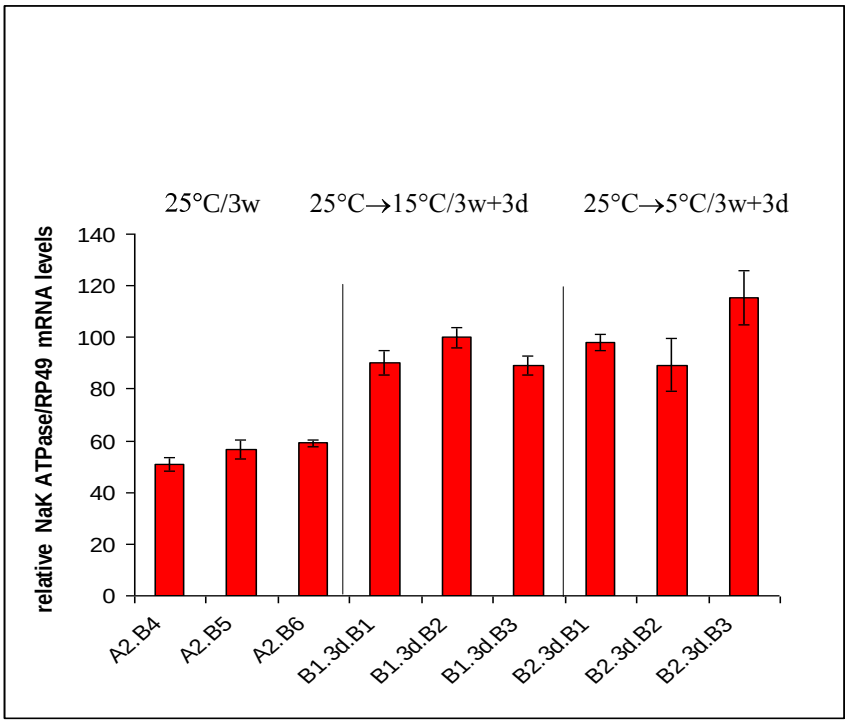
The same two samples as above were processed by RotorGene 6 Analysis.

C_T analysis:

	Rp49	NaK ATPase
sample 1	15,5	18,0
sample 2	15,5	20,0

C_T of sample 2 was delayed after the C_T of sample 1 by about 2 PCR reaction cycles → the NaK ATPase transcripts were more abundant in the sample 1 than in the sample 2 (2^{CT} = 2^2 = 4-fold more abundant).

Fig. 8. Graph example of relative Na^+K^+-ATPase and *Rp49* mRNA levels under 3 acclimation conditions: 25°C/3w, 25°C→15°C/3w+3d and 25°C→5°C/3w+3d of *C. costata.*



Display of relative abundance of Na^+K^+-ATPase transcripts in 9 different samples of *C. costata* CNS tissue as presented in Fig. 4. Note relatively small technical variation (bars showing S.D. of C_T values within each doublet) and also relatively small variations among three independent replications of each experimental acclimation variant (A2, B1, B2).

2.5.5 Data evaluation

- averages of doublets were calculated
- averages of three replications were calculated
- to analyze *D. melanogaster* results, the unpaired two-tailed t-test was used. The mean relative abundances of transcripts at 25°C/4d vs. 15°C/11d were compared.

- To analyze *C. costata* results, the unpaired two-tailed t-tests were used to compare mean relative abundances of transcripts at 25 ºC/3w ("basal" value) vs. each acclimation variant analysed separately. This approach (individual tests) was prefered over the more strict multiple comparison test (ANOVA followed by Tukey post hoc tests).

3. Results

Cloning results

PCR results

Approximately 900 bp long PCR product was obtained. This product was then ligated into the vector, in which the product was multiplied and than isolated from plasmids. Finally, the product was prepared for sequencing.

BLAST results - Searching for a similarity

- (to know what the BLAST is, see chapter 2.4.15)
- The "nucleotide blast" (NCBI BLAST) method was used to compare the *C. costata Vha55* to its nearest homologue
- As a nearest homologue to *Vha55*, the V – ATPase B subunit gene of mosquito *Aedes aegypti* was found – homology 88%. The second nearest sequence is 55 kDa large B subunit, coding the V- ATPase of *D. melanogaster* – homology 87%.

