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The role of adenosine signaling pathway in regulation of metabolic reserves

Ph.D. Thesis

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Annotation

This thesis presents a study of possible role of the extracellular adenosine signaling pathway in the regulation of metabolism of energetic reserves in *Drosophila melanogaster*. I focus on a study of metabolism of carbohydrate reserves and show a connection of adenosine signaling pathway to regulation of glucose homeostasis. Results of hemolymph and whole larval and pupal carbohydrates concentration measurements are shown together with concentration of other metabolites. They suggest that glucose homeostasis is impaired in the *adgf-a* mutant, which is accompanied by impaired metabolism of energetic reserves. The work is based on genetic screening for dominant suppressors of larval lethality caused by a loss of function mutation in the main larval adenosine deaminase, ADGF-A. Possible interactions of adenosine signaling pathway and regulation of immune response are disscussed.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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Monika Žuberová

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Finally I would like to apologize to all my experimental animals.

List of papers and author's contribution

The thesis is based on the following paper:

Zuberova M, Fenckova M, Simek P, Janeckova L, Dolezal T (2010): Increased extracellular adenosine in Drosophila that are deficient in adenosine deaminase activates a release of energy stores leading to wasting and death. Disease models & mechanisms 3(11-12):773-84.

Monika Žuberová designed and completed the detailed deletion screening, dietary response experiments (except for some experiments involving AKH and AdoR) and measurements of whole larval and pupal energetic reserves. She prepared samples for HPLC-MS and participated on the preparation of the manuscript.

Contents

1. Introduction

1.1. The role of extracellular adenosine as a stress signaling hormone

Adenosine is intracellular metabolite that is produced in higher amount and released outside the cells undergoing metabolic stress, when the oxygen demand/supply is not balanced and thus the rate of ATP utilization/regeneration is not balanced. The most probable source of the adenosine in these cells is dephosphorylation of AMP in order to shift equilibrium of following reaction in order to regenerate more ATP: 2 ADP ź ATP + AMP (Bruns 1991). Extracellular adenosine is an ancient signaling molecule from plants to animals (Abbracchio 1996) and its signaling role is used to protect the tissue undergoing metabolic stress against damage. Therefore it has been referred to as a local regulatory or ñretaliatoryò metabolite that has extended its sphere of action beyond the cell that generates it (Newby 1984 and 1985).

In general, the action of adenosine can be designated as protective and homeostatic. This central paradigm of adenosine research is based on hypotheses that it increases blood flow during hypoxia (Berne 1963) and that it controls energy supply/demand balance by reducing energy demand and increasing energy supply (Bruns 1991).

Extracellular adenosine is now considered as an important stress signaling molecule. It dramatically increases during conditions associated with multiple organ failure, which is the cause of 50-80% of all deaths in surgical intensive care units (Hasko et al., 2002). During activation of systemic stress, it is released from sympathetic nervous system (Sperlagh et al., 2000). It is also released in the vicinity of immune cells during both systemic and cellular stress. During hypoxia, it is released from various tissues (Buck, 2004).

The role of extracellular adenosine as a signaling molecule is in many ways indistinguishable from that of a hormone and it is sometimes called a óstress hormoneô (Hasko et al., 2002). However, its usually short half-life in the circulation makes it rather a local hormone.

Extracellular adenosine has some effect in every mammalian tissue (Linden 2001). The effect on vasoconstriction was desribed already in 1929 by Drury and Szent-Györgyi. Adenosine modifies hormone release (Nyce, 1999) and thus oxygen and metabolic balance in tissues (Berne, 1963; Costa &Biaggioni, 1998), immune responses (Sitkovski and Lukashev, 2005) and signaling in the nervous system (Dunwiddie and Masino 2001; Masino & Dulla, 2005).

1.2. The role of extracellular adenosine in regulation of immune response

ATP leaks out from damaged cells (e.g. in the site of inflammation) and it is successively dephosphorylated by a cascade of ecto-enzymes to adenosine. The increasing of both ATP and adenosine regulate onset of the acute inflammatory response, the fine-tuning of ongoing inflammation and its eventual downregulation through purinergic receptors (Bours et al., 2006). This regulation is quite complex and many questions regarding the role of ATP and adenosine persists.

ATP could be also released from endothelial cells in response to specific inflammatory stimuli, e.g. bacterial products (Bodin and Burnstock, 1998).

The range of effects of adenosine on immune and inflammatory functions, on functions of peripheral blood cells and on regulation of hematopoiesis is reviewed in Hasko et al., (2008), Cronstein et al. (1996) and in Hofer et al. (2011) and are not a particular scope of this work.

1.3. Adenosine receptors

The extracellular adenosine regulates cellular functions by binding to G-protein coupled adenosine receptors that generally regulates intracellular cAMP (Latini and Pedata, 2001).

In mammals, four subtypes of adenosine receptors had been identified: A_1, A_{2a}, A_{2b} and A_3, which are variously distributed throughout the body. Almost every cell expresses one of four adenosine receptors. Some cells could even express more subtypes (e.g. astrocytes express all four of them; Boison et al., 2010). Each subtype of adenosine receptor activates different set of second messengers: A_1 and A_3 are coupled to Gi proteins, and thus stimulate K^+ channels, reduce voltage dependent Ca^{2+} channels, and inhibit adenylate cyclase and cAMP formation. A_{2a} and A_{2b} are coupled to Gs proteins and stimulate adenylate cyclase. In addition, in some cells, e.g. in human kidney epithelial cells, the A_{2B} receptor is also coupled to the calcium-mobilizing G protein subunit, $G\breve{U}_q$ (Murphree et al., 2002; Gao et al., 2007).

The existence of four receptors with different functions but overlapping patterns of expression, together with their pervasiveness, pose difficulties for deciphering the extracellular adenosine actions on different cells. In addition, expression of individual receptors may change in dependence on the functional state of the investigated cell population. Moreover, it is complicated due to a cross talk among various G-protein coupled receptors (Fredholm et al., 2000; Werry et al., 2003) and by a fact that not only

adenosine is a natural agonist of these receptors but also inosine (the product of adenosine degradation) can act as partial agonist at A_1 and A_3 (Fredholm et al., 2001).

A better understanding of the adenosine signaling pathways would help in the development of strategies for the treatment of various human diseases, such as tachycardia, sleep disorders, immune and inflammatory disorders (for a review see Jacobson and Gao, 2006).

1.4. Adenosine deaminases and regulation of adenosine concentration

The concentration of extracellular adenosine is normally maintained at very low levels. It is quickly taken up by cells, however main regulator is an enzyme adenosine deaminase (ADA) that catalyzes an irreversible conversion of adenosine to inosine.

Two types of enzymes with adenosine deaminase activity had been described: ADA1 and ADA2-like proteins (or ADGFs).

ADA1 (ñclassicalò adenosine deaminase) is present in all procaryotes and eucaryotes. There are two types of ADA1: an intracellular soluble monomer is present in all cells (and most studies are done for this form, especially on lymphocytes; Aldrich et al., 2010). The same enzyme may also appear as an ecto-ADA (bound to the membrane glycoprotein CD-26/dipeptidil peptidase IV) that probably regulates the level of extracellular adenosine (Franco et al., 1997; Richard et al., 2002).

ADA1 catalyzes irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine. Deoxyadenosine is a cytotoxic metabolite released by various cell populations that undergo programmed cell death, it can kill cells through a mechanism that includes disturbances in deoxynucleotide metabolism. Congenital absence of human or mouse ADA1 is accompanied by greatly elevated levels of its substrates adenosine and deoxyadenosine and by many developmental defects. It is one of the possible causes of Severe Combined Immunodeficiency Syndrome (SCID; in humans it accounts for about 20% of all types of SCID; Aldrich et al., 2010) that is one of the most severe human immunodeficiencies accompanied with loss of all types of lymphocytes as well as pathological changes in other tissues (Buckley et al., 1997). Without an intervention, affected newborns die on opportunistic infections during few months.

Drosophila contains only one gene with a similarity to Ăclassicalñ adenosine deaminases, but its product is most likely inactive (Zurovec et al., 2002).

ADA2 subfamily is slightly different from ñclassicalò adenosine deaminases. The founding member of this subfamily is IDGF (Insect-Derived Growth Factor; Homma et al.,

1996) and its homologs have been described in various organisms. Mammalian ADA2 is a secreted and its natural substrate is only adenosine. The coding gene for mammalian ADA2 corresponds to human gene *CECR1* (cat eye syndrome critical region protein 1; Riazi et al., 2000). *CECR1* is localized in a small region of human chromosome 22, which duplication results in Cat Eye Syndrome (characterized by hypoplastic kidney, congenital heart malformation and anomalous pulmonary venous connection) and deletion of this region causes DiGeorge syndrome. Six genes with sequence similarity to *CECR1* subfamily have been identified in Drosophila: *ADGF-A*, *-A2*, *-B*, *-C*, *-D* and *-E*. ADGF-A and ADGF-D exhibit strong adenosine deaminase activity and are mitogenic for certain cells in cultures (by depletion of the extracellular adenosine, which has been shown to block proliferation) from which the name for all six genes originates: Adenosine Deaminase Related Growth Factors (Zurovec et al., 2002).

1.5. Strategies to study adenosine signaling in mammals

Extracellular adenosine could act in two ways: 1) by cell uptake and 2) through adenosine receptor. Most studies on receptor-mediated effect of elevated adenosine in mammals are based on injection of adenosine together with Dipiridamol = a drug inhibiting cellular uptake of adenosine. Effects of the stimulation of individual receptors are studied by their selective activation/blocking by agonists/antagonists in cell culture or by injection.

1.6. *Drosophila* model for studying adenosine signaling effects

Drosophila melanogaster has been established as a model to study the role of extracellular adenosine signaling pathway in vivo by mutating ADGFs (Dolezal et al. 2003) and adenosine receptor (Dolezelova et al., 2007). Comparing to mammals, there is only one copy of adenosine receptor and only one active form of adenosine deaminase belonging to ADA2 subfamily, although encoded by six genes with different expression patterns. ADGF-A, a main Drosophila adenosine deaminase, is expressed in gut, brain and in hematopoietic organ. Hemocytes, Drosophila ñblood cellsò, are main regulators of adenosine (Dolezal et al., 2005), similarly to mammals.

Loss of function of main larval adenosine deaminase (*adgf-a*; Dolezal et al., 2005) causes:

1) an increase of hemolymph adenosine (to 1.14 ± 0.26 µM versus 0.08 µM in the wild-type) and deoxyadenosine (1.66 ± 0.99 µM versus undetectable level in the wild-type),

2) delay in developmental, block of pupation and larval/pupal lethality,

3) hematopoietic defects (increased of number of circulating hemocytes and differentiation of lamellocytes in pre-wandering larvae),

4) disintegration (disappearance) of larval fat body, which was ascribed to action of precocious metamorphosis,

5) development of melanotic tumors (capsules), probably around fat body.

All these effects are overall termed as ñ*adgf-a* mutant phenotypeò. Hematopoietic defects are rescued by an overexpression of ADGF-A in lymph gland suggesting that the extracellular adenosine somehow influences hematopoiesis. Dietary 20-hydroxy ecdysone restores the pupation frequency to almost wild-type level, showing that the *adgf-a* larvae are responsive to action of ecdysone and suggesting that there is an impaired release of hormones from ring gland (Dolezalet al., 2005), where the AdoR receptor is expressed.

Drosophila adenosine receptor is encoded by CG9753 (further mentioned as AdoR; Dolezelova et al., 2007). AdoR shares a structural similarity with mammalian receptors. It is most similar to A_2B receptor by: it is coupled to the G-protein subunits Gs and Gq and activates cAMP and calcium signaling. Loss of function of AdoR has no effect on fly viability, but overexpression *in vivo* causes lethality or severe developmental anomalies that in certain cases mimic effects of the *adgf-a* mutation (Dolezelova et al. 2007).

The double *adoR adgf-a* mutant shows a less extreme phenotype than the *adgf-a* mutant (Dolezal et al., 2005) confirming that the *adgf-a* mutant phenotype results from overstimulation of adenosine signaling pathway. Mutation of *AdoR* suppresses hematopoietic and developmental defects. However, exerted effect of increased hemolymph adenosine on disintegration of the fat body seems to be independent (or not only dependent) on signaling through the AdoR. Such an adenosine receptor-independent pathway or effects had been suggested also in mammals.

1.7. Genetic interaction of adenosine signaling with Toll signaling pathway

Cactus is a Drosophila inhibitor of NF-əB in Toll signaling pathway, one of the antimicrobial immune response pathways identified in Drosophila. The *cactus null* mutation leads to constitutive activation of Toll pathway. Phenotype of the *cactus null* mutant larvae is similar to *adgf-a*. They have over-proliferated hemocytes which encapsulate fat body. They have slower larval development, with 60% larval lethality, as well as a thin body-shape phenotype (Qiu et al., 1998).

The forced expression of ADGF-A under *hs* promoter can partially rescue the effects of overactive Toll signaling, suggesting that adenosine might function downstream of Toll signaling to control its effects (Dolezal et al., 2005)

Similar phenotype of the *adgf-a* and *cactus* larvae and the interaction of Toll and adenosine signaling pathway suggested connection of adenosine signaling and the regulation of immune response. Therefore one of the aims of the deletion screening we performed was to uncover genes connected to hematopoiesis or activation of immune response.

2. Objectives of this work

1) To identify suppressors of the *adgf-a* mutant phenotype that is caused by hyper-activation of the adenosine signaling pathway by continuing in a deletion screening.

2) To further characterize the identified suppressors in order to uncover cellular responses activated by the extracellular adenosine.

Results & discussion

The following text of results and discussion is divided in two parts and is composed from unpublished data and from data published in following paper (which forms last part of supplements):

Zuberova M, Fenckova M, Simek P, Janeckova L, Dolezal T (**2010**): Increased extracellular adenosine in *Drosophila* that are deficient in adenosine deaminase activates a release of energy stores leading to wasting and death. *Disease models & mechanisms* 3(11-12):773-84.

Summary of publication in DMM (Zuberova et al., 2010):

Extracellular adenosine is an important signaling molecule in neuromodulation, immunomodulation and hypoxia. Adenosine dysregulation can cause various pathologies, exemplified by a deficiency in adenosine deaminase in severe combined immunodeficiency. We have established a Drosophila model to study the effects of increased adenosine in vivo by mutating the main Drosophila adenosine deaminase-related growth factor (ADGF-A). Using a genetic screening, we show here that the increased extracellular adenosine in the *adgf-a* mutant is associated with hyperglycemia and impairment in energy storage. The adenosine works in this regard through the adenosine receptor as an anti-insulin hormone in parallel to adipokinetic hormone, a glucagon counterpart in flies. If not regulated properly, this action can lead to a loss of energy reserves (wasting) and death of the organism. Because adenosine signaling is associated with the immune response and the response to stress in general, our results mark extracellular adenosine as a good candidate signal involved in the wasting syndrome that accompanies various human pathologies.

3. Part I. of results & discussion - Genetic screening

3.1. ñLow resolutionò genetic screening for dominant suppressors of the *adgf-a* phenotype

To identify genes associated with the effects of elevated adenosine, a deletion screening for dominant suppressors of the *adgf-a* mutant phenotype was performed using Bloomington deficiency kit. A total of 49 deletions for chromosome X and 73 deletions for chromosome II were screened and 11 regions that suppress the *adgf-a* phenotype were found (Table S1A). Chromosome III was not screened, because the *ADGF-A* gene is on chromosome III, thus it would be necessary to recombine every single deletion to the *adgf-a* mutation.

Rescue effects of these identified deletions were characterized as an increase of non-tubby pupae in the vials and their earlier appearance, which was often accompanied by further successful development through metamorphosis to adult flies (Fig. 1). Parent flies were crossed and allowed to lay eggs into vials for 2 days. All progeny non-*Tubby* larvae were homozygous for the *adgf-a* mutation. One half of non-*Tubby* larvae did not contain the deletion and showed the *adgf-a* mutant phenotype (larval and pupal lethality). The second half of non-*Tubby* larvae contained the deletion and could show a mutant or rescued phenotype (suppression of the lethality). Thus the rescue effect of the tested deletion was manifested by increasing amount of non-*Tubby* larvae and pupae in the vial. All *Tubby* larvae were heterozygous for the *adgf-a* mutation and thus show a wild type phenotype.

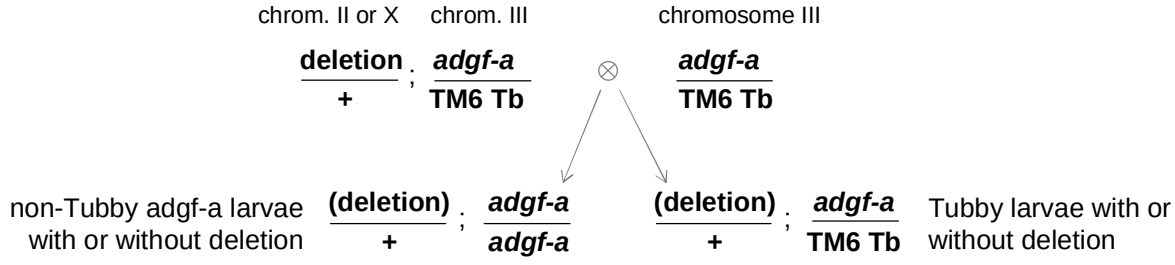

***Figure 1*. The crossing scheme used for the low resolution deletion screening**

All non-Tubby larvae resulting from crossing of TM6B balanced *adgf-a* heterozygots were homozygous in *adgf-a* and 50 % of them were bearing the tested deletion, which was on chromosome II or X.

3.2. ñDetailedò screening for dominant suppressors of the *adgf-a* phenotype

I started my PhD project by confirming the results of the low-resolution screening and by continuing with a detailed deletion and insertional screening of these 11 regions. My aim was to find particular genes with the *adgf-a* phenotype suppressing effect.

The genetic crossing that was used for the low resolution screening did not allow to distinquish between the *adgf-a* larvae with or without the screened deletion, which was necessary for a better characterization of the rescue effect of a particular deletion on the *adgf-a* mutant phenotype (e.g. proportion of melanotic tumors, the rate of fat body disintegration or the number of hemocytes). In the detailed screening both the deletion and the *adgf-a* mutation were balanced with balancers expressing GFP (Fig. 2). This allowed us to distinguish between *deletion/+; adgf-a* (GFP negative) larvae and the others (GFP positive). I put 20-30 GFP-negative first-instars per vial and searched for an increase of pupariation, an increase in number of pupae that further continued in development, a faster larval development/earlier appearance of pupae, a disappearance of melanotic tumors/fat body disintegration/*adgf-a* pupal phenotype ï thin pupae with a curvature of the abdomen and bad tracheal eversion. All these effects are further in text described as a ñrescueò, meaning the suppression of the *adgf-a* phenotype. As controls, I used *adgf-a* larvae (GFP negative) and *adgf-a*/*TM3 Act-GFP Ser* (GFP positive; further referred to as *wt* = wild type; see stock list S1), both of them resulted from a crossing of two independent *adgf-a* mutant lines - *adgf-a*^{karel}/*TM3 Act GFP Ser* and *adgf-a*^{gerda}/*TM3 Act GFP Ser*.

