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Role of adipokinetic hormone during starvation in *Drosophila*

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Role of adipokinetic hormone during starvation in *Drosophila*

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ABSTRACT

The role of adipokinetic hormone (Drome-AKH) in maintaining the levels of basic nutrients, under starvation conditions, was studied using *Drosophila melanogaster* mutants with AKH deficiency (*Akh*^{1}) and AKH abundance (*EE-Akh*). Our results showed lipids as the main energy reserve in *Drosophila*, and their physiological level and metabolism were shown to be under the control of AKH. AKH abundance in the body resulted in lower levels of triacylglycerols and diacylglycerols than in the controls, probably due to a more intensive metabolism; interestingly, there was a disproportional representation of fatty acids in triacylglycerols and diacylglycerols in *Drosophila*. Lower level of glycogen and its partial control by AKH suggest its lesser role as the storage substance. However, maintenance of free carbohydrate level in *Drosophila* seemed to be critical; when glycogen stores are exhausted, carbohydrates are synthesized from other sources. Protein levels and their alterations, under starvation, did not seem controlled by AKH. AKH-deficient flies were more resistant while AKH-abundant flies were more sensitive to starvation; females were found to be more resistant than males, regardless of the AKH level, probably due to higher body mass and higher amount of nutrients. However, in accordance with the level of all nutrients, that of AKH also gradually decreased with prolonged starvation.

1. Introduction

Deficiency of food represents a heavy stress for all animals, including insects. They must, therefore, mobilize their energy stores to eliminate, or at least reduce, the impact of such an adverse situation, and to survive until conditions become favorable and food is available in sufficient amount. During starvation, metabolic hormones are activated; they trigger catabolic reactions producing energy-rich metabolites that ensure homeostasis of biochemical and physiological functions in the body. In mammals, the level of nutrients in blood – predominantly that of glucose – is very precisely monitored and quickly equilibrated by the activity of several metabolic hormones, such as the pancreatic hormones insulin and glucagon. On the other hand, insects can tolerate large fluctuations of hemolymph sugar and other metabolite levels (Chapman, 1998), since similar homeostasis mechanisms are less accurate in their bodies. Although insects possess insulin and glucagon (Buch et al., 2008; Bednářová et al., 2013), their role in the maintenance of a hemolymph nutrient level is not completely understood; besides, they are thought to play other roles in insect metabolism. The level of nutrients in hemolymph of insects is controlled predominantly by adipokinetic hormones (AKHs), synthesized either in the corpora cardiaca, a small neuroendocrine gland connected to the brain, or in the corresponding corpora cardiaca cells of dipteran ring glands, from where AKHs are released into the hemolymph when required. While AKHs functionally resemble mammalian glucagon (Alquicer et al., 2009; Bednářová et al., 2013), structurally they are quite different; AKHs comprise of eight to ten amino acids only (Gäde et al., 1997). The AKH signal transduction pathway at the cellular level is well documented (Gäde and Auerswald, 2003). Generally, these hormones are typical examples of neuropeptides with complex roles in the control of insect metabolism, including the mobilization of different energy reserves such as lipids, carbohydrates, and/or certain amino acids (Gäde et al., 1997; Gäde and Goldsworthy, 2003). Additionally, AKHs are pleiotropic in nature, with many activities associated with their metabolic role (Kodrík, 2008), including the stimulation of neuronal signaling (Milde et al., 1995), increase of muscle tonus (O'Shea et al., 1984), and stimulation of general locomotion (Socha et al., 1999). These hormones are also known to regulate the starvation-induced foraging behavior of *Drosophila melanogaster* (Lee and Park, 2004), participate in the activation of antioxidant mechanisms (Kodrík et al., 2007, 2015; Večeřa et al., 2007), enhance food intake and digestive processes in insect gut (Kodrík et al., 2012; Bil et al., 2014; Bodláková et al., 2017, 2018), and interact with the cellular and humoral immune system (Goldsworthy et al., 2002, 2003).

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In *Drosophila*, there is a single *Akh* gene that encodes a 79-amino acid (AA)-long hormone precursor. This precursor includes the active AKH octapeptide (Drome-AKH: pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp-NH_2), the 22-AA N-terminal signal peptide, and 49-AA AKH-associated peptide with unknown function (Schaffer et al., 1990; Noyes et al., 1995; Gáliková et al., 2015). Drome-AKH is thought to be involved in the control of carbohydrate and lipid metabolism in *Drosophila* (Lee and Park, 2004); however, the real situation is probably more complicated. Isabel et al. (2005) reported that AKH has little effect on the lipid levels in *Drosophila* hemolymph due to the absence of a proper lipophorin (apolipoprotein III being absent), specialized in the hemolymph lipid transport. Accordingly, these authors claimed that *Drosophila* adults use carbohydrates as their exclusive source of energy during flight, despite their relatively small glycogen reserve. This limitation might be compensated by the carbohydrate store in a special part of *Drosophila* crop (a blind-ended extension from the esophagus), which may be utilized as needed (Isabel et al., 2005). On the other hand, importance of lipids in energy metabolism in *Drosophila* was reported by Grönke et al. (2005, 2007), who suggested a dual control of lipolysis in these: one via AKH signaling pathway, and another via activity of Brummer lipase, a homolog of human adipocyte triacylglycerol lipase.

Gáliková et al. (2015) have recently proven that AKH does not play a critical role in energy mobilization during larval development of *Drosophila*, although it regulates both fat and hemolymph nutrient levels in adults. These authors also suggested that lipid accumulation in fat bodies of AKH-deficient adult flies might be a result of increased cellular uptake of hemolymph carbohydrates and enhanced lipogenesis. Interestingly, AKH seems to primarily regulate lipid and not glycogen stores in *Drosophila* (Gáliková et al., 2017).

Several previous studies (Gáliková et al., 2015; Sajwan et al., 2015; Zemanová et al., 2016) have proved that absence of AKH in the *Drosophila* body may increase survival rate during starvation. Although surprising, it could be explained by the probable inability of loss-of-function flies to stimulate either the proper mobilization of nutrients from fat body into hemolymph, or their subsequent utilization in tissues, thereby retaining energy stores longer.

This study aimed to characterize the role of AKH in the metabolism of basic nutrients in *Drosophila* during starvation. To that end, mutants with either dysfunction or enhanced production of AKH were used, and characteristics such as nutrient level, mortality, AKH level alterations, etc. were studied.

2. Material and methods

2.1. Experimental flies

The fruit flies *D. melanogaster* were maintained in glass vials with standard cornmeal/yeast/sucrose/agar diet at 25 °C and 12:12 L:D cycles. All assays were carried out on larvae of the 3rd instar, 3-day after the larval hatching, and on 3-day old adult males and females kept at the same temperature and light conditions. The fly strains used in this study were as follows: (1) the controls (white) w^{1118} (Bloomington Centre); (2) Akh^1 mutant with a three-base deletion in the *Akh* gene on the chromosome III resulting in lack of the third amino acid in AKH octapeptide prepared by the transcriptional activator like effector nucleases (TALEN) mutagenesis (Sajwan et al., 2015); (3) *EE-Akh* 'rescue' mutant fly which ectopically express *Akh*, prepared as described previously (Sajwan et al., 2015); briefly, the original Akh^1 mutant was combined with the *Act-Gal4* driver and UAS-Akh transgene by crossing w^{1118}; *Act-Gal4/CyO*, *Act-GFP*; Akh^1 flies to w^{1118}; *UAS-Akh*$^+$; Akh^1 ones; resulting adult flies showed more than an order of magnitude higher expression level of *Akh* transcript than w^{1118} flies. The mutants were backcrossed to w^{1118} for eight generations for isogenization.