Fig. 9. Alignement of amino acid sequences of *A.*. *aegypti* Vha55 (NCBI GenBank) and its *C. costata* structural homolog (obtained in this study)

```
>  ref|XM_001651408.1|  Aedes aegypti strain Liverpool
Length=1491

 GENE ID: 5567068 AaeL AAEL005798 | ATP synthase subunit beta vacuolar
[Aedes aegypti] (10 or fewer PubMed links)

 Score = 1103 bits (597),  Expect = 0.0
 Identities = 815/921 (88%), Gaps = 12/921 (1%)
 Strand=Plus/Plus

Query  2     GCCGGTCTGCCCCACAACGAAATTGCTGCCCAGATTTGTCGTCAAGCTGGTTTGGTTAAG  61
             |||||| |||| ||||| ||||| ||||||||||||||||||| || ||| |||| |||
Sbjct  538   GCCGGTTTGCCTCACAATGAAATCGCTGCCCAGATTTGTCGTCAGGCCGGTCTGGTCAAG  597
Query  62    CTGC-CCGGCAAATCGGTGCTGGATGATCAC-ACTGATAACTTTGCCATTGTTTTTGCTG  119
             |  | ||||||| |||||| |||| || ||| |  || |||||||||||| || || || |
Sbjct  598   C-ACACCGGCAAGTCGGTGTTGGACGAGCACGA-GGACAACTTTGCCATCGTGTTCGCCG  655
Query  120   CTATGGGTGTTAACATGGAAACGGCACGTTTCTTCAAGCAAGATTTCGAGGAGAATGGTT  179
             |||||||||| |||||||||| || ||||||||||||||| |||||||| ||||| ||||
Sbjct  656   CTATGGGTGTCAACATGGAAACTGCCCGTTTCTTCAAGCAGGATTTCGAAGAGAACGGTT  715
Query  180   CCATGGAGAACGTGTGCT-TGTTCTTGAACTTGGCCAACGATCCAACTATTGAGCGTATT  238
             | |||||||||||||| | ||||||||||||||||||||||||| || || || |||||
Sbjct  716   CTATGGAGAACGTGTG-TCTGTTCTTGAACTTGGCCAACGATCCGACCATCGAACGTATC  774
Query  239   ATTACACCACGTCTGGC-CTTGACCGCTGCCGAATTTTTGGCCTATCAGTGCGAGAAGCA  297
             || || || |||||||| | |||| || || || || |||||||| ||||||||||||||
Sbjct  775   ATCACTCCGCGTCTGGCGC-TGACGGCGGCTGAGTTCTTGGCCTACCAGTGCGAGAAGCA  833
Query  298   CGTGTTGGTCATCTTGACAGACATGTCCTCTTATGCTGAAGCTTTGCGTGAGGTGTCCGC  357
             |||||||||||||||| || |||||||| |||||||||| ||||||||||||||| ||
Sbjct  834   CGTGTTGGTCATCTTGACCGATATGTCCTCGTATGCTGAAGCCTTGCGTGAGGTGTCGGC  893
Query  358   TGCCCGTGAAGAGGTGCCCGGTCGTCGTGGTTTCCCAGGTTACATGTACACTGATTTGGC  417
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Sbjct  894   TGCCCGTGAAGAAGTGCCTGGACGTCGTGGTTTCCCAGGTTACATGTACACCGATTTGGC  953