When the rescue region was restricted to few genes, I started to screen for rescue effect of *P*-element or piggyBac insertions instead of deletions. The crossing scheme was the same as in figure 2 but deletions were replaced by insertions.

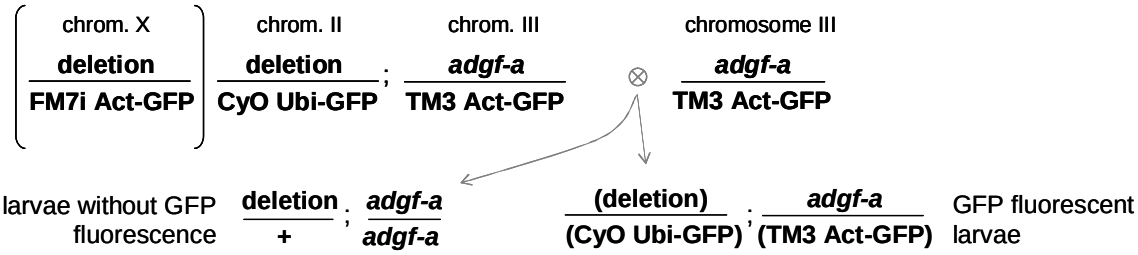

Figure 2. **The crossing scheme used for the detailed screening**

Studied mutations on each chromosome were in parental generation balanced with GFP marked balancer under actin (*Act-GFP*) or ubiquitin (*Ubi-GFP*) promoter. All GFP-negative larvae in F1 generation were heterozygous for tested deletion and homozygous for *adgf-a* mutation.

3.3. Optimization of the detailed screening

The very first outcome of the detailed screening was an identification of two *P*- element insertions rescuing the *adgf-a* mutant phenotype: *P{EP}PhKgamma*^{EP779} in coding region of gamma subunit of phosphorylase kinase (PhKɔ; CG1830) and *P{RS3}CG14478*$^{CB-6372-3}$ in protein coding gene of unknown function CG14478 (further mentioned as Monar). Both insertions are further mentioned as ***phKɔ*** and *monar*. Details regarding the identification of these two *P*-element insertions are provided in supplements.

The rescue effect of *P*-element insertion on the *adgf-a* phenotype is manifested as a decrease of lethality in all scored developmental stages: wandering 3rd instar, prepupa, pupa, pharate imago, eclosed imago and imago that was alive for a minimum of 3 days (Fig. S6). All larvae about 3-4mm that were on the surface of the diet or on the side of a vial were considered as wandering 3rd instars. All pupated animals were considered as prepupae. All pupated animals that continued in pupal development and formed adult head structures, which are visible through the pupal case, were considered as pupae (Fig. S8).

Ethanol washing: From the beginning of my work with the *adgf-a mutants*, I had problems with bacterial contamination in the vial. Sometimes food in vials was completely overgrown by bacteria which interfered with larval development. Therefore, I started washed embryos in 96 % ethanol before larval hatching. This prevented overgrowth of bacteria and increased survival of the *adgf-a mutants*.

Most of the *adgf-a* animals that were not washed in ethanol stopped their development as prepupae (light columns in figure 8E). These prepupae were thin, they had not fully everted tracheae and they showed curved abdominal parts (Fig. 9). After washing with ethanol, more of the *adgf-a* larvae pupated and more of prepupae continued in development and some of them successfully finished metamorphosis giving rise to pharate imagoes.

The rescue effect of suppressing mutation was better manifested on unwashed embryos (e.g. the percentage of pupated animals hatched from unwashed embryos was 10 % for *adgf-a*, 20 % for *monar*;*adgf-a*, 60 % for ***phKɔ***; *adgf-a*, while from embryos washed with ethanol it was 40 % for *adgf-a*, 70 % for *monar*;*adgf-a* and 60 % for ***phKɔ***; *adgf-a*). The *wt* larvae pupated at about 80 % after ethanol washing and at 60 % without ethanol washing. All *wt* pupae successfully finished metamorphosis and emerged into adults (data not shown).

For all further experiments I started to wash the embryos in ethanol. Later I also started to use antibiotics in combination with ethanol washing, because sometimes in some vials

bacterial contamination emerged even after the embryo washing. The addition of antibiotics had no additional effect on mutant viability, unless there was bacterial contamination.

Phenotype of rescued animals: third instar larvae of *monar*; *adgf-a* or *phKɔ*; *adgf-a* were more similar to *wt* larvae than to *adgf-a* larvae. However, late wandering larvae still showed disintegration of fat body, formation of melanotic tumors and increased circulating hemocytes. Their pupae had fully everted tracheae and were not as thin as the *adgf-a* pupae, but still had curvature of the abdomen. The rescue effect of homozygous *P*-element insertions was more profound than that of heterozygous insertions. The *monar*; *adgf-a* larvae pupated at the same time as *wt*, whereas the *phKɔ*; *adgf-a* larvae pupated at the same time as *adgf-a* (all these rescue effects are summarized in Fig. S6F).

The rescue effects shown in Fig. S6 were manifested on the cornmeal diet with 1-hour thermal hydrolysis of the yeasts (see Fig. S2 for the details) and are specific for these particular conditions. Later I found that the phenotype of the *adgf-a mutants* strongly depends on many factors. The most important ones are: the composition of the diet, bacterial or other contamination and the genetic background, in which the *adgf-a* mutation is placed. The other possible factors are discussed in following text and are summarized in Tab. S3. Some factors changed with every new experiment and with every new batch of the diet, although one tried to keep them constant. So it was always necessary to compare *adgf-a* and rescued larvae reared on the same batch in the same time.

Identification of these two suppressors had influenced my further work during PhD fellowship: I started to characterize imbalances in carbohydrate metabolism in *adgf-a* mutants and I also started to characterize the gene *Monar*, while still continuing in screening.

3.4. Connection of adenosine signaling pathway to carbohydrate metabolism

Phosphorylase kinase (PhK) regulates glycogen synthesis and degradation. It is composed of Ŭ, ɓ, ɔ and ŭ subunits. The ɔ subunit has catalytic activity, the other three subunits have regulatory function (Bahri et al., 1994; Brushia and Walsh, 1999). We assumed that rescue *P{EP}PhKgamma[EP779]* insertion (*phK*) impairs the expression of PhKɔ subunit and thus it impairs function of PhK (phosphorylation of glycogen synthase and glycogen phosphorylase; see Fig. 3), which favors glycogen synthesis. It suggested that *adgf-a* mutant suffers from tendency to switch into glycogen degradation.

Most of the 3rd instar *adgf-a* larvae die before pupation, they progressively slow down their locomotion and their fat body disintegrates. Based on *phK* rescue, we hypothesized that this is because *adgf-a* mutant use up more energy reserves to maintain some physiological processes that are not maintained in *wt* under normal condition. We hypothesized that this **a)** results in lower efficiency of glycogen (and other energy reserves) synthesis and **b)** faster consumption of reserves during wandering phase, **c)** is the cause of a delay in the development (because the *adgf-a* larvae are not able to deposit enough energy into body growth) and **d)** of fat body disintegration in wandering phase (due to autophagy of fat body cells) and **e)** that the cause of the *adgf-a* larval and pupal lethality is total depletion of energetic reserves.

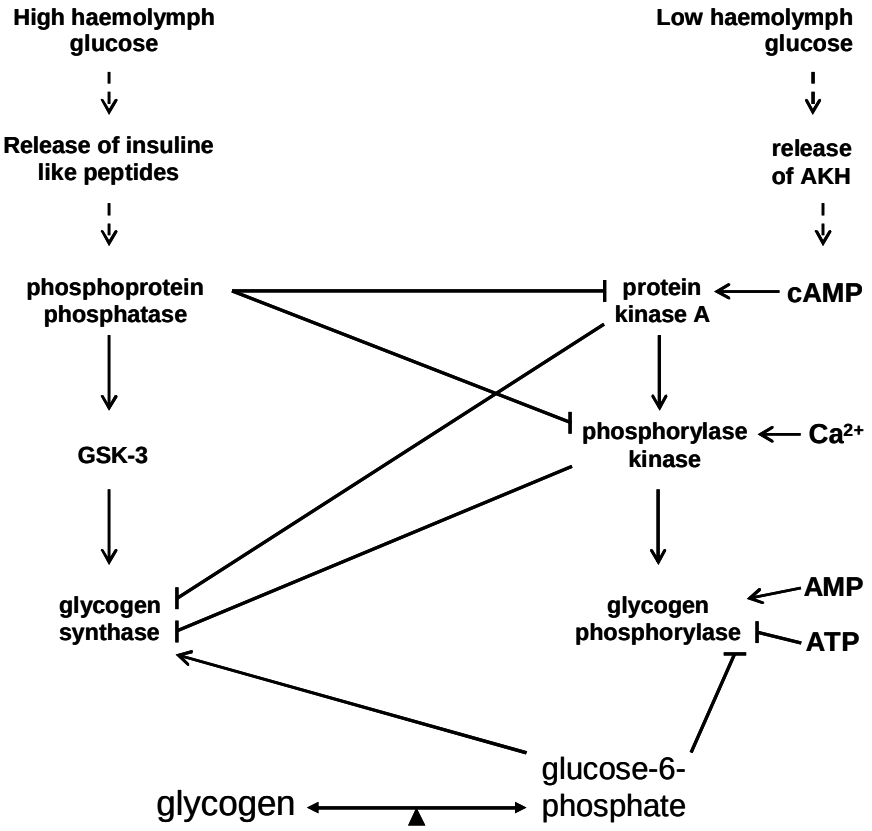

***Figure 3*. The simplified scheme of regulation of the glycogen synthesis / degradation**
Switching from glycogen degradation to synthesis depends on actual shortage or surplus of glucose. When hemolymph glucose increases, its uptake by cells and building up the glycogen is stimulated by an action of insulin-like peptides. On the other hand, when hemolymph glucose decreases, its release from fat body is stimulated by action of adipokinetic hormone (AKH). Both pathways when activated inhibit each other to prevent futile cycle of glycogen degradation and synthesis. Both hormonal regulation and concentration of glucose (and other factors, e.g. Ca^{2+} release or ATP/AMP ratio) strike a balance between glycogen synthesis/degradation

3.5. The sensitivity of the adgf-a larvae to the quality of the diet

I tested, if it is possible to rescue *adgf-a* mutant development by increasing of the concentration of the sugar in the diet. Our assumption was that the increase of dietary sugar will result in increase of hemolymph sugar which will result in shifting of the balance between glycogen synthesis and degradation towards its synthesis (Fig. 3). In order to test this, I prepared set of yeast diets consisting of lyophilized yeasts, agar, water and different concentration of sucrose (see Tab. S2). On the diet without added sucrose, most of the *adgf-a* larvae die in early second instar and none of them pupate (Fig. 3A). Increase of the dietary sucrose improves survival of the *adgf-a* mutant: On the diet supplemented with 5 % sucrose, 32 % of the first instar *adgf-a* larvae survive to pupa formation and 7 % of the first instar larvae eclose. On the 10 % sucrose diet, 56 % of first instar larvae pupate and 43 % of them eclose. The same effect is reached by supplementation of the diet with glucose rather than sucrose (data not shown). Surviving of the *adgf-a/TM3 Ser GFP* (*wt*) larvae is not influenced by 0-10 % sugar concentration in the diet.

The sensitivity of the *adgf-a* larvae to low dietary sugar concentration is suppressed by a rescue *P{EP}PhKgamma[EP779]* insertion and by a mutation of *AdoR* (Fig. 10B). On the diet supplemented with 0 % sucrose, the rescue effect is manifested only by homozygous mutations (phK and adoR in figure 10B). On 5 % sucrose diet, the rescue effect is obvious also in heterozygous mutations (phK/+ and adoR/+).

For the comparison, Fig. 3A shows survival on our standard corn meal diet (CM-60). The corn meal diet consists of 4 % yeast, while the yeasts diets consists of 8 % yeast (see Tab. S2). This is because when I was starting my experiments with yeast diets, I have found out that the development of the both *wt* and *adgf-a* larvae reared on the yeast diet with the same concentration of sugar and yeasts as in our corn meal diet is one day delayed, while the developmental time on 5s8y (5 % sucrose, 8 % yeasts) diet is similar to that on CM-60.

Interestingly, the total amount of the *adgf-a* pupae was slightly higher on 5s4y (5 % sucrose, 4 % yeasts) than on 5s8y. Based on this, I concluded that survival of *adgf-a* larvae is improved with both increasing sugar concentration and decreasing yeast concentration. To test this assumption, I manipulated the concentration of dietary yeasts and sucrose in range of 0-20% for sucrose and 2-20% for yeasts (Fig. 5).

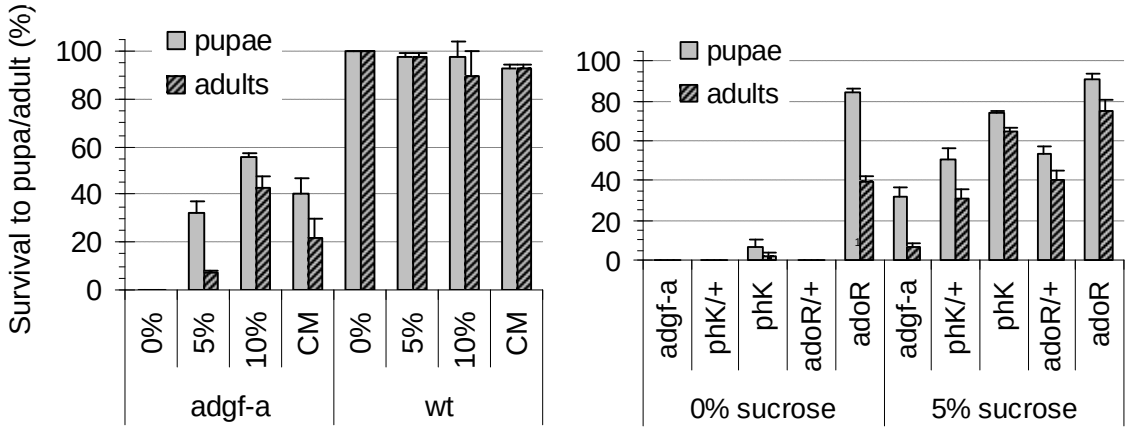

***Figure 4*. Survival of *wt* and *adgf-a mutants* to pupa or adult stage**

A) Survival of the *adgf-a* and *wt* animals on the corn-meal (CM-60) and yeast diets with different concentration of sucrose (0 %, 5 % and 10 %). **B)** Survival of the *adgf-a*, *phK*/+;*adgf-a* (phK/+), *phK*;*adgf-a* (phK), *adoR*/+;*adgf-a* (adoR/+) and *adoR*;*adgf-a* (adoR) animals on yeast diets supplemented with 0 % and 5 % sucrose.

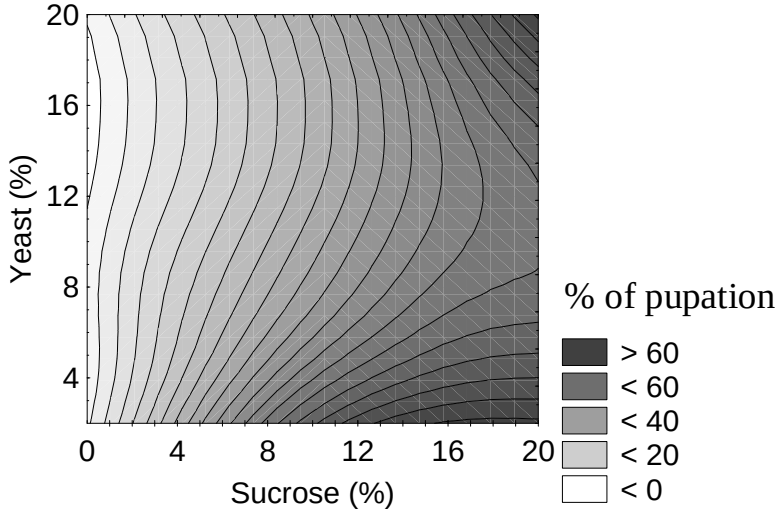

***Figure 5*. 3D contour plot (spline fit) of successful pupation of the *adgf-a* larvae plotted against sucrose and yeast content in the diet**

Independently, these data were used to construct General Linearized Model (distribution normal, link function log). The model included factors sucrose (P = 0.009), yeast content (P = 0.015) and their interaction (P = 0.034) and this model describes these data in following way: sucrose has positive effect on pupation and yeast has negative effect, while both high sugar and yeast leads to higher pupation i.

Diets were prepared in all combination of 0, 2, 4, 6, 8, 10, 12, 20 % sucrose and 2, 4, 6, 8, 10, 12, 20 % yeasts.

Results shown in 3D contour plot are not much convincing. The plot area bends and it seems that the *adgf-a* animals pupate less on 8 %, 10 % and 12 % yeast than on 20 % yeasts. However, later I found that surviving of the *adgf-a* larvae depends neither only on sugar nor yeast concentration, but also on the quality of the thermal hydrolysis of the yeasts during preparation of the diet: The more intensive the hydrolysis is, the worse is the survival of the *adgf-a* larvae. I was not aware of this in time of preparing diets for the 3D contour plot. I prepared single batches of pure yeast diets of desired yeast concentration (2, 4, 6, 8, 10, 12, 20 %) by cooking yeasts in water in a microwave oven for approximately 10 minutes. After cooking, each batch was divided into eight groups and supplemented with desired amount of sucrose (0, 2, 4, 6, 8, 10, 12, 20 %). The diet foamed during cooking and it was necessary to repeatedly interrupt cooking, so it is hard to say how long it was exactly cooked. It is possible that I cooked 8 %, 10 % and 12 % batches for a longer time and 20 % batch for a shorter time than the 2 %, 4 % and 6 % batches, this could explain the bending of the plot area.

Shifting of the sugar/yeasts ratio influences the developmental time of both *wt* and *adgf-a* larvae (not shown). However, neither the duration of thermal hydrolysis in range of 20-120 min nor manipulating with sugar/yeast ratio in observed range influences the total amount of *wt* pupae (not shown). We could only guess which compound is missed or harms the *adgf-a* mutant larvae after the thermal preparation and whether this has a direct connection to the adenosine metabolism.