2.2. Starvation survival experiments

For the mortality experiments the adult males and females were kept in glass vials with 0.5% agarose in water in groups of 20 flies at standard conditions. Numbers of dead flies were evaluated in 12-h periods until all flies died (max. 156 h).

For the physiological/biochemical determinations (see below) the above mentioned starving flies were taken at 10-h intervals for the period of 60 h. Only living flies were used for these experiments.

2.3. Spectrophotometric determination of nutrients

2.3.1. Lipid determination

Adult fly bodies were individually homogenized in 150 µl chloroform: methanol (2:1) mixture according to Folch et al. (1957), and Košťál and Šimek (1998). The extraction was repeated twice, extracts were joined together, evaporated in Speed Vac centrifuge (Jouan RC 10.22) and residues of evaporation used for the total lipid determination. This was done by the sulpho-phosho-vanillin method according to Zöllner and Kirsch (1962), as modified by Kodrík et al. (2000). The optical densities at 546 nm, measured in a spectrophotometer (UV 1601 Shimadzu, Japan), were converted to µg lipids per fly with the aid of a calibration curve based on known amounts of oleic acid.

2.3.2. Free carbohydrate determination

Adult fly bodies were individually homogenized in 100 µl 70% ethanol (Ohtsu et al., 1992), extraction was repeated twice and joined extracts evaporated. The residues were diluted in 40 µl of distilled water and then used for quantification of carbohydrate level by the anthrone method (Carroll et al., 1956) that was modified by Socha et al. (2004). For the calibration curve known amounts of glucose were used.

2.3.3. Glycogen determination

The sediments after the extraction of free carbohydrates by 70% ethanol from the adult fly bodies (see previous paragraph) were used for the glycogen determination using the method of Ohtsu et al. (1992). Briefly, each sediment was extracted by 100 µl 0.1 M Tris-HCl buffer, pH 7.8, the solution evaporated and the residue dissolved in 20 µl distilled water. This sample was boiled in 130 µl 30% KOH (15 min), cooled down, centrifuged and its 50 µl aliquot mixed with 25 µl 10% Na_2SO_4. After the intensive mixing 150 µl 95% ethanol was added and the resulting sample kept on ice for 30 min. After next centrifugation the sediment was washed with 70% ethanol, the supernatant discarded and resulting sediment dissolved in distilled water. The 40 µl aliquot was taken for the glycogen determination using the anthrone method as described for the free carbohydrate determination in the previous paragraph.

2.3.4. Protein determination

Adult fly bodies were individually homogenized in 250 µl 0.2 M Tris-HCl buffer, pH 7.8, centrifuged and the supernatants (50 µl) taken for determination of 'soluble proteins'. Each sediment was extracted again in 100 µl 0.2 M Tris-HCl buffer, pH 7.8 containing 1% Triton, and after the next centrifugation, the 50 µl aliquot was used for determination of 'insoluble proteins'. The quantification of both protein groups was done by the Bicinchoninic Acid Protein Assay Kit (Sigma Aldrich) (Stoscheck, 1990). The protein aliquot (50 µl) was mixed with 1 ml of bicinchoninic acid containing $CuSO_4.5H_2O$ according to the manufacturer's instructions. The bovine serum albumin standard curve was used to convert the optical densities of the samples measured at 562 nm to µg proteins.

Results of nutrient quantification were expressed per mg of body mass (3-day old flies), or just per the whole body (starvation experiments), because all mutants lost body mass at the same rate during starvation (data not shown).

2.4. Mass spectrometry determination of lipids

The lipids were extracted by a chloroform-methanol mixture as mentioned above (see Section 2.3). The extracts were evaporated by Speed Vac (Labconco, USA), dissolved in methanol and mixed with internal standard phosphatidylglycerol (PG) 17:0/17:0 (Sigma Aldrich). High-performance liquid chromatography (HPLC), combined with electrospray ionization tandem mass spectrometry (ESI–MS/MS), was used for analyses of the extracted lipids. A linear ion trap LTQ-XL mass spectrometer (Thermo Scientific, San Jose, CA, USA), coupled to Allegro ternary HPLC system equipped with an Accela autosampler and a thermostat chamber (all from Thermo, San Jose, CA, USA), were employed. The samples (5 µl) were injected into a Gemini column (250 mm × 2 mm i.d. 3 µm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of (A) 5 mM ammonium acetate in methanol, (B) water, and (C) 2-propanol. For details of the analysis see Schneedorferová et al. (2015). The data from HPLC ESI-MS/MS analyses were acquired and processed using Xcalibur software version 2.1 (Thermo Fisher Scientific, San Jose, CA, USA). Identification of particular lipid class and species was achieved by measured mass, retention time, and fragmentation pattern.

2.5. AKH extraction and level determination by ELISA

The larval heads containing central nervous system with the ring gland attached, and the adult heads (males and females separately) with a half of the prothorax were cut off and extracted in 80% methanol. The extracts were used for the determination of the AKH level using a competitive ELISA as described in our previous paper (Zemanová et al., 2016). Briefly, the 96-well microtiter plates (high binding Costar, Corning Incorporated, Corning, New York) were precoated overnight with IgG (anti Drome-AKH polyclonal antibody; a kind gift of Jan Veenstra, Bordeaux University, France), dilution 1:10,000 in coating buffer. After blocking with 3% BSA (bovine serum albumin) solution in dH_2O, the samples were added to specific wells, followed by the biotinylated probe prepared from Drome-AKH using Biotin Long Arm Maleimide (BLAM, Gln-Leu-Thr-Phe-Ser-Pro-Asp-Trp-Lys(Bio)-Gly-NH_2; Vidia, Praha, Czech Republic), diluted in 1 mM PBS (phosphate-buffered saline, pH = 7.5). After the competition for the binding sites on the IgG bound to the plates a streptavidin conjugated with horseradish peroxidase solution (Vector Laboratories, CA, USA), diluted 1:500 in PBS-Tween was added to each well. Finally, the ELISA substrate (3,3′,5,5′-tetramethylbenzidine, Sigma Aldrich) was added, and the visualization of the reaction was stopped by 0.5 M sulphuric acid. The absorbance values were determined in a microtiter plate reader at 450 nm. The known concentration of Drome-AKH was used as a standard which allowed construction of a competition curve and estimation of the AKH content in analyzed samples. The detection limit of this ELISA test was estimated from the standard curve to be about 15 fmol Drome-AKH per well (data not shown).

2.6. Data presentation and statistical analyses

The results were plotted using the graphic program Prism (GraphPad Software, version 6, San Diego, California). The bar graphs and points on the line graphs represent the mean value ± SD; numbers of repetitions are depicted in the Fig. legends. Statistical significance of the results was evaluated using (1) one-way ANOVA followed by Tukey's multiple comparison test (Figs. 1, 3, 4, 6–8); (2) Gehan-Breslow-Wilcoxon test with Bonferroni correction (survival analysis) (Fig. 2); (3) repeated measures (RM) ANOVA (experiment designed as randomized block that is analyzed identically to repeated-measures data in Prism) followed by Tukey's multiple comparison test (Figs. 6–9); (4) unpaired Student's *t*-test at the (Figs. 1, 6–8); (5) paired Student's t-test (Fig. 1).