Query  418   TACCATTTATGAGCGCGCTGGCCGTGTGGAGGGCCGTAATGGTTCTATCACTCAGATTCC  477
              ||||| || || || || || ||||| || || ||||| || || || || ||||| ||
Sbjct  954   CACCATCTACGAACGTGCCGGACGTGTCGAAGGTCGTAACGGATCGATTACCCAGATCCC  1013

Query  478   CATTCTGACTATGCCTAACGACGATATTACCCATCCAATTCCCGATTTGACTGGTTATAT  537
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Sbjct  1014  CATTTTGACTATGCCTAACGACGATATTACCCATCCAATTCCCGATTTGACTGGTTATAT  1073
Query  538   TACCGAAGGTCAGATCTATGTTGATCGTCAGCTGCACAACAGACAGATCTATCCACCAGT  597
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Sbjct  1074  TACCGAAGGACAGATCTACGTCGATCGTCAGCTGCACAACAGACAGATCTATCCACCAGT  1133
Query  598   TAACGTGTTGCCATCATTGTCACGTCTGATGAAGTCTGCCATTGGTGAGGGCATGACACG  657
              |||||||||||||   |||| ||||||||||||||||||| |||||||||||||| ||
Sbjct  1134  GAACGTGTTGCCATCGCTGTCTCGTCTGATGAAGTCTGCCATCGGTGAGGGCATGACCCG  1193
Query  658   CAAGGATCACTCTGATGTGTCCAATCAGCTT-TATGCTTGTTATGCCATCGGTAAGGATG  716
              |||||||||| |||||||||| ||| || || |||||||| |||||||||||||| |
Sbjct  1194  TAAGGATCACTCCGATGTGTCCAACCAG-TTGTACGCTTGTTACGCCATCGGTAAGGACG  1252
Query  717   TACAAGCCATGAAGGCTGTCGTCGGTGAGGAAGCCTTGACGCCTGACGATTTGCTGTACT  776
             |||| |||||||||||||||||||||||||| | || || |||||| ||||||||
Sbjct  1253  TACAGGCCATGAAGGCTGTCGTCGGTGAGGAAGCCCTCACTCCGGACGATCTGCTGTACC  1312
Query  777   TGGAGTTCTTGACAAAATTCGAGAAGAACTTTATCTCGCAGGGTAACTACGAGAACCGCA  836
             |||||||||||||| || |||||||||||||| |||||||||||||||||||||||||||
Sbjct  1313  TGGAGTTCTTGACCAAGTTCGAGAAGAACTTCATCTCGCAGGGTAACTACGAGAACCGCA  1372
Query  837   CCGTCTTCGAATC-CTTGGATATTGGCTGGCAGCTGCTGCGTATCTTCCCCAAGGAAATG  895
             | || |||||||| || ||| |||||||||||||||||||||||||||||||||||||||
Sbjct  1373  CGGTTTTCGAATCGCT-GGACATTGGCTGGCAGCTGCTGCGTATCTTCCCCAAGGAAATG  1431
Query  896   CTCAAGCGTATCCCGGCCTCC  916
             |||||||||||||||||||||
Sbjct  1432  CTCAAGCGTATCCCGGCCTCC  1452
```