3.6. Unification of the genetic background

Two genes for ectonucleotidases (which are supposed to produce adenosine in site of tissue damage) are located in the chromosomal region where is the gene *Monar* (Fig. S5). I tried to rescue the *adgf-a* animals by *P*-element insertions into these ectonucleotidases and I found that they have very negative effect on survival of the *adgf-a* larvae. It was contrary to all expectations and it pushed me to take into consideration the effect of genetic background on overall mutant viability. To solve this problem, I decided to unify genetic background of the *adgf-a* mutation and all the other tested mutations.

To unify the genetic background, I subdued all tested mutations to free recombination for 7 - 15 generations. All mutations that were subdued to free recombination were marked by miniwhite. In each generation, around fifteen red eyes virgins were crossed to about eight males from our *white* stock. After last (7^{th} or 15^{th}) cross, I set up new órefreshedô

stocks from around twenty five virgins and fifteen males. The genetic background of these new stocks is referred as genetic background 2. The genetic background of original *adgf-a/TM3 Ser GFP* stock is referred as background 1.

The genetic background 2 markedly improves survival of the *adgf-a* animals. They are still sensitive to dietary sugar restriction and to length of thermal hydrolysis of dietary yeasts, however this sensitivity is shifted in comparison to background 1. Fig. S7 shows that almost 90 % of the *adgf-a* larvae of background 2 pupate when reared on 5s8y diet that was heated (at 90°C) for 30 minutes, while only 30 % of them pupate on 5s8y diet that was heated for two hours. Only 20 % of the *adgf-a* larvae of background 1 pupate on the same batch of 5s8y diet that was heated for 30 minutes.

Figure 12 also shows that the rescue effect of the *P{EP}PhKgamma[EP779]* insertion into gene coding for PhKɔ in background 1 is no more apparent in background 2. Probably, the increase of the viability in the background 2 wiped out the rescue effect of this insertion. Also the other identified rescue mutations which improved survival of *adgf-a* larvae in background 1 did not significantly improved it in the background 2 (the mutation which were tested in unified background 2 are listed in column ñBoth adgf-a and tested mutationò of Tab. S4B).

Interestingly, the genetic backgrounds of two different collections of the deletions which I used for screening: the Exelixis collection at Harvard Medical School and the DrosDel collection in Vienna, have opposite effects on the *adgf-a* mutant viability. These two collections were made in isogenic background, different for both collections (Parks et al., 2004; Ryder et al., 2004). The genetic background of Exelixis collection worsens the viability of the *adgf-a* animal (it is illustrated in Fig. S3B). The genetic background of DrosDel collection seems to rescue *adgf-a* mutant phenotype in comparison to genetic background 1 (so the rescue by *monar* could be only effect of this genetic background).

4. Part II. of results & discussion - Measurements of larval and pupal metabolites and energetic reserves

Based on the screening and dietary rescue experiments we concluded that carbohydrate metabolism is affected in the *adgf-a* mutant. So, I aimed my effort to characterize the differences in carbohydrate reserves in larval hemolymph and in the whole *adgf-a* and *wt* larvae and pupae. I also measured hemolymph carbohydrates of different mutants and whole larval/pupal triglycerides in *wt* and *adgf-a* mutant.

4.1. Measurement of hemolymph carbohydrates, adenosine and inosine

I measured hemolymph glucose, fructose, trehalose, adenosine and inosine simultaneously in the same sample, by HPLC/MS. Samples of hemolymph were collected by tearing 10-20 3rd instar larvae of desired age, 3 µl of spilled hemolymph were immediately transferred into extraction medium (details are in Zuberova et al., 2010). Circulating hemocytes were removed by centrifugation (2000xG/2 min), because they could be a rich source of measured metabolites.

My preliminary biochemical measurements of hemolymph glucose and trehalose by GAGO-20 kit (Sigma) suggested that their concentration strongly depends on larval age. Therefore, it is necessary to compare the concentration of hemolymph glucose in larvae of the same age. However, the time of larval development of different mutants differs, so larvae of the same age (in hours after egg laying) are in fact of different ñphysiological ageò. For a meaningful comparison it was necessary to compare larvae of the same physiological age. Moreover, the time of larval development not only depends on genotype but also on external factors that change with every new batch of the diet, although one tries to keep them constant. So it is almost impossible to collect repeatedly larvae of the same age. Moreover, differences between measured concentration of hemolymph glucose at every single measurement were sometimes higher than the differences between *adgf-a* and *wt* larvae measured in the same time. Therefore I decided that for further experiments it is necessary to harvest all desired larvae from one batch of the diet and measured all collected samples at one jump.

For HPLC measurement, I decided to use larval size as a marker of physiological age and in order to obtain short-time developmental profile I attempted to harvested larvae of three different ages (smaller 3rd instars of about 3 mm, bigger non-wandering 3rd instars of about 4 mm and wandering larvae). Of course, it is impossible to exactly determine the

larval size and thus to collect the larvae of exactly the same physiological age. However, while comparing short time developmental profiles this is no more necessary.

4.1.1. Differences in hemolymph metabolites between *adgf-a* and *wt* larvae

Adenosine: Consistently with previous observation of Dolezal et al. (2005), adenosine hemolymph concentration in *wt* larvae (blue columns in every graph in Fig. S9) never exceeds 0.2 µM. The concentration of adenosine in *adgf-a* larvae (red columns) is increased and vary between 1.8-2.2 µM, depending on the diet and larval age. Figure 13A shows that the rescue effect of high sugar diet is not caused by decreasing of hemolymph adenosine concentration. Thus, it is rather that high sucrose diet suppresses the effect of adenosine on larval physiology.

It even seems that the concentration of adenosine in hemolymph of larvae reared on higher sucrose diet is higher than in those reared on 0s8y. One possible explanation for this is that increased glucose damages tissues by glycosylation of membrane proteins resulting in ATP release from damaged cells. ATP is then dephosphorylated forming extracellular adenosine. The other possible explanation could be: Because of *adgf-a* larvae growth is faster on a sugar rich diet than on 0s8y, their tissue get more easily into metabolic stress. So, despite they have very high hemolymph glucose, their tissues could get into metabolic stress, in consequence of which is released adenosine into hemolymph. As these *adgf-a* larvae do not possess adenosine deaminase, every little release of adenosine is added to its total amount.

The concentration of adenosine decreases in the *adgf-a* wandering larvae. ADGF-A is main larval adenosine deaminase, but in the late third instars, also ADGF-D is expressed (Zurovec et al., 2002). ADGF-D activity could be cause of adenosine decrease in wandering larvae.

Fructose: The main source of carbohydrates in our experimental diets is sucrose, it is a disaccharide of glucose and fructose. Sucrose is split in the gut on its sugar monomers, which are then absorbed to hemolymph (Nation 2002).

Fig. S9A shows that on sugar supplemented diets, fructose is almost doubled in *adgf-a* in comparison to *wt* prewandering larvae. While on pure yeast diet, it is almost undetectable in both *adgf-a* and *wt* larvae. This suggests that hemolymph fructose originates almost exclusively from the dietary sucrose. Thus the elevated hemolymph

fructose could be a good marker of three possible things: **1)** it could indicate that larvae eat more or **2)** that they have more effective absorption of fructose in the gut or **3)** that they have less effective uptake of fructose from hemolymph into target tissues (see Fig. S12).

Glucose: The concentration of hemolymph glucose is doubled in prewandering *adgf-a* larvae reared on sucrose supplemented diets in comparison to *wt*. On pure yeast diet, the *adgf-a* larvae have not increased the concentration of hemolymph glucose as much as on sugar supplemented diet. It is probably because they do not obtain enough glucose from the diet.

Both glucose and fructose decrease during wandering phase (Fig. S9B), thus it seems that at least part of hemolymph glucose originates from gut absorption. Thus we could extent our previous three statements about elevated fructose also for glucose.

Interestingly, the concentration of hemolymph fructose on sucrose supplemented diet is about 5-times higher than the concentration of glucose, although they are in 1:1 ratio in the diet. This could be caused by a slower uptake of fructose from the hemolymph into target tissues in comparison to glucose (Wang and Wang, 1993), because of different expression/regulation of glucose/fructose transporters or slower processing of fructose by tissues.

Inosine: Inosine is product of adenosine degradation by adenosine deaminase. The *adgf-a* larvae have not active adenosine deaminase, in the consequence of which they have increased hemolymph adenosine. Therefore one would expect inosine to be in very low concentration in *adgf-a* larval hemolymph, but the results were surprising in this point. According to our measurement, the concentration of inosine in larval hemolymph is about one order higher than the concentration of adenosine. This could be caused by the fact that inosine is a mid-product of degradation of protein nitrogen into uric acid (inosine is produced from inosine monophosphate in this pathway).

The concentration of inosine is higher in the *adgf-a* larvae in comparison to *wt* and increases significantly on pure yeast diet (it is about 1.5-2 times higher on sucrose supplemented diets and about three times higher on pure yeast diet in *adgf-a* than in *wt*). Catabolism of inosine and excretion of uric acid is energetically demanding process. Increase of inosine in *adgf-a* larvae could be caused by following possibilities: **1**) The *adgf-a* larvae degrades inosine slower than wt, because they have not enough energy. **2**) The *adgf-a* larvae produce much more inosine than *wt*, so their uric acid synthesis pathway is saturated. It is highly probable that both possibilities play role in the increase of inosine in the hemolymph of *adgf-a* larvae.

Fig. S9B shows that the concentration of inosine increases in the *wt*, *adoR;adgf-a* and *C7>A;adgf-a* (see Tab. S1) wandering larvae, but not in *adgf-a* and *Hml>A;adgf-a* wandering larvae. It seems not to be measurement error, because it correlates with increasing of adenosine. It demonstrates in a how complicated way could increased adenosine be interconnected with metabolic pathways.

Trehalose: Trehalose is the main hemolymph carbohydrate in majority of insect species, it is a disaccharide of two glucose units. Trehalose serves as a transporter of glucose and also as a hemolymph and tissue reserves of glucose. It is a non-reducing sugar, so it could be present in hemolymph in high concentration. In my measurement, it is 20-times higher than glucose and 4-times higher than fructose in the prewandering *wt* larvae on all three used diets (Fig. 9A).

The *adgf-a* larvae reared on sucrose supplemented diet have the trehalose concentration similar to *wt* (about 4000 µM). However, the *adgf-a* larvae reared on pure yeast diet have a half amount of trehalose than on sucrose supplemented diet, which could have the same cause as lower increase of hemolymph glucose in these larvae (see glucose section above and Fig. S9A).

Trehalose increases in *wt* wandering larvae by about 2-times (from 4000 to 8000 µM, Fig. S9B). However, increasing of trehalose in *adgf-a* wandering larvae is not such high as in *wt*. There are two possible explanations. **1)** They are not able to increase their hemolymph trehalose to concentration that was measured in *wt*, because their glycogen reserves are limited (as will be demonstrated in the next chapter). **2)** The *adgf-a* larvae were of different physiological age (wandering for shorter/longer time).

4.1.2. Hemolymph metabolites in genetically rescued *adgf-a* larvae

The *adoR* mutation: It was shown that homozygous mutation of *AdoR* almost completely rescues the development of the *adgf-a* larvae on pure yeast diet (Fig. 4).

Fig. S9B shows that the concentration of glucose, fructose and inosine is the same in both *adoR;adgf-a* and *wt*, although the adenosine concentration is increased in *adoR;adgf-a*. This result is very important. It proves that the increase of hemolymph inosine and the imbalances in carbohydrate metabolism are caused by AdoR signaling, not by direct misregulation of energetic metabolism (ATP/AMP ratio) or metabolism of purines, due to elevated adenosine.

I also tested concentration of hemolymph metabolites in *adoR,adgf-a*/*TM3 Ser Act-GFP* (heterozygotes for both *adgf-a* and *adoR*) and in *adoR,adgf-a*/*adoR* (homozygotes for *adoR* and heterozygotes for *adgf-a*). Both of them have the same concentration of all measured metabolites as *wt* (data not shown).

Forced expression of ADGF-A: Expression of *UAS-ADGF-A* (adenosine deaminase) under control of *C7-Gal4 (C7>A)* or *Hml-Gal4 (Hml>A)* driver almost completely rescues *adgf-a* larval development on the 5s8y diet, similarly to *adoR* rescue. Almost all *C7>A*; *adgf-a* pupae and half of *Hml>A*; *adgf-a* pupae eclose (Fig. 6).

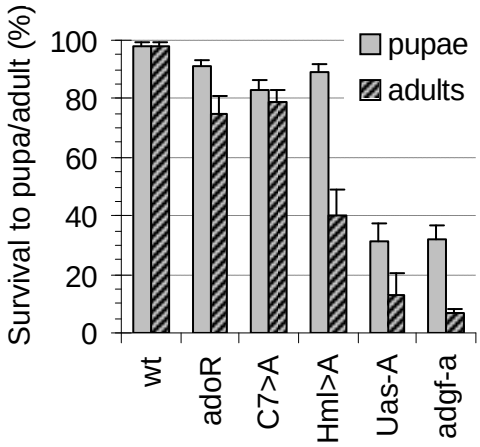

***Figure 6*. Survival of the *wt* and *adgf-a* mutants on 5s8y diet (genetic background 1)**

Graph shows percentage of survival to pupae or to adults of following animals: *wt, adoR*;*adgf-a* (adoR), *C7-Gal4*/*UAS-ADGF-A*;*adgf-a* (C7>A;adgf-a), *Hml-Gal4*/*UAS-ADGF-A*;*adgf-a* (Hml>A;adgf-a), *UAS-ADGF-A*;*adgf-a* (Uas-A) and *adgf-a*.

The expression of *UAS-ADGF-A* in the fat body leads to almost complete clearing of hemolymph adenosine in *adgf-a* mutant (*C7>A*;*adgf-a* in Fig. S9B) what is accompanied by decrease of hemolymph glucose and fructose back to *wt* levels. The effect on inosine concentration is not so profound.

The expression of *UAS-ADGF-A* in hemocytes (*Hml>A*;*adgf-a* in Fig. S9B) causes only slight decreases of adenosine, but it seems to be sufficient for slight decrease of glucose and fructose.

The explanation for different impact of these two drivers on adenosine concentration could be following: The C7 driver is expressed in the fat body. The Hml driver is expressed in hemocytes, which are of much less volume in comparison to fat body, so it is possible that total amount of ADGF-A expressed under control of *Hml-Gal4* driver is lower than that from *C7-Gal4* driver.

Rescue mutations identified in genetic screening: Hemolymph metabolites were measured in following four *adgf-a mutants*: ***en.hypo;adgf-a*** (bearing hypomorphic mutation of engrailed transcription factor en^{spt}; see Tab. S1), ***phKɔ;adgf-a***, ***ahcy13;adgf-a*** (bearing *P*-element insertion in an enzyme that releases adenosine from adenosyl homocysteine) and ***monar;adgf-a***. Results of this measurement are shown in Fig. S10A.

Unfortunately, the evaluation of these data is complicated by following facts: **a)** I did not obtain hemolymph from *adgf-a* larvae for this measurement. However, proportion of the concentrations measured in the *wt* and *adgf-a* larvae should be similar to previous measurement shown in Fig. S9, which could help to evaluate data from Fig. S10. Concentrations of purines are shown in the same unit in both figures, concentrations of carbohydrates are in arbitrary units in Fig. S10. **b)** I obtained only one or two replicates from some larvae (numbers of obtained replicates are given in the graph). However, because of small s.e.m., the number of replicates seems not to be so important. **c)** More serious problem in the evaluation of these data is that I did not measure short time developmental profiles, so I can not be sure whether the *adgf-a mutants* were of the same physiological age as *wt* larvae. It is problematic particularly because of the fact that hemolymph for this measurement (Fig. S10A) was collected from *wt* and *adgf-a mutants* that were 125 hal old and prewandering, while 125 hal old larvae collected for previous measurement (Fig. S9) were already wandering. Thus all larvae collected for this measurement were probably very close to the turning point into wandering phase in which the concentration of every measured metabolite rapidly changes.

The trehalose concentration in the *wt* larvae is about 2-times higher than that in all four *adgf-a mutants* in this measurement (Fig. S10A). Based on the concentration of trehalose in Fig. S9, it seems that the *wt* larvae were already in the turning point into wandering phase, while the *adgf-a mutants* were still in the prewandering phase. The fact that the *wt* larvae in Fig. S10A were almost wandering is also supported by inosine concentration, which is the same as in the wandering *wt* larvae in Fig. S9. Finally, the hemolymph glucose concentration of the *adgf-a* prewandering larvae is 3-times higher than that of the *wt* wandering larvae in Fig. S9, the *monar*;*adgf-a* larvae have the concentration of hemolymph glucose also three times higher than *wt*. The other *adgf-a mutants* in figure 15 seems to have lower concentration of hemolymph glucose than the *adgf-a* larvae, however without having the developmental profile it is only speculation. I show this data mainly because of comparison with *cactus* larvae (Fig. S10B), which were harvested from the same batch of the diet and measured in one jump with the *adgf-a mutants*.

4.1.3. Hemolymph metabolites in *cactus* larvae

Cactus is a *Drosophila* inhibitor of NF-əB in Toll signaling pathway, one of the antimicrobial immune response pathways identified in *Drosophila*. The *cactus null* mutation leads to constitutively activated Toll pathway. Phenotype of the *cactus* null mutant larvae is similar to the *adgf-a* larvae: They have over-proliferated hemocytes in hemolymph, form melanotic tumors, are delayed in development, have 60 % larval lethality and thin body-shape phenotype (Qiu et al., 1998).

Although *cactus* larvae could be rescued by ADGF-A over-expression or *adoR* mutation (similarly as *adgf-a* larvae; Dolezal et al., 2005; Fenckova and Dolezal, 2007), their hemolymph adenosine concentration remains undetectable as in *wt* (Fig. S10B). Concentrations of other measured metabolites are also similar in the *cactus* and *wt* larvae, except for increased glucose in *cactus null*. Increasing of hemolymph glucose by Toll signaling pathway has been already demonstrated by DiAngelo et al. (2009), they have shown that this increase is caused by impaired insulin signaling in the fat body.

The undetectable concentration of adenosine in *cactus* larvae (which have active adenosine deaminase) could indicated that the minimal increase of adenosine can have a big physiological impact (*cactus* mutant phenotype) and that the enormous increase of adenosine in the *adgf-a* larvae is likely to be strongly non-physiological. However, it could also indicated that the carbohydrate metabolism is affected by different mechanism in both *adgf-a* and *cactus* larvae.