The data obtained from the HPLC ESI–MS/MS analyses (areas of

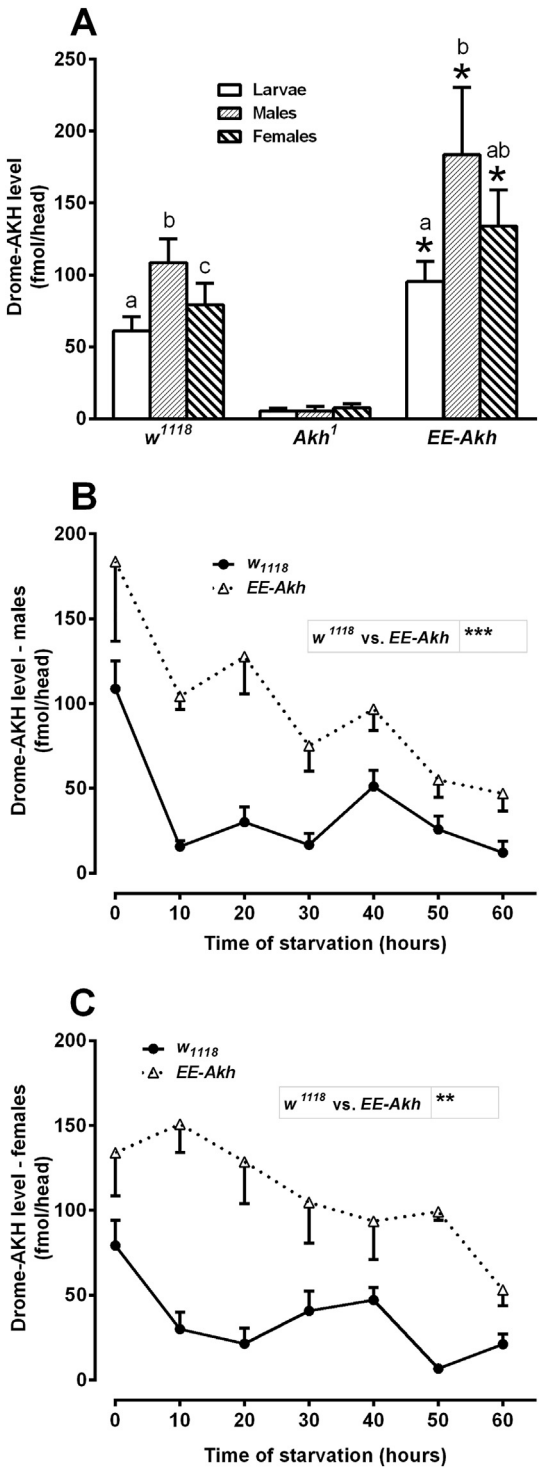

Fig. 1. (A) Level of Drome-AKH (fmol/CNS) in w^{1118}, Akh^1 and *EE-Akh* 3-day old last instar larvae, and 3-day old *Drosophila* adult males and females. (B) Temporal course of the Drome-AKH level in adult males, and (C) in adult females under starvation. Statistically significant differences were evaluated as follows: (A) among the larvae, males and females within the w^{1118} and *EE-Akh* mutants (Akh^1 were not tested due to AKH level under the detection limit) using one-way ANOVA with Tukey's multiple comparison test, the differences at the 5% level are indicated by different letters; between the w^{1118} and *EE-Akh* mutants within the larvae, males and females by Student's *t*-test, the differences at the 5% level are indicated by asterisk; (B, C) between the w^{1118} and *EE-Akh* mutants by paired Student's t-test, the differences at the 1% level are indicated by two asterisks, at 0.1% level by three asterisks; n = 4–7.

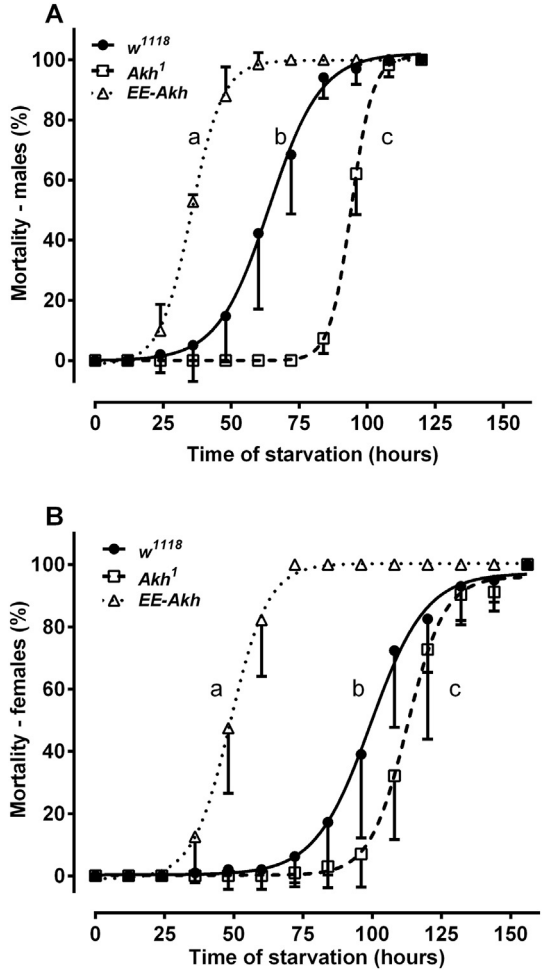

Fig. 2. Mortality of w^{1118}, Akh^1 and *EE-Akh* 3-day old *Drosophila* adult males (A) and females (B) under starvation. Statistically significant differences among the mutants were evaluated by Survival analysis with the Gehan's Wilcoxon test and Bonferroni correction, statistically significant differences at the 1.7% level are indicated by different letters; n = 7–10.

triacylglycerol (TG) and diacylglycerol (DG) peaks; Figs. 3–4) were statistically evaluated using ordination methods as follows: for all data - detrended correspondence analysis (DCA); for linear data - principal component analysis (PCA), redundancy analysis (RDA), Monte-Carlo permutation test (unrestricted permutations, n = 999), and for unimodal data - correspondence analysis (CA), constrained correspondence analysis (CCA), Monte-Carlo permutation test (unrestricted permutations, n = 999). The data was transformed by using internal standard peak area of the particular sample followed by recalculation to the ratio of particular detected lipids. For each, the transformed peak areas were calculated in the deconvoluted total cell and peak area. In the canonical analysis (RDA, PCA) the fly strains represented a categorical predictor. Monte-Carlo permutation test was used for statistical significance determination. Statistic software CANOCO 4.5 (Biometris, Plant Research International, Wageningen UR, Netherlands) was used for the DCA, CA, CCA, PCA, RDA, and Monte-Carlo permutation test analyses.