- After the translation of *Vha55* nucleotide sequence into the „amino-acid language“, the 100% match was found between *C. costata* and *D. Melanogaster*
- The *Vha55* gene of *C. costata* is highly conserved sequence

Fig.10. Alignement of amino acid sequences of *D. melanogaster* Vha55 (NCBI GenBank) and its *C. costata* structural homolog (obtained in this study)

```
ref|NP_476908.1| UG Vacuolar H[+]-ATPase 55kD B subunit CG17369-PB, isoform
B [Drosophila
melanogaster]

 Score =  611 bits (1575),  Expect = 2e-173
 Identities = 305/305 (100%), Positives = 305/305 (100%), Gaps = 0/305 (0%)
 Frame = +2

Query  2    AGLPHNEIAAQICRQAGLVKLPGKSVLDDHTDNFAIVFAAMGVNMETARFFKQDFEENGS  181
            AGLPHNEIAAQICRQAGLVKLPGKSVLDDHTDNFAIVFAAMGVNMETARFFKQDFEENGS
Sbjct  174  AGLPHNEIAAQICRQAGLVKLPGKSVLDDHTDNFAIVFAAMGVNMETARFFKQDFEENGS  233

Query  182  MENVCLFLNLANDPTIERIITPRLALTAAEFLAYQCEKHVLVILTDMSSYAEALREVSAA  361
            MENVCLFLNLANDPTIERIITPRLALTAAEFLAYQCEKHVLVILTDMSSYAEALREVSAA
Sbjct  234  MENVCLFLNLANDPTIERIITPRLALTAAEFLAYQCEKHVLVILTDMSSYAEALREVSAA  293

Query  362  REEVPGRRGFPGYMYTDLATIYERAGRVEGRNGSITQIPILTMPNDDITHPIPDLTGYIT  541
            REEVPGRRGFPGYMYTDLATIYERAGRVEGRNGSITQIPILTMPNDDITHPIPDLTGYIT
Sbjct  294  REEVPGRRGFPGYMYTDLATIYERAGRVEGRNGSITQIPILTMPNDDITHPIPDLTGYIT  353

Query  542  EGQIYVDRQLHNRQIYPPVNVLPSLSRLMKSAIGEGMTRKDHSDVSNQLYACYAIGKDVQ  721
            EGQIYVDRQLHNRQIYPPVNVLPSLSRLMKSAIGEGMTRKDHSDVSNQLYACYAIGKDVQ
Sbjct  354  EGQIYVDRQLHNRQIYPPVNVLPSLSRLMKSAIGEGMTRKDHSDVSNQLYACYAIGKDVQ  413

Query  722  AMKAVVGEEALTPDDLLYLEFLTKFEKNFISQGNYENRTVFESLDIGWQLLRIFPKEMLK  901
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Query  902  RIPAS  916
            RIPAS
Sbjct  474  RIPAS  478
```

3.1.3 Primer design

- Based on BLASTing results, specific upper and lower primers for *C. costata* Vha55 were designed with help of DNAStar software. These primers were used for further transcript quantifications (qRT-PCR).
- **Primer structure:**

Upper Primer: 24-mer 5’ GGC CGT AAT GGT TCT ATC ACT CAG 3’ - (CcVha55F1)

Lower Primer: 22-mer 5’ CCC TCA CCA ATG GCA GAC TTC A 3’ - (CcVha55R1)

3.2 Transcript quantification results

3.2.1 Na^+K^+-ATPase and H^+-ATPase transcripts in *Drosophila melanogaster* CNS and Malpighi tubules

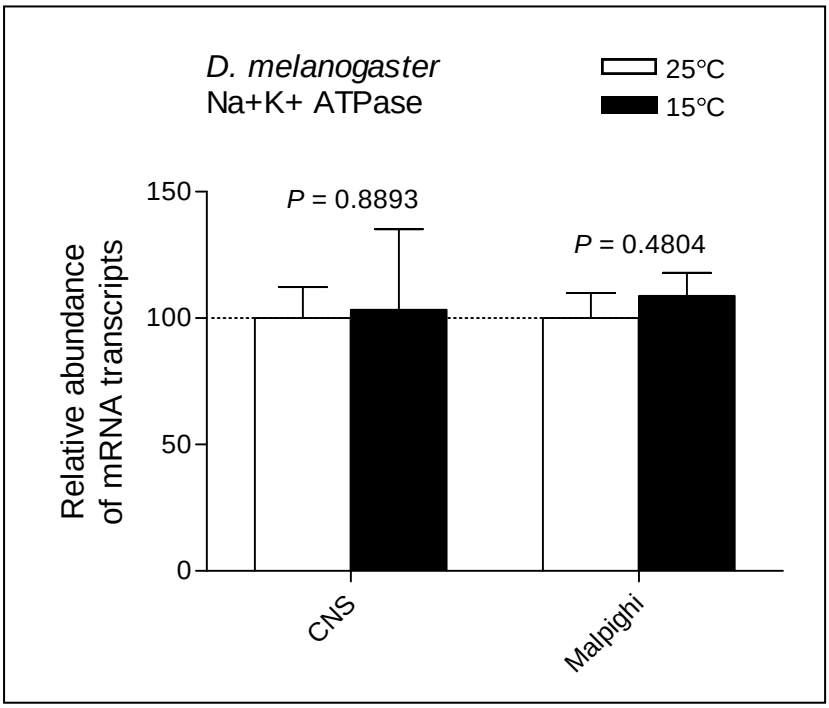

Fig.11. Relative abundance of mRNA transcripts of Na^+K^+-ATPase in *D. Melanogaster*, CNS and Malpighi tubules (each column represents mean of three independent replications ± S.E.). See text for more explanations.