The both *cactus null* ($cact^{E8}/cact^{D13}$) and *hypomorphic cactus* ($cact^{E8}/cact^{IIIG}$) have increased trehalose like the wandering *wt* larvae. It is strange, because they surely were not close to turning point into wandering phase like the *wt* larvae in figure 13. They were collected at 100 or 115 hal. Moreover, they are normally delayed after *wt* larvae. It may suggest that the carbohydrate metabolism is not affected in the same way as in the *adgf-a* larvae. The low concentration of hemolymph fructose and the high concentration of trehalose together suggest that *cactus* larvae had stop feeding and are fasting (like in wandering phase). It would mean that increased hemolymph glucose in the *cactus null* larvae originates from degradation of glycogen reserves.

4.2. Developmental profile of energetic reserves

I measured whole larval and pupal carbohydrates, soluble proteins and triglycerides in the *adgf-a* and *wt* animals reared on CM-30 diet. Results are shown in Fig. S11 (red line is for *adgf-a*, blue line is for *wt*). All larvae were harvested in regular intervals, from the same batch of the diet, starting from 3rd instar larvae, finishing in pupae. Samples for all measured metabolites were collected at the same time points. The first sample from *wt* larvae was collected 96 hours after egg laying (hal). The last sample from *wt* pupae was collected 90 hours after pupation (hap). The *adgf-a* larvae grow slower and pupate later than *wt*, therefore the first sample was collected from 112-hal larvae which size was similar to 90-hal *wt* larvae. Most of *adgf-a* pupae stopped their development after 40 hap, therefore the last sample was collected from 40-hap pupae. Resulting developmental profiles were aligned to time of pupation (white prepupa = time 0 on horizontal axis). In this case, the *adgf-a* larvae pupated at around 170 hal and the *wt* larvae pupated at around 140 hap.

Glycogen, trehalose and glucose were measured by the GAGO-20 kit (Sigma). Soluble proteins were measured by Bradford reagent. Samples for measurement of proteins and carbohydrates were prepared by homogenizing 10-15 larvae or pupae in PBS buffer, followed by centrifugation (10 000 G/5 min). Supernatant was divided into two aliquots. An aliquot for protein measurement was immediately frozen at -20°C. An aliquot for carbohydrate measurement was heated to 70°C/70 min before freezing, in order to deactivate carbohydrate consuming enzymes.

For the measurement of triglycerides, 10-15 animals were homogenized in PBS containing 0.1 % tween, sample was centrifuged (10 000 G/5 min) and supernatant was frozen at -20°C. Triglycerides were enzymatically hydrolyzed to fatty acids and glycerol and the concentration of glycerol was measured by Serum Triglyceride Determination kit (TR0100). Samples for the triglyceride measurement were collected at the same time points as for carbohydrates. However at some time points, the samples for triglyceride measurement were not obtained.

Red line illustrates a development of the most progressive *adgf-a* larvae (about 30 % of all animals). The rest of the *adgf-a* larvae progressively lagged behind, few of them pupated later (about 10 %), but the rest of these larvae died before pupation. These larvae, which never pupate, gradually slow down their crawling, their fat body disintegrates and some of them completely melanize. The white circle outside of red line in time 0 on

horizontal axis is from delayed larvae which were still in the diet while the most progressive larvae started to pupate. The concentration of measured metabolites in these delayed larvae is similar to those on the red line at 15 hours before pupation.

Graphs show that the concentration of every measured metabolite in the *wt* larvae increases in feeding larvae and decreases in wandering larvae and pupae. **Soluble proteins** increase also in wandering phase (Fig. S11D). It is interesting, because they could not have been newly synthesized from the food intake. This could be explained by conversion of water insoluble proteins (e.g. bound to cuticle) to water soluble ones. The *adgf-a* larvae have lower concentration of soluble proteins than the *wt* larvae, almost during the whole development. Most probably, it results from higher amino acids catabolism in the *adgf-a* animals. This is supported by the fact that the other metabolites are higher in the *adgf-a* larvae, when expressed per proteins (Fig. S11G-I), except for glycogen per proteins. It is higher only until late third instars (around 25 hours before pupation; Fig. S11A), because later the accumulation of glycogen in the *adgf-a* larvae is blocked and it never reaches the peak, which is present in the *wt* larvae. I did not measure dry weight of the sampled animals, so I could not exactly compare the mass of the measured metabolites in the *adgf-a* and *wt* animals.

Feeding *adgf-a* larvae did not accumulate as much **glycogen** as *wt*, which had 3-times more glycogen in the beginning of wandering period (30 versus 12 µg; Fig. S11A). However, the *wt* larvae consumed much more glycogen during wandering phase than the *adgf-a* larvae and both the *wt* and *adgf-a* larvae pupated with the same amount of glycogen (about 8 µg per prepupa). The *wt* pupae slow down glycogen degradation shortly after pupation, while the *adgf-a* pupae continue in consumption of glycogen reserves and at 15 hours after pupation they have almost undetectable amount of glycogen reserves. The rapid decrease of glycogen in the *adgf-a* pupae is accompanied by increased **trehalose** (Fig. S11B). The *adgf-a* pupae die shortly after depletion of both glycogen and trehalose reserves.

The concentration of **free glucose** (Fig. S11C) is insignificant in comparison to concentration of glycogen and trehalose (1.5 versus 30 and 20 µg/animal in prewandering peak). Glucose per protein is higher in the *adgf-a* larvae than in *wt* (like glycogen or trehalose), but not in pupae (not shown).

The course of **triglyceride reserves (**Fig. S11E and S11I) is similar to trehalose and glycogen in the both *adgf-a* and *wt* animals. Similarly to glycogen, the *adgf-a* pupae consume their triglyceride stores more quickly than *wt*. Similarly to trehalose, the *adgf-a*

animals have more triglycerides per µg of protein than *wt*. Amount of triglycerides was measured as an amount of glycerol that is incorporated in triglycerides. It says nothing about the length of fatty acids, which is decisive for total energy stored in one molecule of triglyceride. So, the length of fatty acids could shorten during starvation/wandering period, although the total amount of triglyceride molecules remains about the same.

The concentration of **free glycerol** (Fig. S11F) in larvae is relatively high in comparison with glycerol that is incorporated in triglycerides (free glycerol presents about ¼ of that in triglycerides) and it decreases during wandering phase and 14-hours starvation (white and black squares in Fig. S11).

Interestingly, the total amount of glucose and trehalose in hemolymph seems to be negligible in comparison to that stored inside cells: The concentration of hemolymh glucose in the *wt* wandering larva is about **0.02 µg per µl** (~100µM) and the concentration of trehalose is about **2 µg per µl** (~8000µM). While the total amount of glucose per larva is about **1.5 µg per larva** and the total amount of trehalose is **20 µg per larva.** In the wandering larva, there could be around 0.5-1 ul of hemolymph. (I am not sure, whether only glucose or also glucose-(1)6-phosphate are measured by the GAGO kit. By the mass spectrometry, only glucose was measured.) For the comparison: the live weight of wandering larva is about 1 mg, summation of trehalose glycogen and soluble proteins measured in the *wt* wandering larvae is about 200 µg.

4.3. Higher dietary sucrose could help to build up glycogen reserves

I repeated the measurement of pupal glycogen in animals reared on diets with different concentration of sucrose (0s8y, 5s8y and 10s8y). We assumed that larvae reared on high-sugar diet accumulate more glycogen reserves than those on low sugar diet, which helps them to survive the pupal period. For this measurement (and also for experiments described in following two chapters), I used newly prepared *adgf-a/TM3 Ser Act-GFP* stocks, in which the *adgf-a* mutation had been put into new genetic background (background 2), which differs from original background 1 by suppressing lethality of the *adgf-a* larvae. I did not expect it when starting this experiment.

Fig. 7 shows results from my previous measurement of glycogen reserves on CM diet, together with the new measurement on yeast diets: The *adgf-a* pupae of genetic background 1 (red line) consumed their glycogen reserves more quickly in comparison with *wt* (blue line) and stopped their development when they consumed all their glycogen

and trehalose reserves. However, all *adgf-a* pupae of genetic background 2 (separate spots) successfully finished their development and did not deplete their glycogen reserves. This is surely caused by the particular genetic background, not by the absence of corn-meal in yeast diets. Nevertheless, presented data (although very preliminary) suggest that **1**) *adgf-a* larvae reared on the diet with higher concentration of sucrose pupate with higher glycogen content than that reared on the diet with lower sucrose concentration and that **2**) *adgf-a* larvae pupate with lower glycogen content than *wt* in the respective yeast diet.

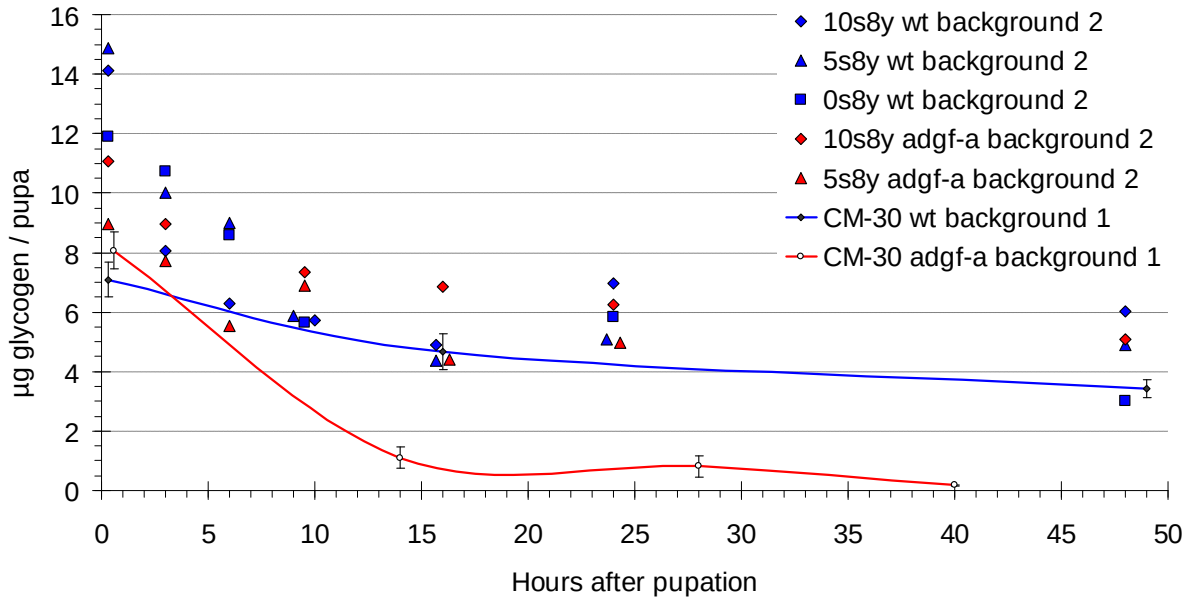

***Figure 7*. Glycogen consumption in the *wt* and *adgf-a* pupae of two different genetic backgrounds, harvested from yeast diets (0s8y, 5s8y and 10s8y; background 2) and from CM-30 diet (background 1)**

All *wt* pupae of both backgrounds and all *adgf-a* pupae of genetic background 2 successfully finished their development. All *adgf-a* pupae of genetic background 1 died at around 40 hours after pupa formation. Each spot present pooled sample of 10-15 pupae, each spot on CM are average of three pooled samples of 10-15 pupae.

The course of glycogen consumption in the both *adgf-a* and *wt* pupae of genetic background 2 is similar to the *wt* pupae of genetic background 1: It is relatively quick in the first 10 hours after pupa formation, then progressively slows down and almost stops, leveling off similar amounts in both backgrounds. The amount of glycogen per pupa of background 2 scatters between neighboring time points, it is higher in some time points than in foregoing ones. The only reasonable explanation of this is that harvested pupae varied in size. This could also explain, why the *wt* prepupae have less glycogen on the

10s8y diet than on 5s8y. It would have been better to express the measured concentration of glycogen per µg of proteins than per animal, however we did not continue with this or other experiments, because of the unexpected improving of survival by the genetic background 2.

4.4. Consumption of reserves in prewandering starved larvae

Our screening suggested that slowing down of glycogen utilization helps the *adgf-a* larvae to pupate and therefore we supposed that the *adgf-a* larvae might consume its stores more quickly during wandering / fasting period than *wt*. To test this assumption, larvae collected approximately 35 hours before pupation were divided in two groups. The first group was immediately sampled (the point from where the dotted arrows start in Fig. S11). The second group was sampled after 14-hours starvation (white square for *adgf-a*, black square for *wt*). Larvae were starved on filter paper soaked with PBS.

The 3rd instars prewandering *adgf-a* larvae finished 14-hours starvation with the same amount of glycogen as *wt* larvae, although they started to starve with lower glycogen content than *wt* (Fig. 16A). It could suggests that **1**) the *adgf-a* larvae consume their glycogen reserves slower than *wt* during starvation or that **2**) there is a critical mass of glycogen reserves, which is not touched until there are other reserves available. The important fact for evaluating this can be that most of the *adgf-a* larvae die shortly after 14 hours of starvation, while most of the starved *wt* larvae finally prematurely pupated. This indicates that the *wt* starved larvae were in particular developmental phase (they have reached critical weight for pupation; Mirth and Riddiford, 2007). The *adgf-a* larvae probably also have reached the critical weight before the beginning of the starvation, but they have probably impaired ecdysone secretion (Dolezal et al., 2005). It could result from impaired nutrient sensing due to impaired insulin signaling in the *adgf-a* larvae (Mirth and Riddiford, 2007). Soluble proteins increase in the starved *wt* larvae in the same manner as in the feeding *wt* larvae (Fig. S11D). However, in the starved *adgf-a* larvae it did not increase as much as in the feeding *adgf-a* larvae. It further supports our conclusion from hemolymph metabolites measurement that the *adgf-a* larvae are forced to process more proteins than *wt*, because they have impaired glucose uptake. Slower glucose uptake could result to slower glycogen degradation by regulatory mechanisms shown in Fig. 3.

To decide, whether the glycogen degradation is faster or slower in the starved *adgf-a* larvae than in *wt*, I measured consumption of glycogen reserves during starvation in shorter

intervals. I collected larvae of different age (92, 100, 108, 116 hal) to made a short-time developmental profile that would allow to compare potential differences more precisely. Collected larvae were sampled after 2, 4 and 8-hours starvation. I did not found significant differences in glycogen, trehalose or protein consumption between the *adgf*-a and *wt* larvae (data not shown), but this could be specific for the particular genetic background used.

The degradation of glycogen is significantly slower in starved *phK*;*adgf-a* larvae than in starved *wt* or *adgf-a* larvae of all studied ages (Fig. 8 shows an results from 108 hal old larvae). It suggests that the *phK* rescue could be mediated by reducing of the speed of glycogen degradation or rather by support of its synthesis.

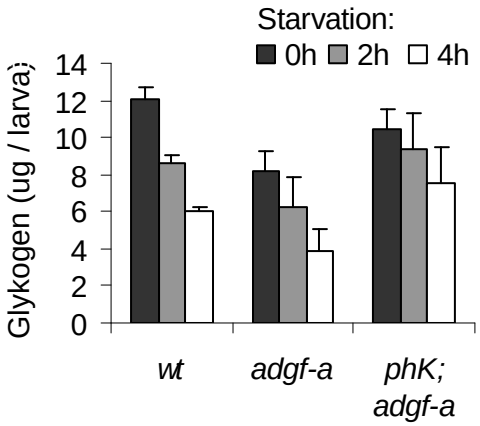

***Figure 8*. Rate of glycogen reserves consumption during starvation (CM-30 diet, background 2)**

The 108 hal old *wt*, *adgf-a* and *phK*;*adgf-a* larvae were subdued to 0, 2 and 4-hours starvation. The *phK*;*adgf-a* larvae show significantly slower glycogen breakdown than *wt* larvae (multiple comparison among regression $P<0.05$, $t=1.89$, $Df=3$), whereas the *adgf-a* and *wt* larvae do not differ.

4.5. Processing of glucose depends on dietary glucose/yeast ratio

We measured the amount of exhaled ^{14}C-labeled CO_2 by *wt* and *adgf-a* animals reared on the yeast diet supplemented with 5 and 10 % glucose with ^{14}C-label. Fig. 9 shows that one animal reared on the diet supplemented with 10 % glucose exhales during the first 200 hours of its life in total 16 µg of CO_2, while animal reared on the diet supplemented with 5 % glucose exhales much less (between 5-8 µg of CO_2 per animal). This indicates that animals reared on the 5 % glucose cover relatively more of their energetic demands from other sources (dietary proteins or fats) than from the dietary glucose.

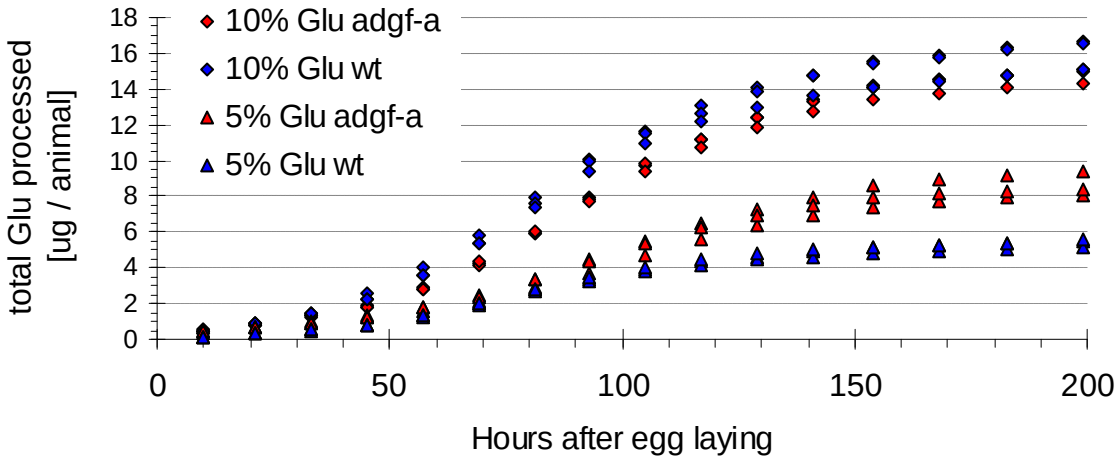

Figure 9. **Dietary glucose consumption in µg per animal, in *wt* and *adgf-a* animals, reared on 5 and 10 % glucose (background 2)**

Larvae (30 per vial) were reared on yeast diet supplemented with 5 % or 10 % ^{14}C labeled glucose. Exhausted CO_2 was trapped in 1M NaOH and measured in 12-hours intervals by liquid scintilation counter. The measured amount of $^{14}CO_2$ was divided by number of pupae in the vial. Results from each vial are shown independently (each spot is for one vial), triplicates were used for each of four combinations of the diet and genotype (blue for *wt*, red for *adgf-a*, diamond for 10 % and triangle for 5 % dietary glucose). Horizontal axis is in hours after embryo laying. Vertical axis shows cumulative amount of processed glucose per animal during the whole development after hatching. The *wt* larvae pupated at around 130-140 hal and the most progressive *adgf-a* larvae at around 140-160 hal.