3. Results

3.1. Level of Drome-AKH

The level of Drome-AKH was negligible in the heads of Akh^1 mutants being below detection limit of the ELISA method employed (Fig. 1A); in *EE-Akh* flies, the AKH level was significantly higher than in control w^{1118} flies (1.6, 1.7, and 1.7 times for larvae, males, and females, respectively). Interestingly, the level was lowest in larvae and highest in males. Monitoring Drome-AKH level in the w^{1118} and *EE-Akh*

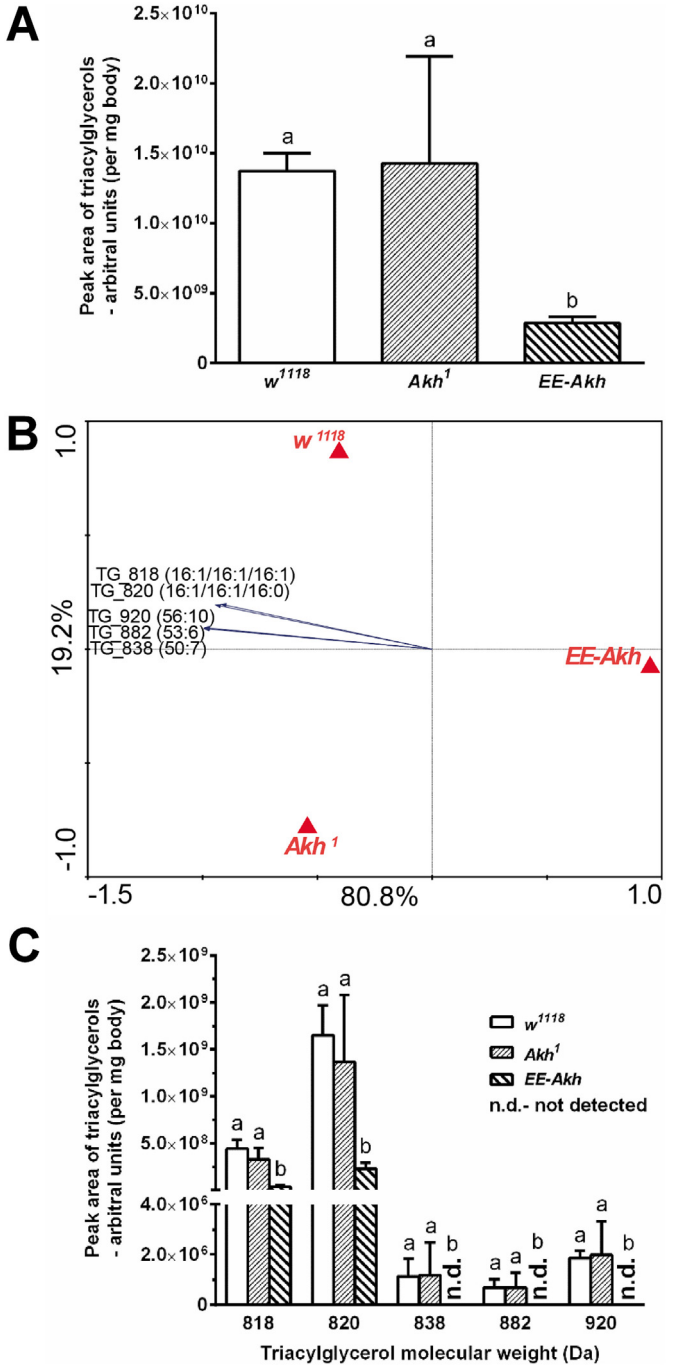

Fig. 3. (A) Relative amount of triacylglycerols (TGs) in w^{1118}, Akh^1 and *EE-Akh* 3-day old *Drosophila* adult males (data obtained from the HPLC/ESI-MS analysis). (B) The RDA diagram of detected TGs in particular mutants expressed as nominal variables: five TGs species depicted in the diagram were the most responsible for the TG profile differences. (C) Relative amounts of the individual TGs participating in the differences mentioned in (B). Statistically significant differences were evaluated as follows: (A) among the mutants using one-way ANOVA with Tukey's multiple comparison test, the differences at the 5% level are indicated by different letters; (B) among the TG profiles in particular mutants using Monte-Carlo permutation test – the test proved significant differences at the 0.1% level; (C) among the mutants within the individual TGs using one-way ANOVA with Tukey's multiple comparison test, the differences at the 5% level are indicated by different letters; n = 6–8.

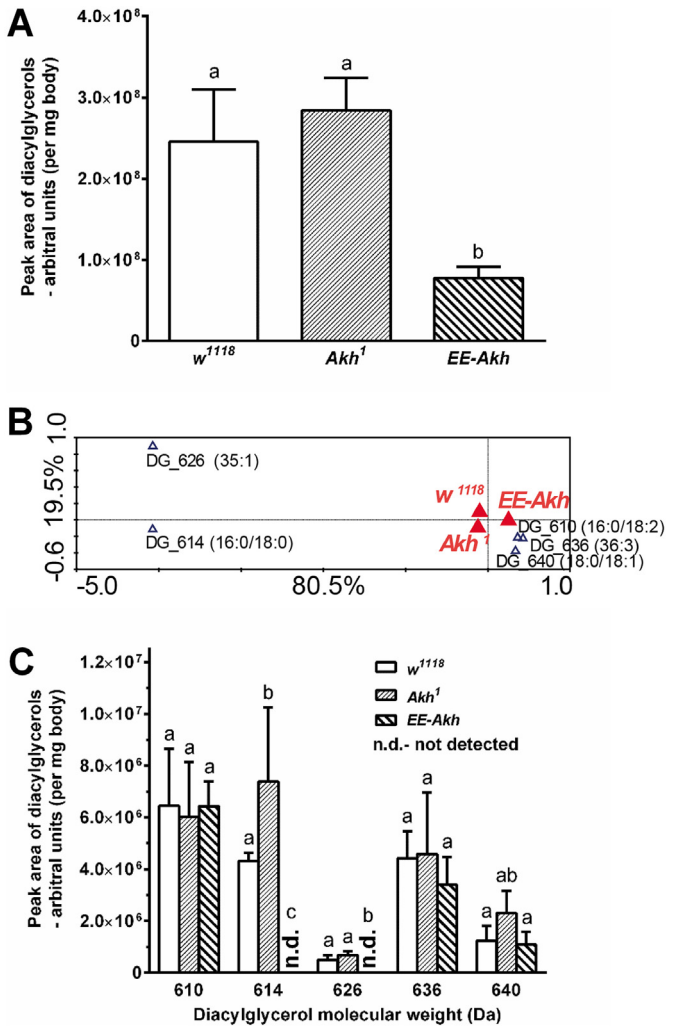

Fig. 4. (A) Relative amount of diacylglycerols (DGs) in w^{1118}, Akh^1 and *EE-Akh* 3-day old *Drosophila* adult males (data obtained from the HPLC/ESI-MS analysis). (B) The CCA diagram of detected DGs in particular mutants expressed as nominal variables: five DGs species depicted in the diagram were primarily responsible for the DG profile differences. (C) Relative amounts of the individual DGs participating in the differences mentioned in (B). Statistically significant differences were evaluated as follows: (A) among the mutants using one-way ANOVA with Tukey's multiple comparison test, the differences at the 5% level are indicated by different letters; (B) among the DG profiles in particular mutants using Monte-Carlo permutation test – the test proved significant differences at the 0.1% level; (C) among the mutants within the individual DGs using one-way ANOVA with Tukey's multiple comparison test, the differences at the 5% level are indicated by different letters; n = 6–8.

adults, during starvation, showed its continuous decline with occasional fluctuations (Fig. 1B, C); the course of decline significantly differed between w^{1118} and *EE-Akh* flies across the sexes.