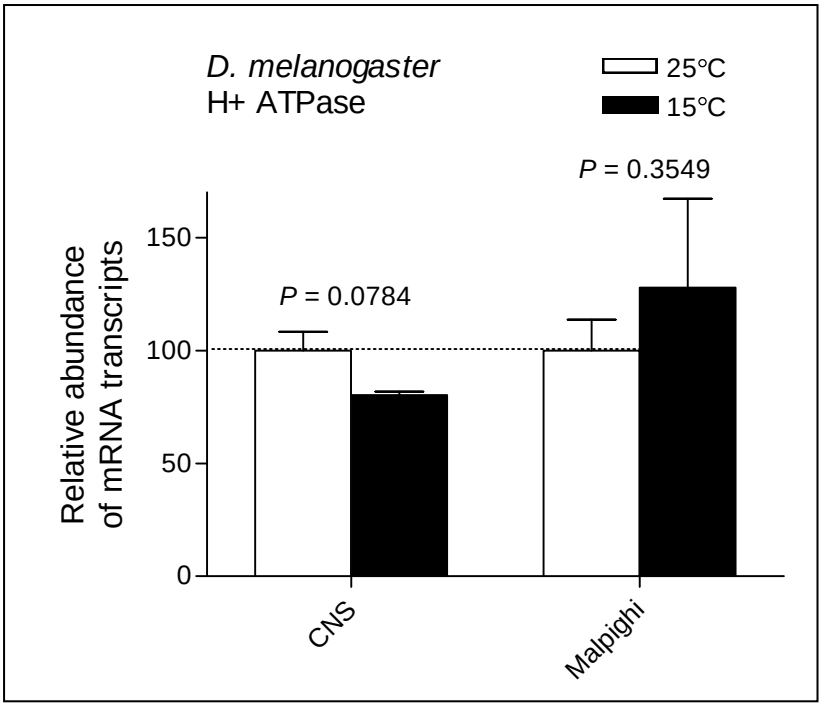

Fig.12. Relative abundance of mRNA transcripts of H^+-ATPase in *D. Melanogaster*, CNS and Malpighi tubules (each column represents mean of three independent replications ± S.E.). See text for more explanations.

The relative abundance of mRNA transcripts of genes coding for Na^+K^+-ATPase and *Vha55* (B) subunit of H^+-ATPase were examined of *D. melanogaster*. No differences in mRNA levels were observed in Malpighian tubules and CNS, when the flies were reared at temperature of 25°C and 15°C.