It seems that *adgf-a* larvae on 5 % glucose process more dietary glucose than *wt* between 100 and 150 hal. However, it is probably caused by the fact that there might had been more living (breathing) animals in the vial than I thought. The amount of alive/dead animals was evaluated by their locommotor activity or by continuing of pupal development. I hope that there were not breathing microorganisms, because diet was supplemented with Methylparaben (Nipagin; 1.5 %) and Penicilin-Streptomycin (100 µl of solution containing 10 000 units/ml of penicillin and 10 mg/ml streptomycin was put on the surface of the diet together with first instar larvae). Moreover, the course of exhaled CO_2 increases as larvae grow and decreases after pupa formation. It is in consistency with the results of glycogen and triglyceride measurements, which consumption is high during wandering period and slows down shortly after pupa formation. In this case *wt* larvae pupated between 130-140 hal and the most progressive *adgf-a* larvae between 140-160 hal. About 95 % *adgf-a* larvae pupated on 10 % glucose and about 50 % on 5 % glucose.

These data suggest that larvae reared on 5 % glucose probably do not eat more in order to obtain the same amount of glucose as larvae reared on 10 % glucose. They process all dietary nutrients unselectively as they come. This supports the possibility, that excessive load of nitrogen excretion pathway (uric acid synthesis) may interfere with impaired adenosine deamination and that the *adgf-a* larvae die in the time when they accumulate too much of these waste products from yeastós proteins or purines degradation. This time comes earlier when *adgf-a* larvae are reared on a diet with low sugar / high yeasts ratio.

Starvation in humans is accompanied with decreased uric acid excretion and its increased concentration in plasma (Lloyd et al., 1970), probably due to increase catabolism of proteins for a gluconeogenesis. I proposed that carbohydrate starvation of cells in *adgf-a* larvae leads to the same effect. In order to verify these speculations, it would be necessary to measure the concentration of uric acid.

Measuring of the total amount of exhausted CO_2, not only of that originating from glucose, would show whether *adgf-a* animals have higher energetic demands or if they have only the problem to build up their reserves.

5. Summary and conclusions

Five potential suppressors of the *adgf-a* phenotype have been identified in genetic screening (the first three listed connect e-Ado to energetic metabolism): **1**) Glycogen phosphorylase and **2**) phosphorylase kinase (PhK) regulate glycogen degradation. I have shown that rescue mutation of PhK worsens glycogen degradation. Activity of **3**) citrate synthase could regulate amount of metabolites processed in the citric acid cycle and thus consumption of energy. **4**) Invected & Engrailed are transcription factors with similar function (Gustavson et al., 1996). **5**) Gene *CG14478* (*Monar*) seems to be a novel nuclear receptor (judged by a presence of both DNA binding and ligand binding domain) and I have shown that it may be localized in both nucleus and cytoplasm (data not shown in this thesis). However, the rescue effect of *monar* mutation seems to be caused by particular genetic background used.

The results of genetic screening suggests that increased extracellular adenosine (e-Ado) in larvae and pupae affects mainly energetic metabolism. On the other hand, other interesting processes affected by extracellular adenosine may have been missed by the screening, because the complications with genetic background pushed us to abandon the genetic screening without finishing it in full detail. The strong effect of genetic background on phenotype of *adgf-a mutants* is probably a result of the impaired energetic metabolism, which is strongly influenced by interactions of many genes. It could also be the cause of strong dependence of *adgf-a mutants* on the quality of the diet. The confirmation of identified suppressors of *adgf-a* mutant phenotype (e.g. by RNAi or other *P*-element insertions) loses the sense, until we are able to decipher the influence of different experimental conditions on this phenotype. The strong and opposite influence of particular genetic backgrounds on survival of *adgf-a mutants* could be exploited for identification of genes that participate on the manifestation of the *adgf-a* phenotype, by comparing the gene expression in genetic backgrounds that improve survival with that which worsen it.

The survival of the *adgf-a mutants* depends on concentration of dietary sugars and on duration of thermal preparation of the diet, regardless of the genetic background. Increasing of dietary sugars suppresses the lethality of *adfg-a mutants* and increases both

pupation and the number of pupae that successfully finish their pupal development. Decreasing of dietary sugars or extension of yeast thermal hydrolysis worsens survival of the *adgf-a* mutant, while it has no effect on *wt*. The survival dependency on duration of thermal hydrolysis suggests that products of this hydrolysis interfere with amino acids or purine metabolism. The survival dependency on dietary sugar concentration strongly supports the results of the genetic screening, pointing mainly to effects on energetic metabolism.

Metabolism of energetic reserves is impaired in *adgf-a mutants* and strongly depends on particular developmental stage. The *wt* larvae rapidly consume their triglyceride and glycogen reserves in the wandering phase, but minimize their consumption after pupation. The *adgf-a* larvae are not able to accumulate as much glycogen and triglyceride reserves as *wt*, but they seem to save most of their glycogen and triglyceride reserves during the wandering phase, which allows them to pupate with the same level of these reserves as *wt*. However, the *adgf-a* pupae rapidly increase consumption of their reserves and die shortly after depletion of carbohydrate reserves.

Dietary sugars accumulate in hemolymph of *adgf-a* larvae (it was glucose and fructose in our experimental conditions). Based on following three reasons, I concluded that free glucose/fructose in larval hemolymph originates most probably from the diet, not from glycogen stores degradation: **A**) Larvae reared on pure yeast diet have undetectable concentration of hemolymph fructose and the concentration of their hemolymph glucose not increased as much as on the sucrose supplemented diets. **B**) Both *adgf-a* and *wt* wandering larvae have lower concentration of hemolymph glucose/fructose than their feeding siblings. Thus the elevation of glucose/fructose in the *adgf-a* larvae could indicate that they: **1**) consume more food, which allows them to absorb more glucose/fructose, **2**) have more effective absorption of glucose/fructose in the gut or **3**) have less effective uptake of hemolymph glucose/fructose (absorbed into hemolymph from the gut) into target tissues. **4**) Combination of all of these three possibilities.

Ad 1) Feeding larvae are robots for eating, *wt* larvae probably eat as much as possible, thus it seems impossible that *adgf-a mutants* could increase their eating.

Ad 2) It would be possible, but does not seem probable, that *wt* larvae absorb only a part of dietary sugars and thus that the *adgf-a* larvae could make the absorption of sugars from the gut more effective.

Ad 3) **Considering all my data, the most likely cause of the increased hemolymph glucose/fructose is a blocking of their uptake by target tissues.** This could be caused by insulin resistance of target tissues or by lower insulin production. The situation in mammals is complicated by the presence of more adenosine receptor subtypes. Ismail et al. (1976) show that adenosine blocks insulin production in isolated rat islets of Langerhans, which is mediated by A1 receptor. The other works show that e-Ado mediates insulin resistance and that this effect is mediated by A2B receptor (e.g. Figler et al., 2011). The only *Drosophila* adenosine receptor ï AdoR ï is more similar to A2B receptor, therefore it seems more likely that the worsed glucose uptake caused by AdoR signaling is caused by decreased sensitivity to insulin-like proteins in feeding *Drosophila* larvae.

The presumption that AdoR mediated response in feeding larvae is identical to that mediated by A2B, is further supported by the finding that A1 signaling facilitates glucose transport in mammals (**Green** 1987). The facilitation of glucose transport in *Drosophila* would result in decrease rather than increase of hemolymph glucose in feeding larvae. Although, it is possible that the response to AdoR signaling differs by tissue to tissue and that in some tissues it may be more similar to action of A1 receptor, while in others to the action of A2 receptor.

At least some of the *adgf-a* larval tissues are fasting for glucose, due to blockage of its uptake and are forced to process more amino acids in gluconeogenesis pathway. It probably leads to overloading of the uric acid synthesis pathway and to accumulation of inosine and other intermediary product of this pathway (e.g. hypoxantin), which could interfere with increased adenosine in *adgf-a* larvae and further worsen their survival.

High sugar diet probably facilitates passive (insulin independent) transport of glucose into cells (because of higher concentration gradient in system gut-hemolymph-target tissue).On the low sugar diet, the transport of glucose into cells is more difficult and it pushes larvae to process more amino acids. It leads to **a)** accumulation of more intermediary products of uric acid synthesis, **b)** slower growth and **c)** glucose tissue starvation, regardless of increased gluconeogenesis.

The glucose tissue starvation in wandering larvae leads probably to autophagy, which we could observe on disintegration of the fat body. High sugar diet allows to build up more glycogen reserves, which allow to wandering larvae survive without autophaging themselves. It also allows to produce more trehalose in the fat body, which may be accessible for all the other tissues independently of insulin-like proteins.

Speculative role of AdoR-mediated signaling in increasing of metabolic rate: Based on deletion screening, we speculated that feeding *adgf-a* larvae are not able to build up their glycogen reserves, because they consume more energy than *wt* to keep processes which are directed by the AdoR signaling. We proposed that these processes prepare cells to stress situations and that responses include mobilization of hemocytes. We further speculated that for this reason, non-feeding *adgf-a* animals (wandering/fasting larvae and pupae) consume their glycogen reserves faster than *wt*. Therefore we measured glucose turnover and speed of glycogen degradation in starved prewandering larvae and we did not find significant differences between *adgf-a* and *wt* animals (however these results could be influenced by particular genetic background of tested animals, which may reduces the effect AdoR signaling). However, these results do not refuse, that the AdoR signaling does not increase the metabolism and energy consumption of some tissues. They only say, that consumption of glycogen is not increased in the *adgf-a* larvae. It is possible that *adgf-a* larvae have higher energetic demands and that they are forced to cover them from other sources, which could further contribute to overloading of uric acid synthesis pathway. It is also possible that AdoR signaling mediates opposite effect in larvae (blocking glycogen synthesis) than in pupae (increasing degradation). Increased degradation of glycogen and following consumption of all carbohydrate reserves in the *adgf-a* pupae supports our assumption that AdoR signaling leads to increased energy consumption. Pupae have to store excreted uric acid inside the pupal case, thus it is possible that for this reason is the processing of amino acids blocked and glycogen and fats reserve consumption are favored in pupae. It is also possible that feeding/wandering larvae have somehow blocked glycogen consumption when it falls to a minimal amount that allows survival of metamorphosis in normal conditions (in *wt* larvae) and that both these mechanisms are independent on AdoR signaling.

My results suggest that increase in dietary sugar may supports glycogen accumulation, it could be caused by increase in insulin production (Ismail et al, 1976) or by facilitation of insulin independent glucose transport. Already 10 hours after pupation, all pupae decrease

their glycogen reserves to similar level, regardless how much of glycogen they had in time of pupa formation. Therefore, it seems that the amount of glycogen in the beginning of metamorphosis influences the rate of its degradation in *wt* pupae. It further supports the assumption that pupae favor glycogen consumption.

Speculative role of AdoR signaling in regulation of glycogen metabolism: Degradation of glycogen in *Drosophila* is promoted by AKH (Isabel et al., 2005). AdoR is expressed in ring gland, the organ which produces AKH (Dolezelova et al., 2007). Therefore it seemed highly possible, that AdoR signaling stimulates AKH production. However, ablation of AKH producing cells does not rescue *adgf-a* mutant phenotype (Michaela Fenckovaôs contribution to Zuberova et al., 2010). It shows that the increasing of hemolymph glucose and the blocking of glycogen synthesis is not mediated by AKH. AdoR signaling increases of intracellular cAMP and Ca^{2+}, both of them stimulate glycogen degradation and block its synthesis. Therefore, we propose that the AdoR signaling could directly regulate glycogen metabolism in fat body, independently on AKH (see Fig. 3 and S12. It is further supported by the fact that AdoR is expressed also in the fat body (Michaela Fenckovaôs contribution to Zuberova et al., 2010) and by the fact that the rescue mutation in phosphorylase kinase slows down the efficiency of glycogen degradation and thus it probably facilitates its synthesis.

Interaction of adenosine signaling and Toll pathway in regulation of glucose homeostasis: Chronic infection is accompanied by increased hemolymph glucose and wasting in both Drosophila and mammals (Dionne et al.,2006; Lazzaro and Galac, 2006). This is similar to effects of increased extracellular adenosine or constitutively activated Toll-immune response pathway. The participation of AdoR signaling on the lethal phenotype of larvae with constitutively activated Toll signaling pathway has also been demonstrated (Fenckova and Dolezal, 2007). Strong expression of ADGF-A protein in melanotic capsules (Novakova et al., 2011) suggests a protective role of ADGF-A in condition of activated immune response. Based on this, we speculated that adenosine signaling is downstream of Toll signaling pathway and that it plays important role in regulation of immune response by increasing of hemolymph glucose.

Therefore, the measurements of metabolites in *cactus null* mutant are particularly interesting: We have found that e-Ado is undetectable, at least in the tested stage, while the hemolymph glucose concentration is increased. It suggest that **1**) the enormous increase of adenosine in the *adgf-a* mutants is not necessary for releasing AdoR signaling and that

small or local increasing (undetectable by our HPLC protocol) is sufficient for manifestation of *cactus null* phenotype. However, it could also suggest that **2**) increased glucose concentration is not mediated via AdoR signaling in *cactus* mutant.

Ad 1) I propose that adenosine may be released from fat body, when the Toll pathway is activated, and that it mediates insulin resistance of fat body cells. This proposition is in consistency with DiAngelo et al. (2009).

Ad 2) Other possible interaction of AdoR and Toll signaling pathway is schematized in Fig. 10. It suppose that the proliferation and differentiation of hemocytes in *cactus* mutant is a direct effect of Toll signaling in the lymph gland while the similar phenotype in the *adgf-a* mutant is most likely a secondary reaction to fat body disintegration. When the fat body disintegration is suppressed in the *adgf-a* mutant by high-sucrose diet, hematopoietic defects are not present (Zuberova et al., 2010). The *cactus* mutants are not rescued by high dietary sucrose (Fenckova unpublished). Thus the increased glucose in the *cactus* and *adgf-a* mutants may be stimulated by different mechanisms and this may be supported by my results of hemolymph trehalose measurement.

Both models are highly speculative and need further investigation.

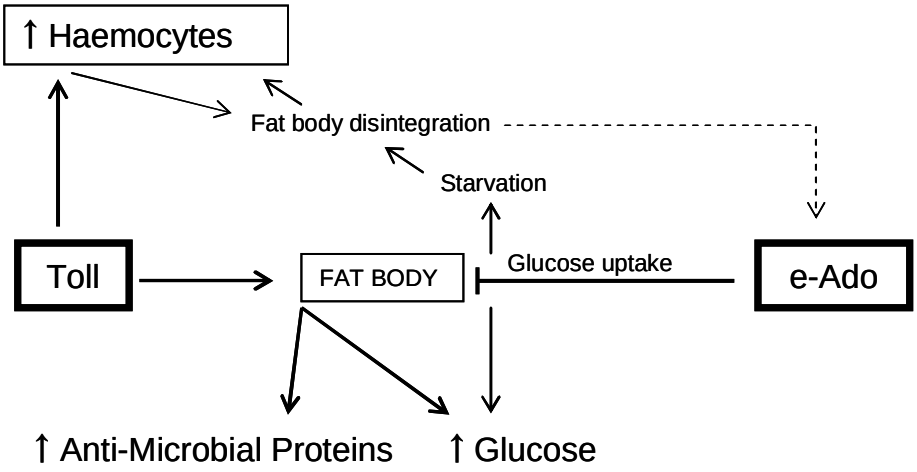

Figure 10. **The possible interaction of Toll pathway and adenosine signaling pathway**

Toll pathway trigger two responses in fat body production of antimicrobial peptides and release of reserves (DiAngelo et al., 2009), and it also trigger the proliferation of hemocytes. Heamocytes are main regulator of extracellular adenosine, which mediates similar effect on hemolymph glucose (probably by blocking its uptake). When adenosine is not regulated (like in the *adgf-a* mutant), it probably leads to glucose starvation of some tissues. Fat body disintegration results from autophagy of starved cells. It activates phagocytosis and encapsulation of fat body cells in both *cactus* null and *adgf-a* mutant. During this inflammatory like response is released adenosine, what may multiply this circle.

6. The main impact of this work

1) **This work shows that e-Ado is an important stress hormone with anti-insulin effects in *Drosophila*, which role is remarkably similar to that in mammalian systems.**

2) **This work shows that *Drosophila* can be used as a model to study energy reallocation in stress.**

   However, all complications that I came across show that *adgf-a* mutant is not as an easy model to study adenosine signaling as we have thought (because the effect of activated AdoR signaling may depends on ongoing developmental program). Therefore, it would be better to transfer these studies into adults in the future. However, three different ADGFs are expressed in adults (Zurovec et al., 2002), which are supposed to have adenosine deaminase activity, which fact could pose another complication.

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8. Supplements

***Table S1*. Abbreviations used when reffering to different genotypes**

wt	= *adgf-a*$^{gerda(karel)}$/*TM3 Act GFP Ser*	
adgf-a	= *adgf-a*^{karel}/*adgf-a*^{gerda}	
***adgf-a* mutants -** animals which are homozygous for *adgf-a* mutation and which can but do not have to bear other mutation (*deletion*, *P*-element insertion, *adoR* é)		
phK	*P{EP}PhKgamma*^{EP779}	insertion of transposable element into coding region of ɔ subunit of phosphorylase kinase
monar	*P{RS3}CG14478*$^{CB-6372-3}$	insertion of transposable element into coding region of gene *CG14478* (mentioned in this work as *Monar*)
adoR	mutation of adenosine receptor AdoR	
C7>A	*C7-Gal4*/*UAS-ADGF-A*	forced expression of adenosine deaminase in fat body
Hml>A	*Hml-Gal4*/*UAS-ADGF-A*	forced expression of adenosine deaminase in hemocytes
ahcy	***P{EP}Ahcy13***^{EP1007}	insertion of transposable element into coding region of adenosyl homocysteinase
en.hypo	*en*^{spt}	hypomorphic mutation of engrailed transcription factor
cactus null	*cact*^{E8}/*cact*^{D13}	loss of function mutation of Cactus
cactus hypo	*cact*^{E8}/*cact*^{IIIG}	hypomorphic function mutation of Cactus

Table S2. **Abbreviations used when referred to different diets and their recipes**

CM-60 = corn meal diet, yeasts heated for 60 minutes at 90°C

CM-30 = corn meal diet, yeasts heated for 30 minutes at 90°C

0s8y = no sucrose, 8 % yeasts

2s8y = 2 % sucrose, 8 % yeasts

5s8y = 5 % sucrose, 8 % yeasts

10s8y = 10 % sucrose, 8 % yeasts

5s4y = 5 % sucrose, 4 % yeasts

Preparation of cornmeal diet:

- 8 % corn meal is heated in the water at 90°C for 2 hours
- 5 % sucrose, 4 % lyophilised yeast and 1 % agar are added for the last hour (CM-60) or for last thirty minutes (CM-30) of heating

Preparation of yeasts diet:

- yeast, sugar and agar (1.2-1.6 %) of desired concentration were heated at 90°C for desired time

Table S3. **Factors influencing the survival of *adgf-a mutants***

Common protocol: Flies of desired genotype were put into fly chamber and let to lay eggs on juice plate. Eggs were collected before hatching and washed in 96 % ethanol, then in water and placed on a new plate with desired diet. The genotype of hatched was distinquished based on GFP expression and first instar larvae of desired genotype were put by 20-30 into vial.