3.2. Resistance to starvation

A starvation resistance experiment with 3-day-old w^{1118}, Akh^1, and *EE-Akh Drosophila* adult flies was performed, and expressed by percentage of mortality. As suggested in our earlier paper (Sajwan et al., 2015), significant difference was recorded among the fly groups (Fig. 2). The *EE-Akh* flies, with ectopically expressed Drome-AKH and AKH over-production (Fig. 1A), showed the highest mortality during starvation. This trend was confirmed by Akh^1 flies deficient in Drome-AKH production; they were the most resistant to starvation and survived food deprivation for the longest time. Additionally, we recorded statistically significant differences between males and females for all tested groups (Gehan-Breslow-Wilcoxon test with Bonferroni correction at 0.1% level). The LT_{50} (lethal time$_{50}$ = the time when 50% mortality was observed) was 64.0, 94.2, and 35.4 h for w^{1118}, Akh^1, and *EE-Akh* males (Fig. 2A), and 99.4, 112.6, and 48.8 h for the females (Fig. 2B), respectively. Based on these results, we decided to monitor the effect of starvation on several biochemical/physiological parameters over a 60-h period (see below).

3.3. Effect of AKH absence/presence and starvation on the level of nutrients

HPLC/ESI-MS analysis of the whole body chloroform-methanol extracts from the experimental w^{1118}, Akh^1, and *EE-Akh Drosophila* adult males revealed the presence of about 230 various glycerolipid species, mostly triacylglycerols (TGs; 106 species), diacylglycerols (DGs; 28 species), and phospholipids (PL; 99 species) (data not shown). They were identified based on the measured mass, ionization behavior in positive and negative ESI modes, retention time, and fragmentation patterns obtained in MS/MS experiments. Nevertheless, most of them were present in negligible amounts, without substantial effect on total lipid level, in the experimental flies. Those present in larger quantities are described in the following paragraph.

Relative quantification of identified TGs showed identical levels in w^{1118} and Akh^1 flies, but significantly lower level in *EE-Akh* flies (Fig. 3A). The RDA test (Fig. 3B) depicted five TG species with molecular weights (MWs) 818, 820, 838, 882, and 920 Da that were the most responsible for differences in TG profiles. Relative amounts of particular TGs in the experimental fly groups are shown in Fig. 3C; some of the TG species (838, 882, and 920 Da) were not present in the *EE-Akh* mutant at all. On the other hand, two TGs (with MW 818 and 820 Da) were the most abundant in all three fly groups; they contained only two fatty acids (FAs) palmitoleic (16:1) and palmitic (16:0). Similar effect, as in the absence/abundance of Drome-AKH, was recorded for the level of DGs, which represents a transport mode of insect lipids. DG levels were almost identical in w^{1118} and Akh^1 flies while significantly lower in *EE-Akh* flies (Fig. 4A). Five ammonium adducts of DGs with molecular masses 610, 614, 626, 636, and 640 Da were the most responsible for the differences (Figs. 4B, C), and the most abundant ones contained palmitic (16:0), stearic (18:0), and linoleic (18:2) acids. Disproportionate representation of individual FAs in all the TGs and DGs detected is shown in Fig. 5. Results revealed that DGs with C18 and C14 FAs were preferentially mobilized prior to those with C16 in the w^{1118} males; highest disproportion was recorded for 18:0 (stearic acid) – the difference being almost three-fold. On the other hand, the 12:0 (lauric acid) was about 20 times more frequent in TGs than in DGs;

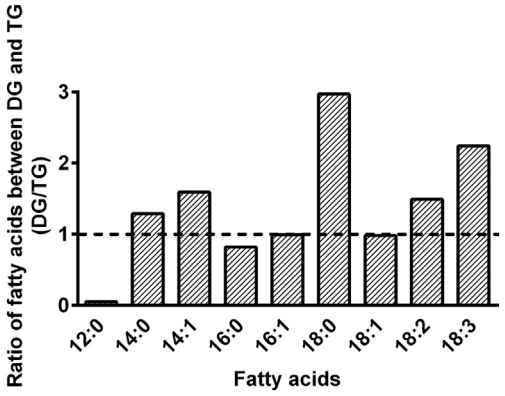

Fig. 5. Ratio of the main fatty acids (FAs) between diacylglycerols (DGs) and triacylglycerols (TGs) in w^{1118} males. Fatty acids with value < 1 predominate in TGs, with value > 1 predominate in DGs. Referred FAs: lauric (12:0), myristic (14:0), myristoleic (14:1), palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3).

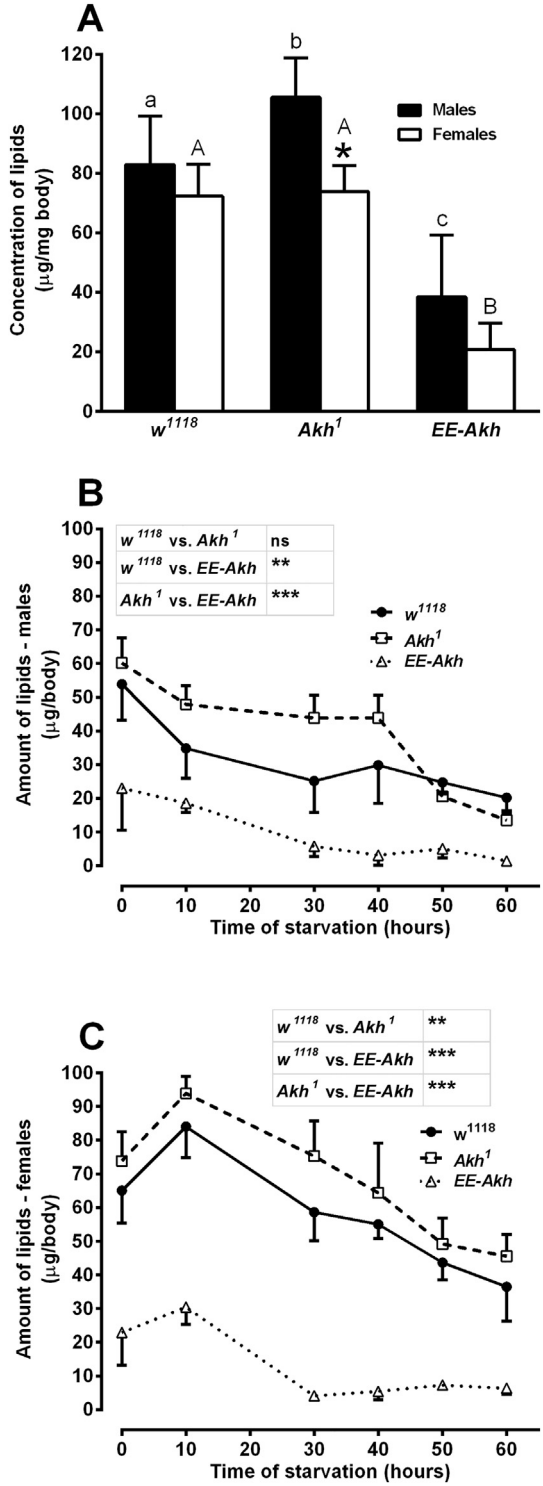

Fig. 6. (A) Concentration of total lipids (µg/mg) in w^{1118}, Akh^1 and *EE-Akh* 3-day old *Drosophila* adult males and females. (B) Temporal course of the total lipid amount (µg/body) in adult males, and (C) in adult females under starvation. Statistically significant differences were evaluated as follows: (A) among the mutants using one-way ANOVA with Tukey's multiple comparison test, the differences at the 5% level are indicated by different letters (lower case letters for males and upper case letters for females); between the sexes by Student's *t*-test, the differences at the 5% level are indicated by asterisk; (B, C) among the mutants by RM ANOVA (randomized block design) followed by Tukey's multiple comparison test, the differences at the 5% level are indicated by one asterisk, at 1% level by two asterisks and at 0.1% level by three asterisks; n = 5–8.