3.2.2 Na^+K^+-ATPase and H^+-ATPase transcripts in *Chymomyza costata* CNS

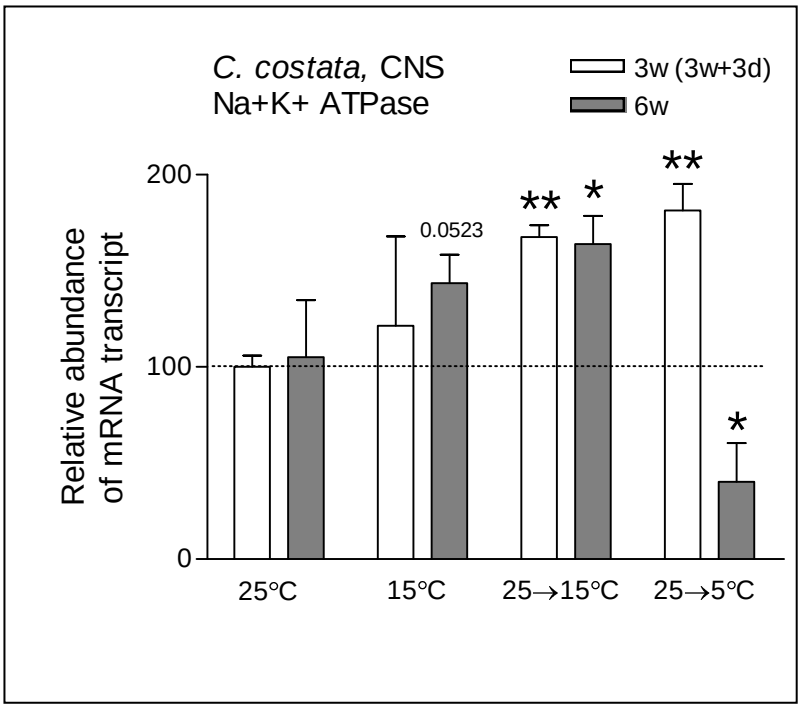

Fig.13. Relative abundance of mRNA transcripts of Na^+K^+-ATPase in *C. costata*, CNS (each column represents mean of three independent replications ± S.E.). See text for more explanations.

The relative abundance of mRNA transcripts of gene coding for Na^+K^+-ATPase was examined of *C. costata*, central nervous tissue. No significant difference was found between two samples (3 weeks and 6 weeks) taken at constant temperature of 25°C. In the temperature of 15°C, moderately higher levels of transcripts were observed (statistically not significant, however). More apparent, ca. 1,5-fold increase (statistically significant) was seen after the transfer from 25°C to 15°C. After the transfer from 25 to 5°C, an increase was observed initially (3d after transfer) but later (3w after transfer), a clear decrease was found.

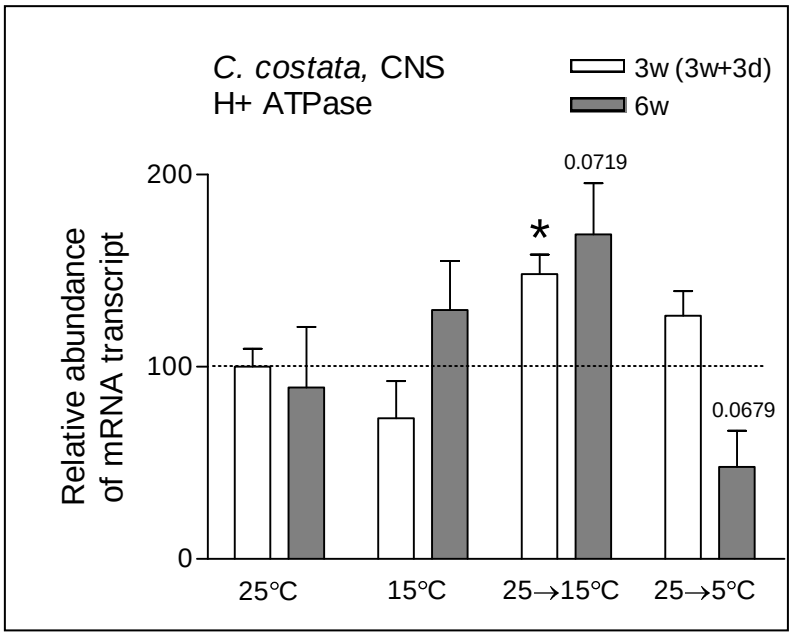

Fig.14. Relative abundance of mRNA transcripts of H^+-ATPase in *C. costata*, CNS (each column represents mean of three independent replications ± S.E.). See text for more explanations.

The relative abundance of mRNA transcripts of gene coding for H^+-ATPase was examined of *C. costata*, central nervous tissue. No significant differences between samples, measured in 3 weeks and 6 weeks at 25°C were found. In the temperature of 15°C, a general trend toward increse was observed (statistically significant only in the case of the transfer from 25°C to 15°C). After the transfer from 25 to 5°C, ca. 2-fold decrease was observed in 6 weeks (statistically not significant, however).