Sucrose or glucose concentration: When I started with these experiments, I used 0s8y or juice plate for collecting the first instar larvae into all types of diets, and I found that the survival of *adgf-a* mutants development was strongly influenced by prolonged staying on the plate (in range from one to six hours after first instar hatching!), before I transferred them into vials: Prolonged staying on a high sucrose plate improves surviving on low sucrose diet and vice versa prolonged staying on low sucrose plate worsen surviving on high sucrose diet. Therefore, I started to collect them from the plate with the same diet as was final diet in the vial.

Time of thermal preparation, which was tested only on the time of the yeasts hydrolysis, but probably also quality of corn meal hydrolysis play a role (here I overlook the fact that every package of corn meal probably differs). Prolonged heating worsen the *adgf-a* phenotype.

Bacterial contamination, which I overcame by embryo washing in ethanol before larval hatching. Later I also started to add penicillin-streptomycin on the surface of the diet. I apply 100ul of the solution containing: 10000 units of penicillin/ml & 10 mg streptomycin/ml, per vial few hours before first instars were placed into this vial.
Washing in ethanol had significantly positive effect on mutant viability. Antibiotics have no additional positive effect.

Density of the diet, that is most influenced by the concentration of agar. I established that the 1.2-1.6 % concentration of agar is the best in 8 % yeast diets. In too runny diet, larvae easily deep into and probably suffer from anoxia. Sometimes they survived worst, sometimes better on the 1 % diet, and it probably depended on how much they eat away the diet. The 2 % agar made the diet too dense and the survival get worsen. When I started my experiments, I used 4 % plate for collecting the first instar larvae, because from more dense plate is the collection easier, and I found that their development was negatively influenced by prolonged staying on this dense diet (in range from one to six hours after first instar hatching!), before I transferred them into vials.

Age of the diet, probably because it is getting much dense and some compounds degrade. For normal fly husbandry even six week old diet is good. However, survival of *adgf-a* larvae is worst on one week old diet than on freshly prepared. Therefore, I used for all experiments maximally three days old diets. But usually, I used one day old diet ï neither younger, nor older.

Other factors: The survival of the *adgf-a mutants* in our experimental conditions differed between every experiment even when one tries to keep all above mentioned conditions constant. Therefore, I made effort to characterize which other factors could influence viability of the *adgf-a mutants*: Sterilization of embryos in 100 % ethanol takes usually 30 seconds, however extending this procedure up to 2 minutes has no effect on larval viability. Collecting of thirty first instar larvae into vial takes usualy one or two minutes. During this period, they are exposed to GFP excitation light (wave lenght ~ 400 nm) in order to distinguish GFP-positive and negative larvae (see figure 2). However, the exposition to GFP excitation light for eight minutes has not effect on survival of the *adgf-a* larvae. Remaining most probable condition that could influence survival of the *adgf-a mutants* and that can vary between every experiment are: oscilation of surrounding temperature, humidity or hypoxic conditions in the vial.

Table S4. **Summarized results of genetic screening**

(next page)

A) The **ñLow resolutionò screening** was performed by my supervisor Tomas Dolezal and my predecessor Lucie Janeckova in laboratory of Peter Bryant (Irvine) in the year 2005 and resulted in identification of 11 deletions that rescued *adgf-a* mutant phenotype.

B) I started the **ñDetailedòscreening** in the year 2006 and I have identified two *P*- element insertions: *P{EP}PhKgamma*^{EP779} (***phKɔ***) and *P{RS3}CG14478*$^{CB\text{-}6372\text{-}3}$ (*monar*). Later, I have employed support of three bachelor students: Jana Kadlecová (2007-2008; generation of *Df(1)6830*) and screening of *Df(1)Exel6239* region), Lucie Jonátová (2009; screening of *Df(2L)E110*) and David Hartmann (2010-2011; detailed screening of *Df(2L)dp-79b, Df(2R)Px2* and *Df(2R)ES1*). The results of detailed screenig are divided into two columns: **Genetic background 1** lists rescue mutation which were identified before unification of the genetic background. G**enetic background 2** lists tested mutations which were placed into uniffied backround (none of these mutation show rescue effect after this procedure). References to pictures relevant to particular region are in last column.

Tab. S4A **Low resolution screening** regions identiffied by Bloomington deficiency kit		**Tab S4B** **Detailed screening** by deletions an P-element insertions		
Deficiency (Bloom. Stock No.) Deleted segments (**genes ***) Sequence coordinates		Genetic **background 1**	Genetic **background 2**	
Chromosome X	**Df(1)Exel6239** (BL7713) 5F2;6B2 **(~ 25**) X:6,238,366..6,410,985-6,432,046	restricted to 3 genes (*CG3847*, *CG3446*, *kdn*) by overlap with *Df(1)ED8630* Hot candidate: *kdn*=**citrate synthase**	*P{RS5}kdn*$^{5\text{-}SZ\text{-}3667}$	Fig. S1.
	Df(1)Exel9050 (BL7759) 10D5;10D6 (**2-4**) X:11,589,014..11,600,755	***P{EP}PhKgamma***^{EP779} in gamma subunit of phosphorylase kinase	*P{EP}PhKgamma*^{EP779} *P{RS5}PhKgamma*$^{5\text{-}SZ\text{-}3097}$ *PBac{RB}FucT6*^{e02394}	Fig. S2. Fig. S6 Fig. S8
	Df(1)ED7294 (BL8035) 13B1;13C3 (**25-30**) X:15,069,448..15,344,331		*P{EP}Ahcy13*^{EP1007} in Adenosylhomocysteinase	
	Df(1)Exel6255 (BL7723) 20A1;20C1 (**22**) X:21,390,230..21,919,501	Not tested		
Chromosome II	**Df(2L)dp-79b** (BL3133**)** 22A2-3;22D5-E1 (**many**)	restricted to *Df(2L)Exel6006* ** Candidate: GlyP = **glycogen phosphorilase**		Fig. S3.
	Df(2L)E110 (BL490) 25F3-26A1;26D3-11 (**many**)	No identified supressor		
	Df(2L)C' (BL4959) h35;40A1 (**many**)	Not tested		
	Df(2R)en30 (BL1145**)** 48A3;48C8 (**31**)	***P{w[+mW.hs]=en2.4-GAL4}e16E*** in invected hypomorphic mutation of engrailed ***en***^{spt}	*P{w[+mW.hs]=en2.4-GAL4}e16E* *en*^{spt}	Fig. S4.
	Df(2R)robl-c (BL5680) 54B17-C4;54C1-4 (**17**)	Identiffied supressor: ***P{RS3}CG14478***$^{CB\text{-}6372\text{-}3}$ Candidates: ectonucleotidases (CG4827, CG30104)	*P{RS3}CG14478*$^{CB\text{-}6372\text{-}3}$ *P{EPgy2}veil*^{EY02176} *P{XP}CG30104*^{d00824}	Fig. S5. Fig. S6.
	Df(2R)Px2 (BL2604) 60C6;60D9ð 10 (**62**)	No identified supressor **		
	Df(2R)ES1 (BL3157) 60E6-8;60F1-2 (**18**)	No identified supressor **		

* Number of predicted genes in the region covered by the deletion

** Tested deletions were in original genetic background, but were crossed to *adgf-a* mutation that wad been placed into background 2

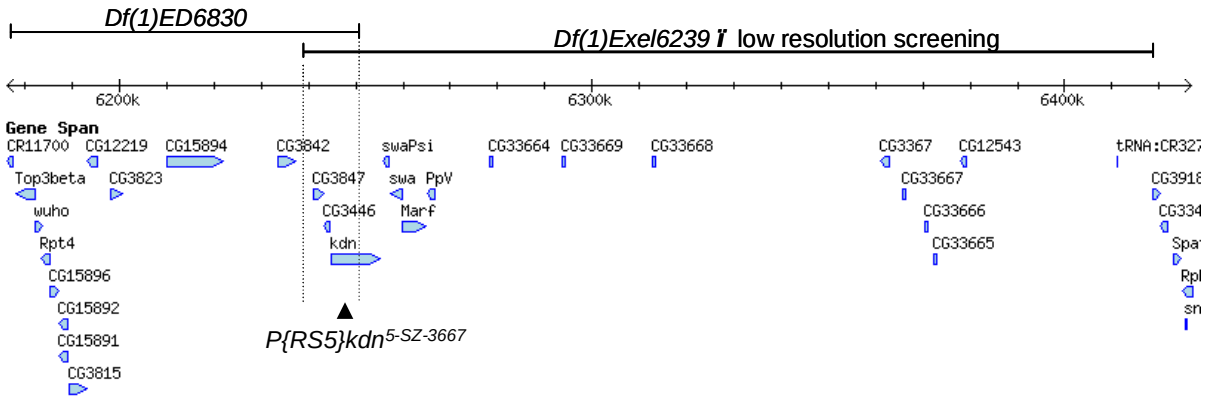


***Figure S1*. The layout of genes in the rescue region Df(1)Exel6239**
and the tested insertion P{RS5}kdn$^{5\text{-}SZ\text{-}3667}$. The candidate rescue gene *kdn* lies in an overlap of the *Df(1)Exel6239* rescue region (identified in Low resolution screening) and *Df(1)ED8630* used in the detailed screening.

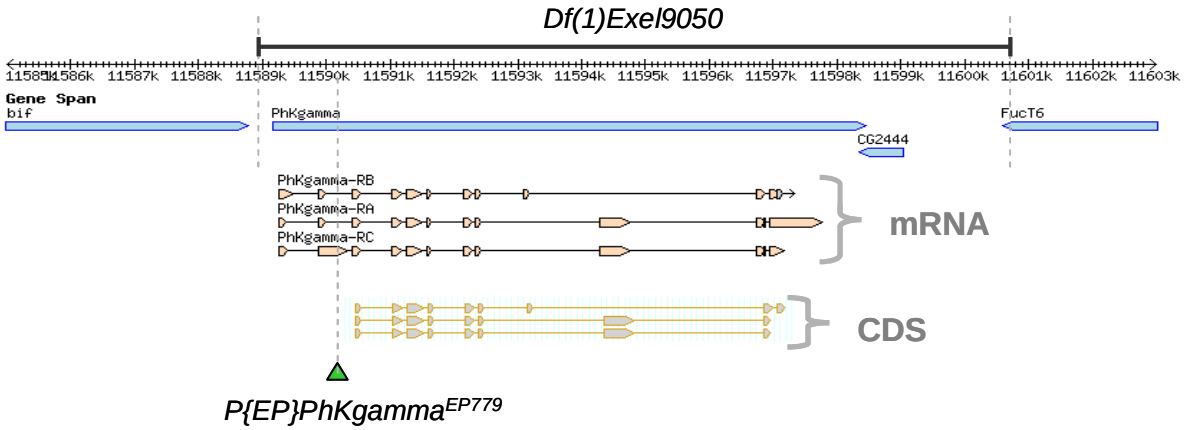
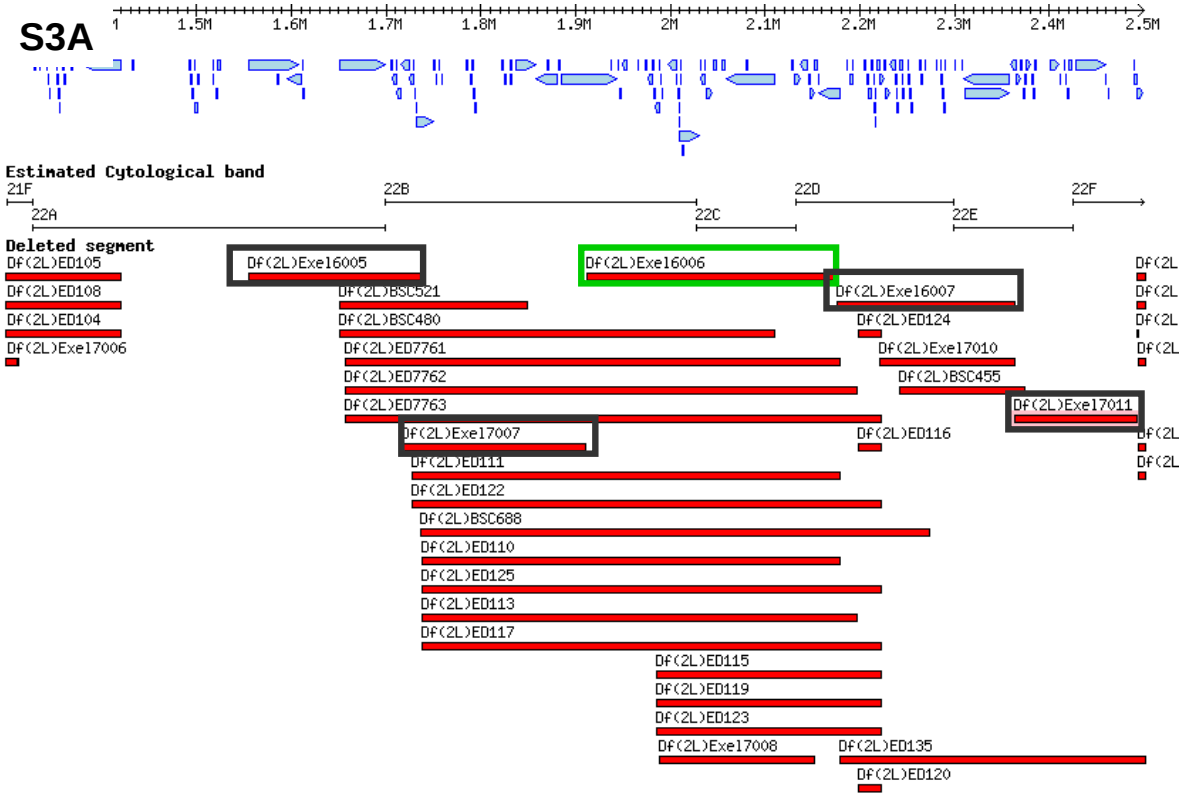


Figure S2*. The layout of genes in the rescue region *Df(1)Exel9050
and the rescue insertion *P{EP}PhKgamma*^{EP779} (*phKƆ*) identified in the detailed screening.



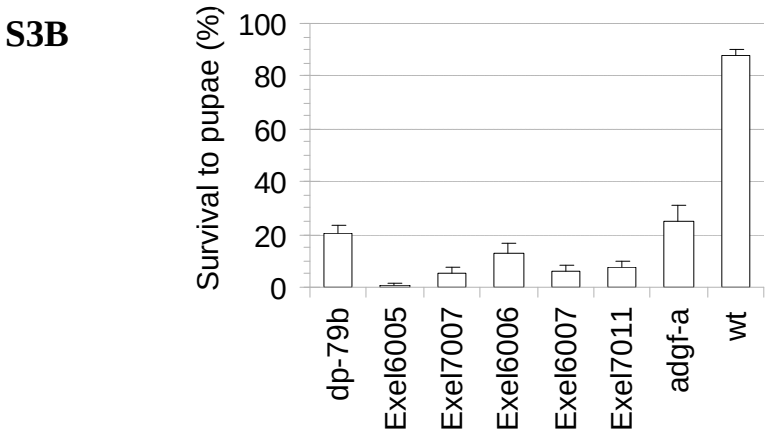


Figure S3. **The layout of genes in the rescue region *Df(2L)dp-79b***

A) The layout of five deletions from Exelixis collection, tested in detailed screening (marked by grey and green frames). In green frame is deletion Exel6006 that was identiffied as supressor and that deletes gene for glycogen phosphorilase.

B) The rescue effect of *Exel6006* is manifested as increase in pupation (of *Exel6006*;*adgf-a* larvaea) in comparison with other tested deletion from Exelixis collection. This graph demonstrates that the genetic background of exelixis collection worsens the survival of *adgf-a* larvae.

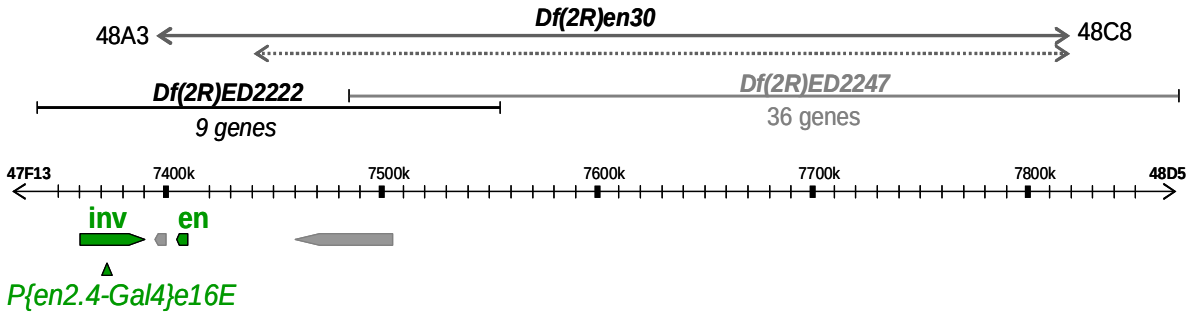


Figure S4. **The layout of genes in the rescue region *Df(2R)en30***

and the recsue insertion *P{w[+mW.hs]=en2.4-GAL4}e16E* that was mapped to gene *invected*. The hypomorphic mutation of *engrailed* (en^{spt}) and deletion *Df(2R)ED2222* were also tested in detailed screening, they improve survival of *adgf-a* mutant. Deletion *Df(2R)ED2247* was not tested.