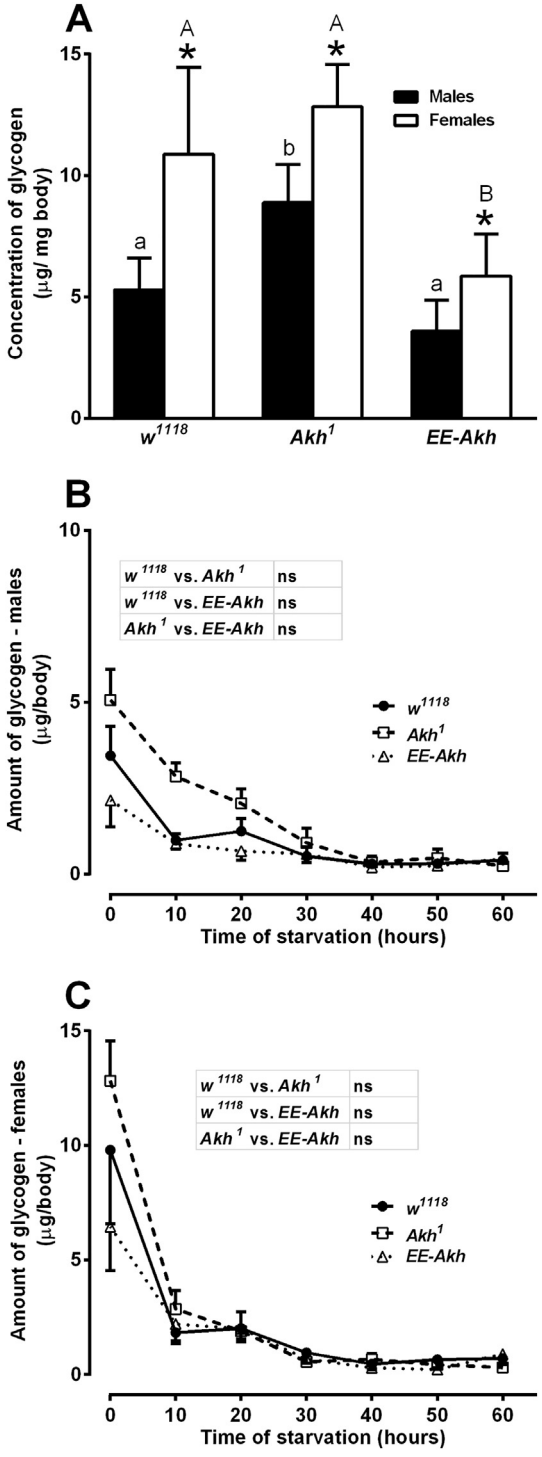

Fig. 7. (A) Concentration of glycogen (µg/mg) in w^{1118}, Akh^1 and *EE-Akh* 3-day old *Drosophila* adult males and females. (B) Temporal course of the glycogen amount (µg/body) in adult males, and (C) in adult females under starvation. Statistically significant differences were evaluated as follows: (A) among the mutants using one-way ANOVA with Tukey's multiple comparison test, the differences at the 5% level are indicated by different letters (lower case letters for males and upper case letters for females); between the sexes by Student's t-test, the differences at the 5% level are indicated by asterisk; (B, C) among the mutants by RM ANOVA (randomized block design) followed by Tukey's multiple comparison test, no differences at the 5% level were recorded; n = 6–8.

nevertheless, lauric acid is not a typical representative of the *Drosophila* FAs (data not shown).

The sulpho-phosho-vanillin method of determination of total lipid content confirmed that lipids play a critical role in *Drosophila* energy metabolism. Their concentration in *EE-Akh* mutants was significantly lower than that in w^{1118} and Akh^{1} flies; this concentration was consistently lower in females than in males, although the difference was significant only in Akh^{1} flies (Fig. 6A). Nevertheless, the total amount of lipids in w^{1118} and Akh^{1} females was higher (1.2 times for both groups) than those in corresponding males, and the same in both sexes in the *EE-Akh* group (compare data from times 0 of all groups in Figs. 6B and C). During starvation, lipids were continuously metabolized, although their utilization statistically differed among the groups, within both sexes, only with the exception of w^{1118} vs. Akh^{1} males (6B, C).

Levels of glycogen stores in *Drosophila* were substantially lower than those of lipids, suggesting not-so-important role of glycogen as a reserve substance in *Drosophila.* Glycogen concentration was significantly higher in 3-day-old females of all three tested groups than in age-matched males (Fig. 7A). The total amount of glycogen in w^{1118}, Akh^{1}, and *EE-Akh* females was higher (2.8, 2.5, and 3.0 times, respectively) than in corresponding males (compare data from times 0 in Figs. 7B and C). Nevertheless, no statistically significant difference in glycogen utilization during starvation was recorded among the tested groups, within both sexes (Fig. 7B, C).

Concentration of free carbohydrates was significantly higher in 3-day-old w^{1118} and *EE-Akh* females than in corresponding males (Fig. 8A). Further, this concentration was significantly higher in Akh^{1} than in *EE-Akh* within males, and vice versa within females; however, the differences were not significantly notable, and probably just reflected the current needs of the organism when carbohydrate levels fluctuate as overall energetic status changes. Comparison of the total amount of free carbohydrates between sexes (compare data from times 0 in Figs. 8B and C) showed their higher level in females than in males: 1.9, 1.7, and 3.1 times in w^{1118}, Akh^{1} and *EE-Akh* flies, respectively. Interestingly, obvious fluctuations in free carbohydrate level were recorded in both sexes, across all fly groups, with a peak around 30 h of starvation (statistically significant at the 5% level in all groups, except for w^{1118} males; tested by one-way ANOVA with Tukey's multiple comparison test). On the other hand, no statistically significant difference in carbohydrate levels was recorded in the course of starvation in the experimental groups, within both males and females.

Protein levels declined in *Drosophila* during starvation (Fig. 9). The drop was more intense for soluble proteins than for insoluble ones in both sexes, and the course of decline during starvation was statistically different in Akh^{1} compared to *EE-Akh* groups; other combinations proved to be insignificant.