3.2.3 Na^+K^+-ATPase and H^+-ATPase transcripts in *Chymomyza costata* Malpighi tubules

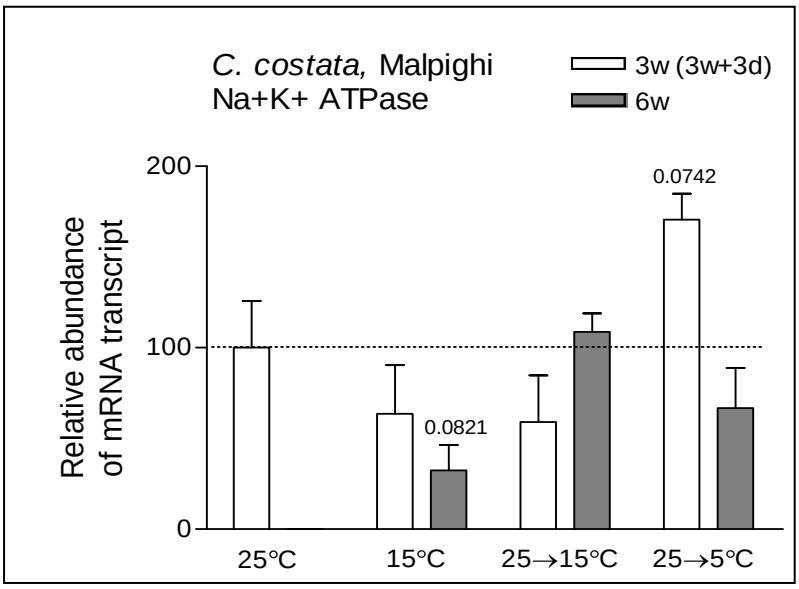

Fig.15. Relative abundance of mRNA transcripts of Na^+K^+-ATPase in *C. costata*, Malpighi tubules (each column represents mean of three independent replications ± S.E.). See text for more explanations.

The relative abundance of mRNA transcripts of gene coding for Na^+K^+-ATPase of *C. costata* was examined in Malpighi tubules. In the temperature of 15°C, an apparent decrease was observed at 3 and 6 weeks (statistically not significant). After the transfer from 25°C to 15°C, a small (insignificant) decrease was observed after 3 days. After the transfer from 25 to 5°C, a statistically insignificant increase 3d after transfer was observed but later (3w after transfer), a decrease was found (statistically not significant).

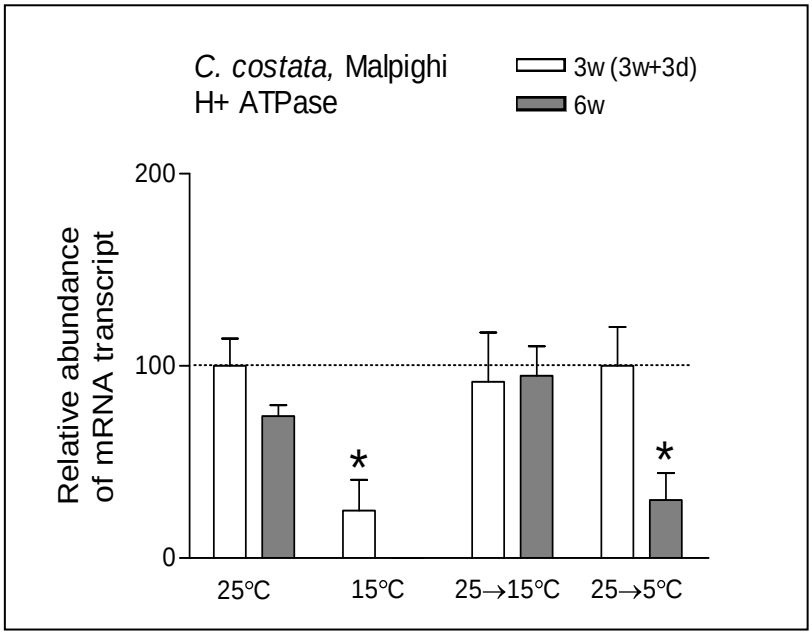

Fig.16.
Relative abundance of mRNA transcripts of H^+-ATPase in *C. costata*, Malpighi tubules (each column represents mean of three independent replications ± S.E.). See text for more explanations.