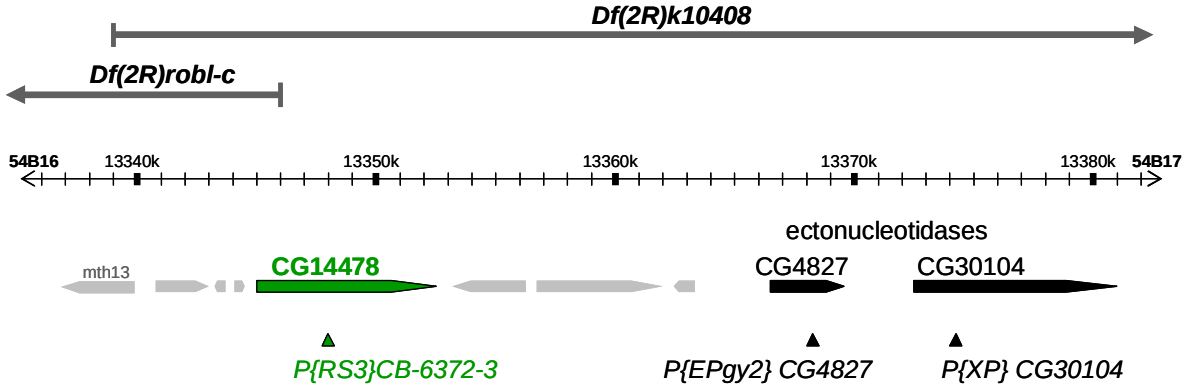
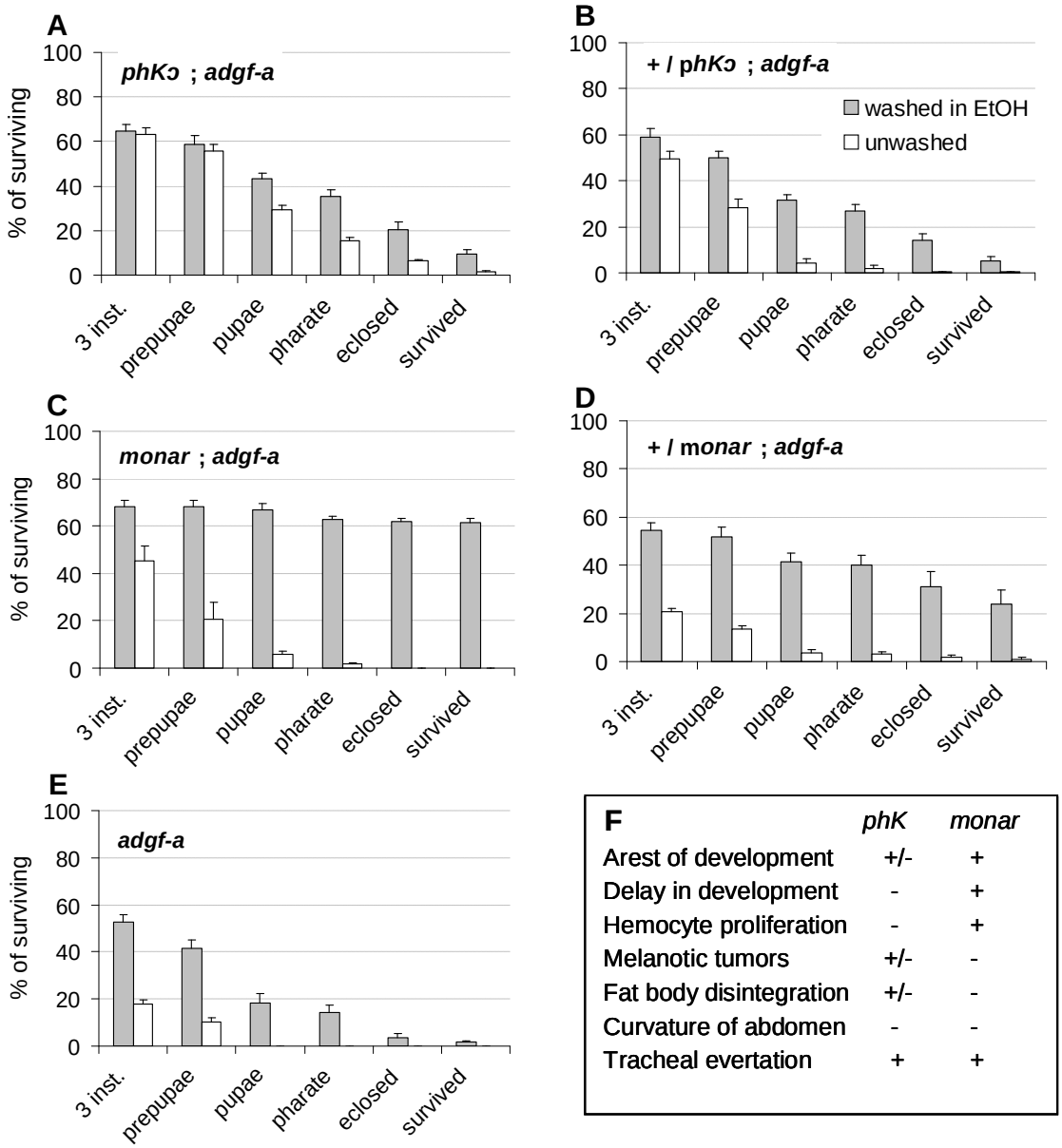


Figure S5. **The layout of genes in the rescue region *Df(2R)robl-c***

and rescue insertion $P\{RS3\}CG14478^{CB\text{-}6372\text{-}3}$ in the overlap of two rescue deletions: *Df(2R)robl-c* and *Df(2R)k10408*. Other two tested insertions in genes for ectonucleotidases: $P\{EPgy2\}veil^{EY02176}$ and $P\{XP\}CG30104^{d00824}$ worsen survival of *adgf-a* larvae, when tested in original genetic backgrounds. None of these three *P*-element insertions modify the survival of *adgf-a* larvae, when tested in uniffied genetic background (background 2).



F	*phK*	*monar*
Arest of development	+/-	+
Delay in development	-	+
Hemocyte proliferation	-	+
Melanotic tumors	+/-	-
Fat body disintegration	+/-	-
Curvature of abdomen	-	-
Tracheal evertation	+	+

***Figure S6*. Effect of rescue mutations on *adgf-a* mutant phenotype, CM-60 diet**

Survival of *phKɔ*; *adgf-a* (**A**), *phKɔ*/+; *adgf-a* (**B**), *monar*; *adgf-a* (**C**), *monar*/+; *adgf-a* (**D**) and *adgf-a* (**E**) animals is shown in percentage of animals that survive to particular developmental stage: wandering 3rd instars, prepupae, pupae (=structured head visible through pupal case), pharate adults, eclosed adults and adults that survived for a minimum 3 days. The *wt* animals pupate at about 80% after ethanol washing and at 60% without ethanol washing, all pupae successfully finished their development and emerged into adults (data not shown).

Dark columns show survival of animals hatched from embryos that were washed in ethanol. Light columns show survival of animals that were not subdued to ethanol treatment. Data are presented as mean of survival percentage ± s.e.m.

Table (**F**) summarizes suppressed (+) and unsuppressed (-) effects of homozygous insertions on mutant phenotype.

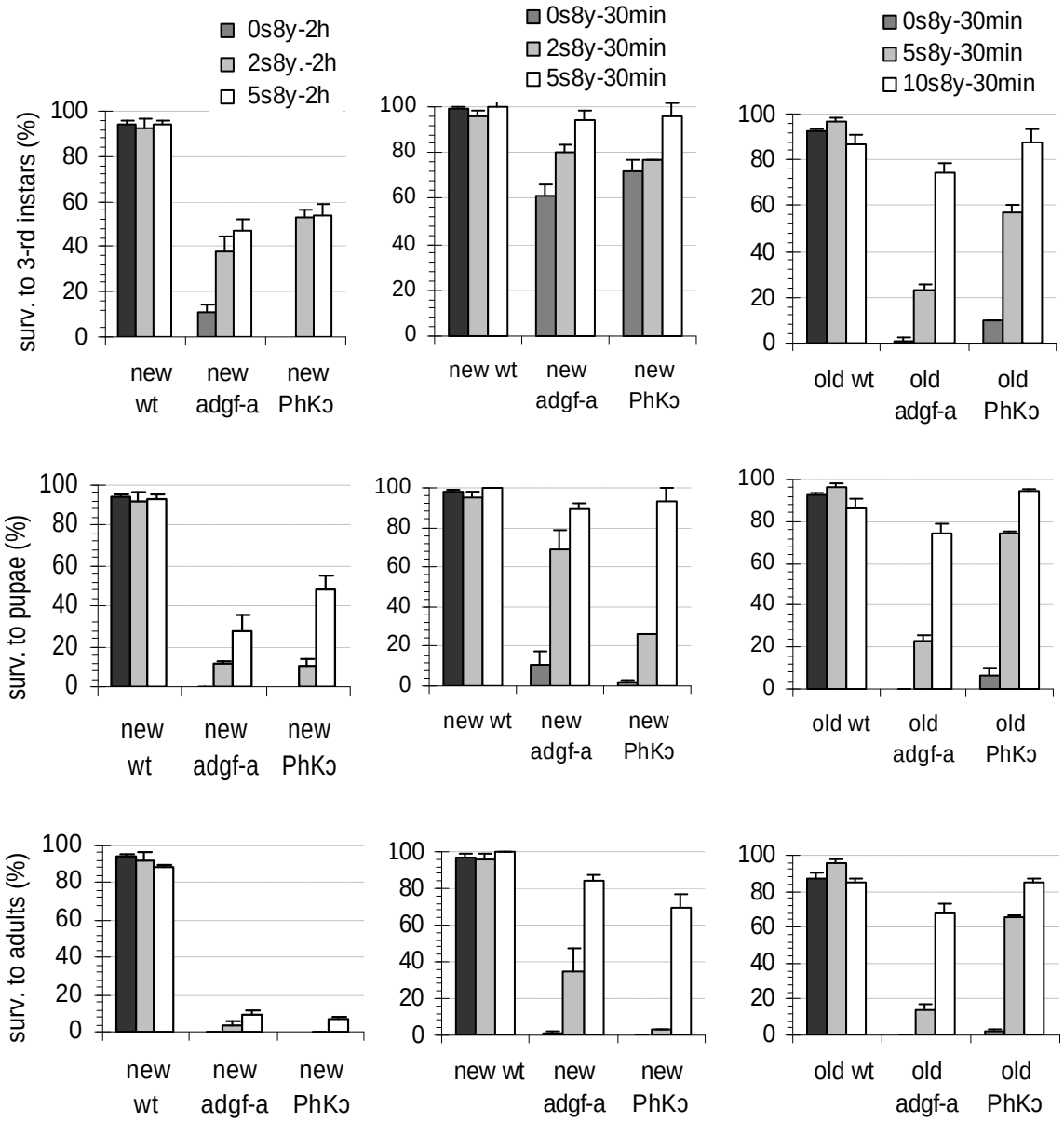


Figure S7. **Modification of survival by different genetic background and different diets**

Graphs show survival to 3rd instars, pupae and adults

of ***wt*** (*adgf-a*/*TM3 Ser Act-GFP*), ***adgf-a*** and **PhKɔ** (*PhK**ɔ**;adgf-a*) animals

of two different genetic backgrounds - background 2 (**New**), background 1 (**Old**)

on different diet - **0s8y**, **2s8y**, **5s8y** and **10s8y**,

heated for different time - **30 min** or **2 hours.**

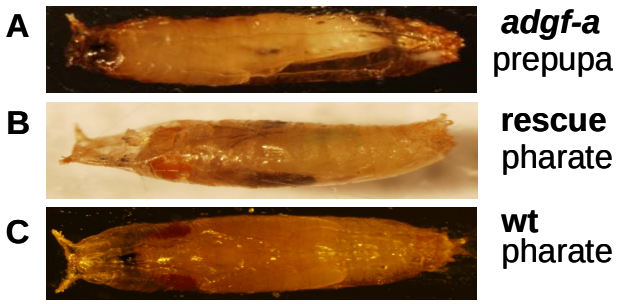


***Figure S8*. Phenotype of the *adgf-a* and rescued animals**

(**A**) Most of *adgf-a* pupae do not have fully everted tracheae and stop their development soon after pupa formation. (**B**) The most of rescued pupae have fully everted tracheae, continue in development, but retain curvature of abdomen, which never occurs at wt pupae (**C**). Shown pupated animals would be considered as prepupa (*adgf-a*), pupa (wt) and pharate imago (rescue).

***Figure S9*. Concentration of larval hemolymph metabolites 1 (next page)**

Result of HPLC-MS measurement of hemolymph inosine, adenosine, glucose, fructose and trehalose in **AdoR adgf-a** (*adoR*;*adgf-a*), **wt** (*adgf-a*/*TM3 Ser Act-GFP*), **C7>A adgf-a** (*C7-Gal4*/*UAS-ADGF-A*;*adgf-a*), **Hml>A adgf-a** (*Hml-Gal4*/*UAS-ADGF-A*;*adgf-a*) and **adgf-a**, reared on **A**) the diets with different sucrose concentration - 0s8y, 5s8y and 10s8y, or on **B)** 5s8y diet.

The *adgf-a* larvae were sampled at 94 and 112 hal (**A**) or at 103, 115 and 130 hal=wandering (**B**). The other larvae were sampled at 90, 102 and 125 hal (= smaller 3rd instars, bigger 3rd instars and wandering).

All columns are average of three replicates ± s.e.m, except for single replicate for *adgf-a* in B.

***Figure S10*. Concentration of larval hemolymph metabolites 2 (page 59)**

Result of HPLC-MS measurement of hemolymph inosine, adenosine, glucose, fructose and trehalose in ***wt*** (*adgf-a*/*TM3 Ser Act-GFP*), **en.hypo adgf-a** (en^{spt};*adgf-a*), **PhKɔ adgf-a** ($P\{EP\}PhKgamma^{EP779}$;*adgf-a*), **Ahcy13 adgf-a** ($P\{EP\}Ahcy13^{EP1007}$;*adgf-a*), **Monar adgf-a** ($P\{RS3\}CG14478^{CB-6372-3}$;*adgf-a*), **cactus hypo** ($cact^{E8}/cact^{IIIG}$) and **cactus null** ($cact^{E8}/cact^{D13}$).

The age of sampled larvae (expressed in hours after laying - hal) and number of replicates (N) are shown in graph.

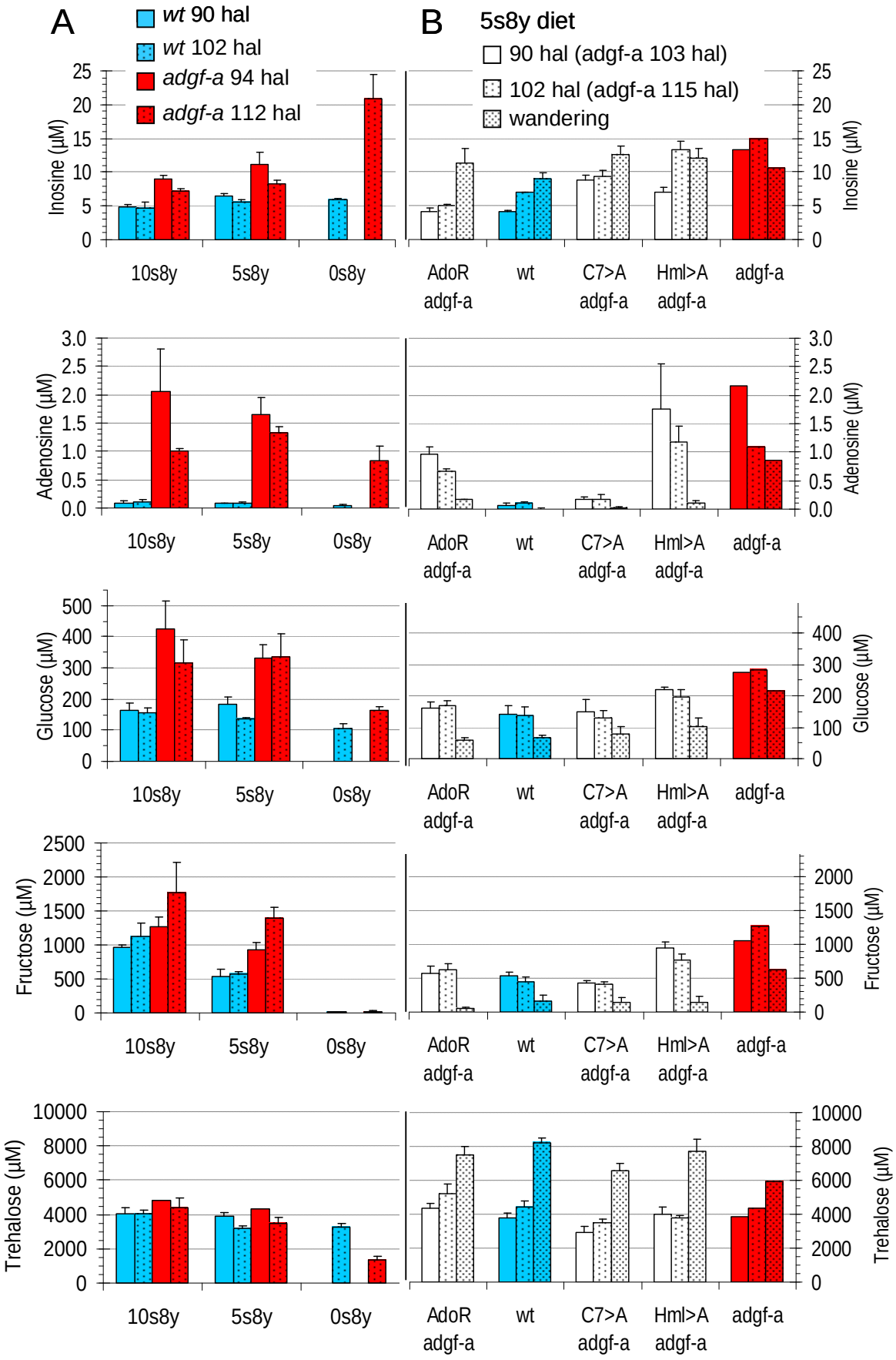


Figure S9. **Concentration of larval hemolymph metabolites 1**
(see legend on page 57)