4. Discussion

Under long-term starvation, *Drosophila,* with higher production of AKH, maintain a level of nutrients in the hemolymph at the expense of stored supplies, which leads to their rapid depletion, while AKH-deficient mutants are not able to control the nutrient mobilization efficiently and therefore do not utilize the reserves so rapidly and are more resistant to starvation (Sajwan et al., 2015; Gáliková et al., 2015; Zemanová et al., 2016). We have confirmed this phenomenon in this study and additionally found that females of all the fly groups were more resistant to starvation and were able to survive for a significantly longer time without food compared to males. One could speculate about more efficient female-specific metabolic pathways as a cause for this survival advantage, but the reasons are probably simpler. The body mass of 3-day old females in all the fly groups was about 1.6 times higher (data not shown) than that of the males, respectively. All the nutrients – lipids, glycogen, and to a lesser extent, also proteins – were responsible for the differences. Thus, it seems that females have more energy rich metabolites available to them and therefore are able to

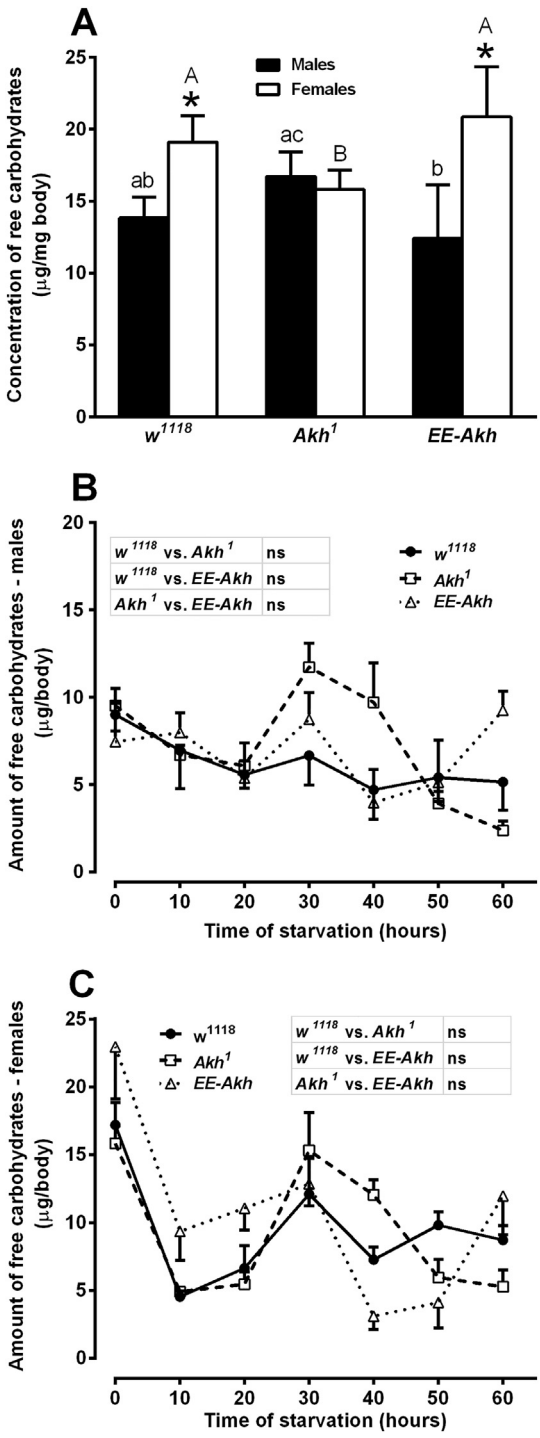

Fig. 8. (A) Concentration of free carbohydrates (µg/mg) in w^{1118}, Akh^{1} and *EE-Akh* 3-day old *Drosophila* adult males and females. (B) Temporal course of the carbohydrate amount (µg/body) in adult males, and (C) in adult females under starvation. Statistically significant differences were evaluated as follows: (A) among the mutants using one-way ANOVA with Tukey's multiple comparison test, the differences at the 5% level are indicated by different letters (lower case letters for males and upper case letters for females); between the sexes by Student's t-test, the differences at the 5% level are indicated by asterisk; (B, C) among the mutants by RM ANOVA (randomized block design) followed by Tukey's multiple comparison test, no differences at the 5% level were recorded; n = 6–8.

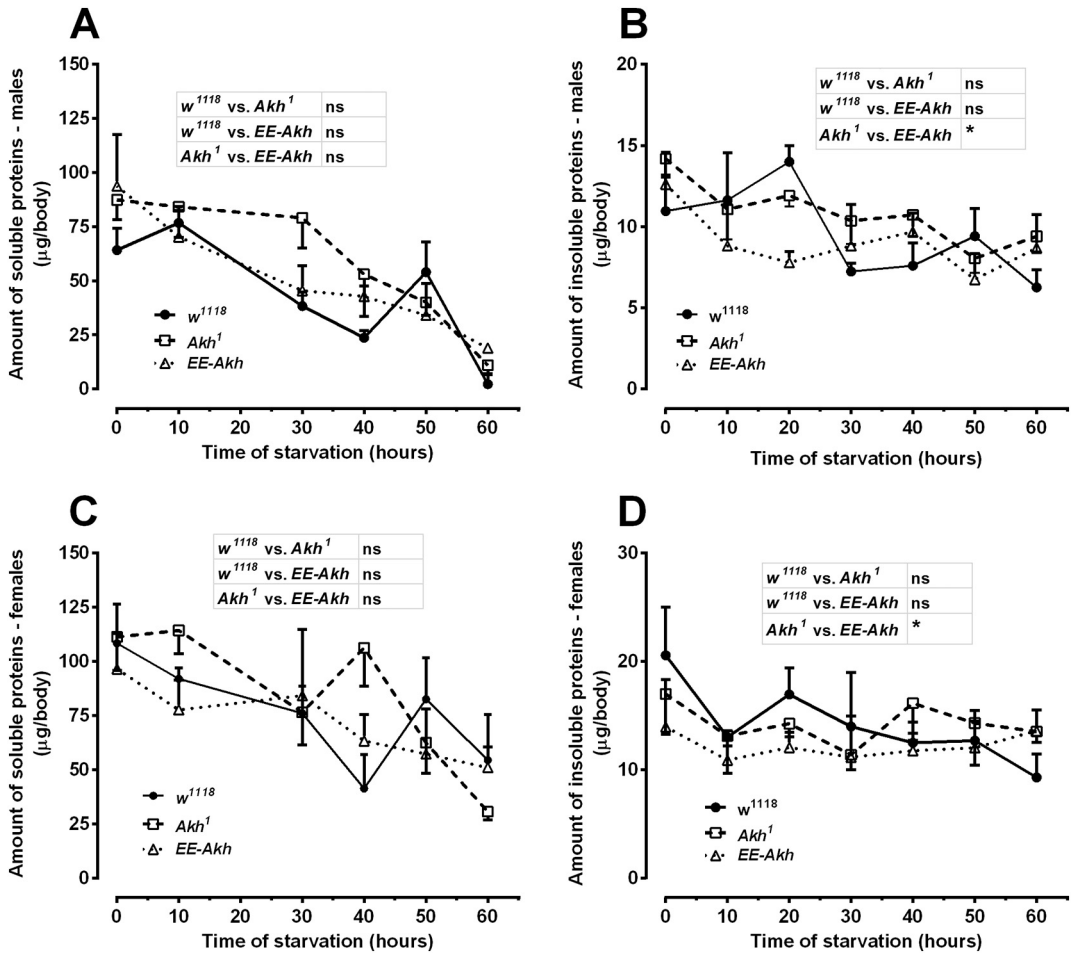

Fig. 9. Temporal course of the amount of soluble (A, C) and insoluble (B, D) proteins (μg/body) in w^{1118}, Akh^1 and *EE-Akh* 3-day old *Drosophila* adult males (A, B) and females (C, D) under starvation. Statistically significant differences among the mutants were evaluated by RM ANOVA (randomized block design) followed by Tukey's multiple comparison test; the differences at the 5% significance level are indicated by asterisk; $n = 5$.

survive for longer without food.