The relative abundance of mRNA transcripts of gene coding for H^+-ATPase of *C. costata* was examined in Malpighi tubules. No significant differences between samples, measured in 3 weeks and 6 weeks at 25°C were found. In the temperature of 15°C, a decrease was observed (statistically significant). After the transfer from 25°C to 15°C, no significant changes were found. After the transfer from 25 to 5°C, a clear decrease was found in 6 weeks (statistically significant).

4. Discussion

Only the transcript quantification results will be discussed here.

4.1 *Drosophila melanogaster*

The relative abundance of mRNA transcripts of Na^+K^+-ATPase and H^+-ATPase genes of *D. Melanogaster* were examined using the qRT-PCR method. Two tissues were studied, CNS and Malpighi tubules. When acclimated to temperatures of 25°C or 15°C, the relative abundance of mRNA transcripts of Na^+K^+-ATPase and H^+-ATPase were invariant in both tissues. Such results conform well with the overall low ability of *D. melanogaster* to cold-acclimate, which is likely related to its tropical origin and evolutionary history.

4.2 *Chymomyza costata*

The relative abundance of mRNA transcripts of Na^+K^+-ATPase and H^+-ATPase genes of *C. costata* were examined using the qRT-PCR method. Two tissues were studied, CNS and Malpighi tubules. *C. costata* revealed the up-regulation of Na^+K^+-ATPase and H^+-ATPase in the central nervous system at the temperature of 15°C and after the temperature transfer from 25°C to 15°C. Such results conform with the prediction of *C. costata* ability to compensate the ion pump functions at low temperatures, but still permissive for growth and development. Similar mechanism of the ion pump up-regulation was described in a common carp, *Cyprinus carpio*. Acclimated *C. carpio* revealed that the expression of Na^+K^+-ATPase is up-regulated in the gills to negate the inhibition effect of sodium pump (Metz et al., 2003).

In contrast, an apparent down-regulation of both ion pumps, Na^+K^+-ATPase and H^+-ATPase was observed in the central nervous system at the temperature of 5°C. Such results conform with the prediction of channel arrest strategy at very low temperatures, which induce the dormancy. Suppression of Na^+K^+-ATPase activity was earlier demonstrated in hibernating mammals (MacDonald and Storey, 1999) and during estivation in foot muscle of the land snail *Otala lactea* (Ramnanan and Storey, 2006).

The relative abundance of mRNA transcripts of Na^+K^+-ATPase and H^+-ATPase in Malpighi tubules showed high variability, which prevented us to find and describe clearly the potential acclimation trends. An (insignificant) up-regulation trend was observed in only one

case, 3 days after transfer to 5°C. In contrast, a down-regulation trend was observed several times at both low temperatures of 15 and 5°C. Perhaps, the ion pumps in Malpighi tubules respond to low temperatures differently than in the CNS. While the up-regulation at 15°C was observed in the CNS, the down-regulation (or no response) was observed in Malpighi tubules at the same temperature. Additional factors, such as (a) very small tissue size (estimated fresh mass of tissue dissected from one larva is less than 1 μg) and, subsequently, very difficult dissection; (b) presence of ion pumps only in the apical part of tubules; (c) very small amount of total RNA and mRNA in each sample contributed to less clear results obtained for Malpighi tubules tissues.

5. Conclusions and future directions

- *Vha55* gene coding for the B subunit of the H^+-ATPase of *C. costata* was cloned and 916 bp-long fragment was sequenced.
- *D. melanogaster* did not reveal any sign of acclimation response at the level of ion pump transcription.
- *C. costata* revealed the up-regulation of both ion pump genes in the CNS at 15°C, and also after the transfer from 25°C to 15°C. In contrast, down-regulation of both ion pump genes was seen in the CNS at temperature of 5°C.
- Because the results for Na^+K^+-ATPase in CNS tissue appear to be the most conclusive, there is a possibility to continue in this work. We plan to verify the results on the level of protein. The questions are: (a) whether the amount of the Na^+K^+-ATPase protein in CNS tissue of *C. costata* changes during cold acclimation (Western blotting); and (b) whether the activity of this pump is changing too (activity measurement).
- The results obtained for Malpighi tubules were not clear (mainly because of the technical difficulties), additional experiments are needed.

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