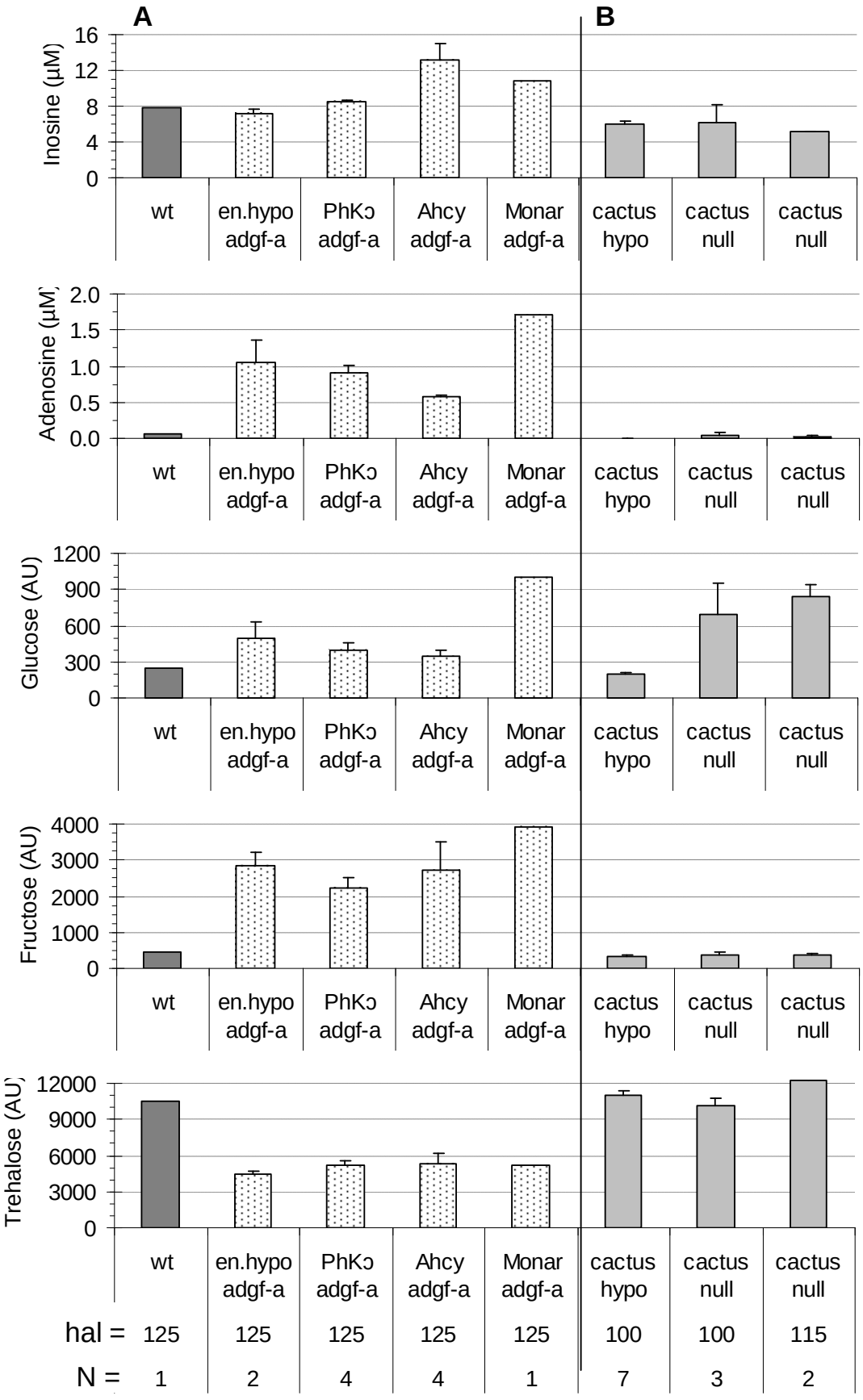


***Figure S10*. Concentration of larval hemolymph metabolites 2**
(see legend on page 57)

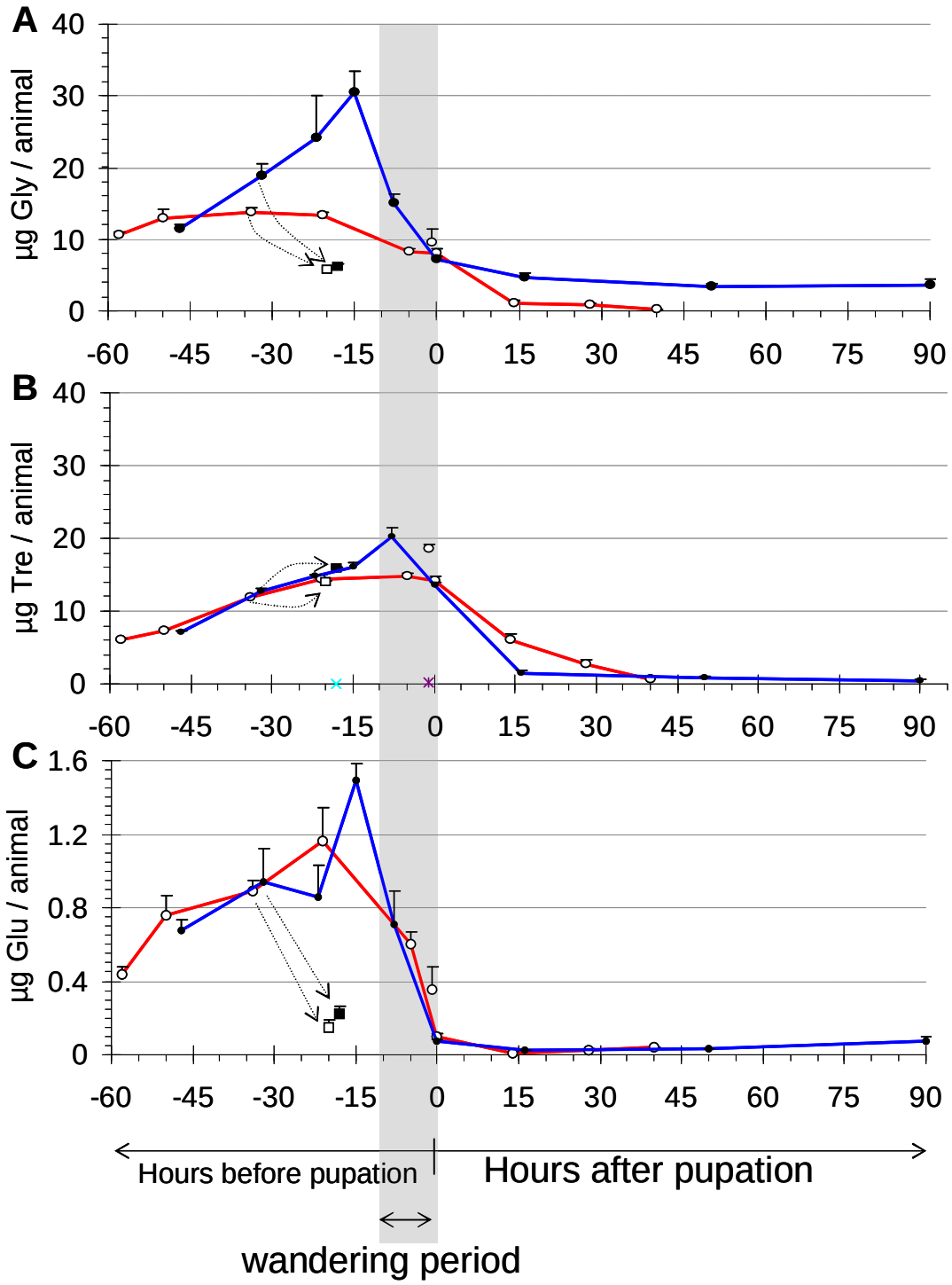


***Figure S11*. The developmental time profile of metabolic reserves**
(see legend on page 63)

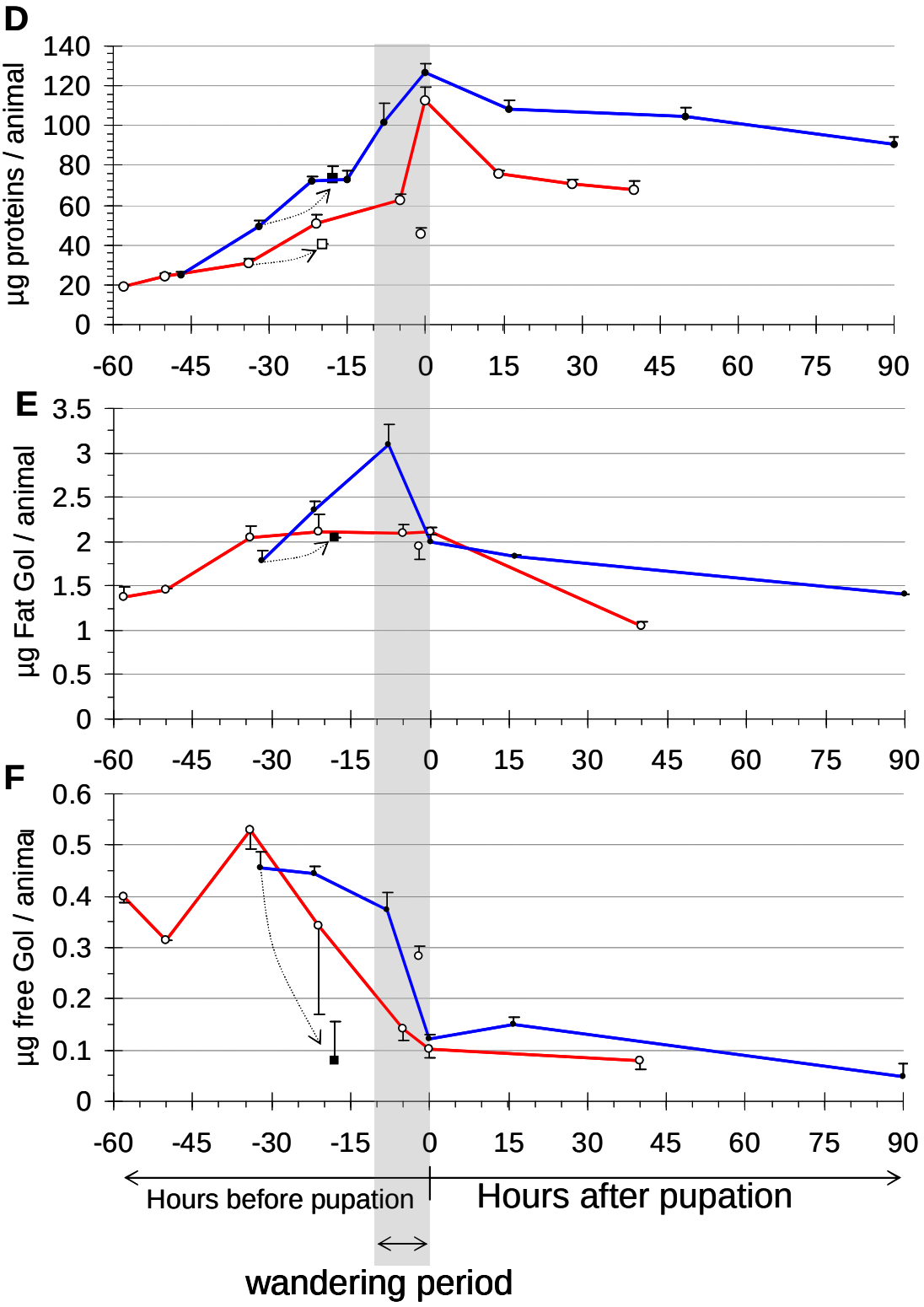


***Figure S11*. The developmental time profile of metabolic reserves**
(see legend on page 63)

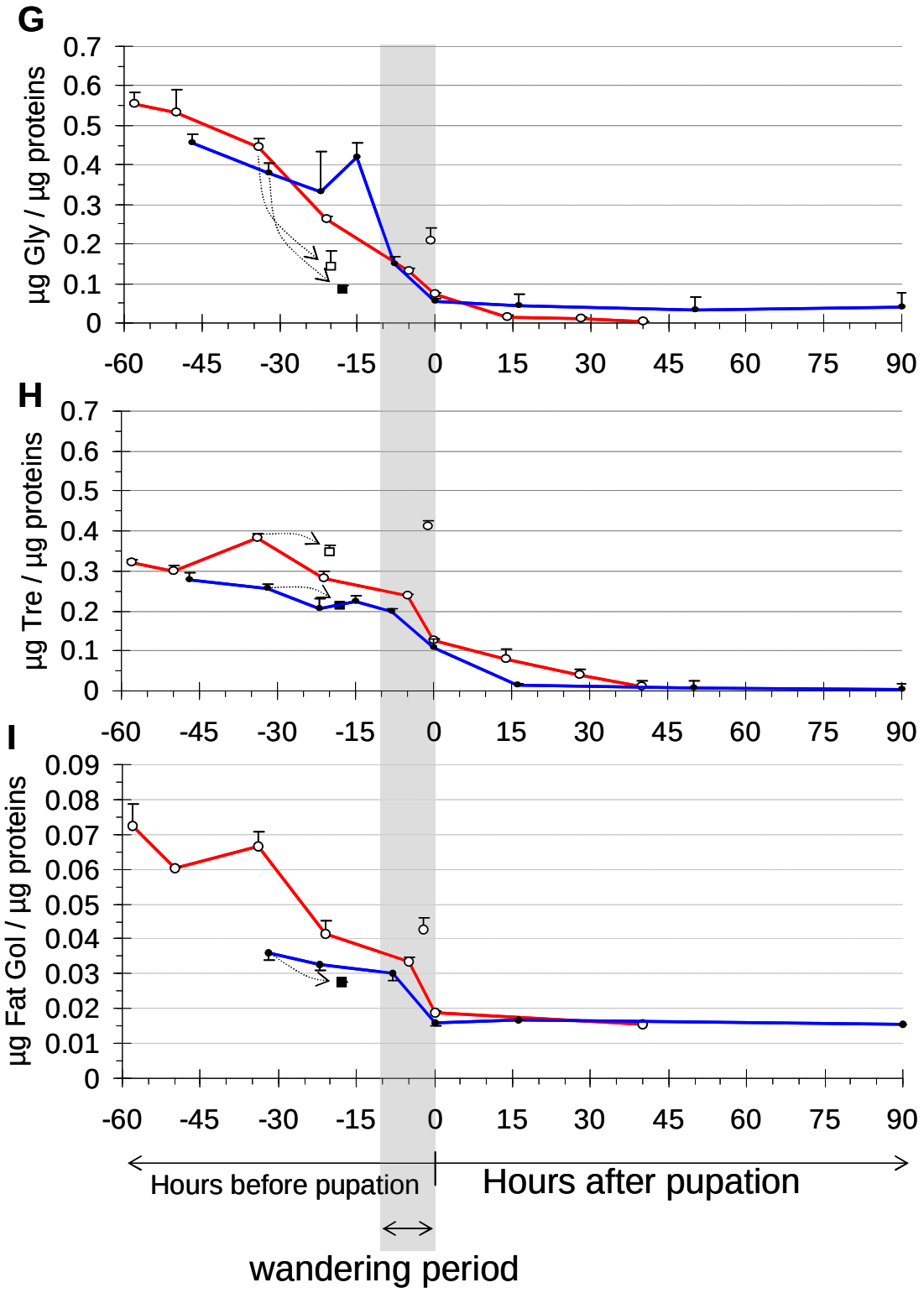


***Figure S11*. The developmental time profile of metabolic reserves**
(see legend on page 63)

***Figure S11*. The developmental time profile of metabolic reserves (page 60 ï 62)**

in *adgf-a* (red) and *wt* (blue) larvae and pupae, aligned on time of pupation (time 0 on horizontal axis). Larvae started to wander about 10 hours before pupation. Squares represent 14-hours starved *adgf-a* (white) and *wt* (black) larvae. The difference of reserves on the beginning and the end of starvation is indicated by dotted arrows. Empty circle outside the red line in time 0 on the horizontal axis represents *adgf-a* larvae that remained in the diet and lagged behind the most progressive larvae that started to pupate in this time.

Measured metabolites are expressed in µg per animal (A-F) or in µg per µg of proteins (G-I).

Glycogen (A, G), **Tre**halose (B, H), **Glu**cose (C), **proteins** (D), **Fat Gol** (E, I) is the glycerol that is incorporated in triglycerides, **Free Gol** (F) is glycerol that is not incorporated in triglycerides.

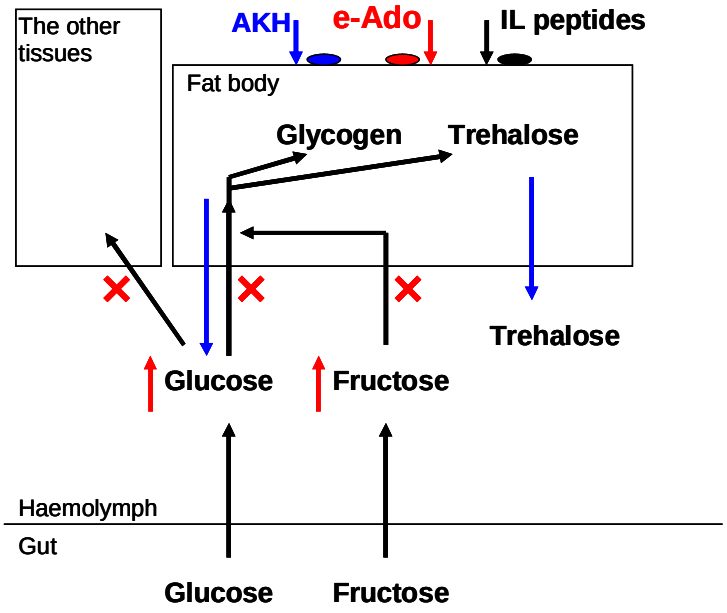


***Figure S12*. Possible regulation of carbohydrate reserves metabolism by e-Ado**

Dietary sucrose is split in gut into its subunit: glucose and fructose, which are absorbed into hemolymph. Glucose could be processed by all tissues but fructose is probably processed only by fat body (in mammals it is processed only in the liver).

Releasing of glucose from fat body reserves is triggered by AKH (blue). Ablation of AKH producing cells does not rescue the sensitivity of adgf-a mutant to low concentration of dietary sugars. So, this sensitivity (accompanied by increase of dietary sugars in hemolymph and low glycogen reserves) is not caused by action of AKH (we speculated, that e-Ado trigger release of AKH; Zuberova et. al, 2010).

We propose that e-Ado acts independently of AKH and block uptake of hemolymph glucose and fructose into fat body and/or other tissues. In the consequence of this, the concentration of hemolymph glucose/fructose increases, but simultaneously at least some tissues are restricted in glucose. Insulin-like peptides stimulate glucose and fructose transporters (black arrows). By blocking these transporters, e-Ado behaves like anti-insulin hormone.

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