Akh and *Akh-receptor* mutants accumulate higher amounts of lipids and carbohydrates in their bodies (Bharucha et al., 2008; Baumbach et al., 2014a, 2014b) and are slightly heavier (Sajwan et al., 2015) than the corresponding controls. In agreement with this, Gáliková et al. (2015, 2017) clearly showed that mutations in *Akh* and *Akh-receptor* genes resulted in obese flies, and over-expression of those genes resulted in a reduced lipid level in the fly bodies. Accordingly, in this study, both male and female *EE-Akh* flies accumulated substantially lower levels of lipids; however, increased lipid accumulation has been recorded only in Akh^1 male flies, while, for females, the lipid level was almost identical in both groups. Nevertheless, a crucial role of AKH in the control of *Drosophila* lipid metabolism was confirmed during starvation experiments. Naturally, the lipid levels declined with increasing duration of food deprivation; however, the intensity of lipid utilization significantly differed among the mutants (with the exception of w^{1118} vs. Akh^1 males, where the difference was not significant). Stored lipids in *EE-Akh* flies with higher Drome-AKH level were relatively quickly utilized, being close to zero before the end of the observed period; this obviously resulted in higher mortality in *EE-Akh* flies, as discussed above. On the other hand, the lipid level in Akh^1 flies was the highest among all the tested groups for the longest duration during starvation. It seems evident that the AKH-deficient flies are not able to utilize the lipid reserves properly; however, it is not known whether any other factors are involved allowing higher survival of those flies. If so, it would not be surprising due to the critical importance of lipids for *Drosophila* energy metabolism (Slaidina et al., 2009; Lehmann, 2018).

For structural characterization of lipids, only the males of the studied fly groups were used due to the complexity of the employed analyses. We focused on TGs and DGs as energy rich metabolites. TGs serve primarily as reserve compounds stored mainly in the fat body in insects, and can be mobilized and utilized according to energy demands. DGs are the transport form of lipids and are present mainly in the hemolymph, where they are bound to carrier protein lipophorines (Arrese and Wells, 1994; Chino and Downer, 1982; Arrese et al., 1996). In those DGs, only relatively low number of fatty acids (FAs) - just 8–9, possessing 12–18 carbons (C) in molecules - represent the majority of the FA insect storage (Downer, 1985; Schneider and Dorn, 1994). Mobilization of lipids elicited by AKH activity is specific, in other words, the DGs with characteristic FAs are mobilized preferably (Tomčala et al., 2010; Bártů et al., 2010). In the fire bug, *Pyrrhocoris apterus*, the Pyrap-AKH injection preferably mobilized mostly DGs with C16 and unsaturated C18 FAs (Bártů et al., 2010). In the African migratory locust, *Locusta migratoria*, that possesses three intrinsic AKHs, AKH-specific FA mobilization has been recorded: Locmi-AKH-I preferred mobilization of unsaturated FAs (mostly linoleic acid), while AKH-II and AKH-III preferred saturated FAs (mostly stearic and myristic acids) (Tomčala et al., 2010). Recently, Fukumura et al. (2018) found that the AKH-receptor knock-down mediated by RNA interference ($AKHR^{RNAi}$) decreased the proportion of unsaturated FAs in the cricket *Gryllus bimaculatus* hemolymph, and correspondingly, AKH administration elicited the opposite effect. Interestingly, the ration of saturated and unsaturated FAs in the cricket hemolymph was modified also after the knockdown of a hormone-sensitive lipase by RNAi. It has been

found in this study that two dominant TGs (818 and 820 Da) contained just C16 FAs (palmitic and palmitoleic acids), while two dominant DGs (610 and 614 Da) contained those two FAs plus two C18 FAs (stearic and linoleic acids). Additionally, FA analysis of the whole pool of TGs and DGs revealed preferential mobilization of several C18 and C14 FAs in *Drosophila*. This suggests that the selective mobilization of particular FAs might be more common within insects and is probably species–-specific. However, the mechanism underlying this phenomenon or the reasons for it are not fully understood.

Glycogen definitely represents a less important reserve substance than lipids in *Drosophila*: its level was approximately an order of magnitude lower in these flies, but it was present in significantly higher amounts in the studied adult females than males. Some differences in glycogen level among 3-day old flies of w^{1118}, Akh^{1}, and *EE-Akh* mutants were observed; however, no significant changes of glycogen level among mutants were observed during starvation. Therefore, the role of AKH in control of its level in *Drosophila* is not entirely clear (see also below). Recently, it has been reported that the mobilization of fat body glycogen is AKH-independent in larvae (Gáliková et al., 2015), and as suggested by Yamada et al. (2018), the glycogen level is regulated by carbohydrate availability in a tissue-autonomous manner. Nevertheless, these facts do not reduce the importance of glycogen for maintaining a constant level of carbohydrates (trehalose, glucose) in *Drosophila* larval hemolymph. Yamada et al. (2018) have also found a correlation between the carbohydrate level and level of fat body glycogen during larval starvation. The present study showed a relatively quick depletion of the glycogen reserves (unlike lipids with the exception of *EE-Akh* mutants) in the body to almost zero after about 30 h of starvation; however, the level of hemolymph carbohydrates was still relatively high at that time, indicating that the carbohydrate level may be maintained at the expense of non-glycogenic sources (see also below).

As noted for glycogen, the levels of free carbohydrates measured in the whole body also showed some differences among the 3-day old adult mutants, but these differences probably also reflected the current needs of the organism and not only the AKH-dependent status. In several previous studies (Lee and Park, 2004; Kim and Rulifson, 2004; Isabel et al., 2005; Sajwan et al., 2015), the level of free carbohydrates was determined in larval hemolymph: their lower level was recorded in AKH-deficient larvae, and higher level, in larvae with normal or higher AKH production. The authors explained these results by difficulties in the mobilization of glycogen stores in the former larvae with the absence of natural Drome-AKH, and by facilitated mobilization by the AKH abundance in the latter group. Unfortunately, determination of free carbohydrates (as well as any other substances) in adult fly hemolymph is very difficult - almost impossible to perform routinely – due to the low amount of the hemolymph in adult fly bodies. Therefore, we have measured free carbohydrate levels in the whole bodies, where the results were apparently affected by carbohydrates present inside the cells. This was probably one of the reasons why no differences in free carbohydrate levels within the mutants during the starvation were recorded. As already mentioned above, around the 30th hour of starvation, an obvious peak of the carbohydrate level was recorded in all the studied fly groups. The true reasons for that are unclear, thus, one can only speculate that almost total depletion of glycogen reserves at that time might play a role. To restore a deepening disruption of free carbohydrate homeostasis in the body, the metabolism has to switch to the utilization of another storage substrate which might be responsible for the increase of the carbohydrate level; the involvement of other signaling pathways or controlling mechanisms (Lehmann, 2018) also cannot be excluded.

As expected, the levels of Drome-AKH in all the tested stages (larvae, males, females) of the Akh^{1} mutant were negligible (for details see Zemanová et al., 2016). Further, in *EE-Akh* mutants, the hormonal levels were higher in all the three tested stages (about 1.7 times each) than in the corresponding w^{1118} controls. Interestingly, *Akh* gene expression recorded in the *EE-Akh* flies previously was almost 500-fold higher than that in w^{1118} controls (Zemanová et al., 2016). Thus, it appears that the *Akh* gene expression is not directly correlated with AKH peptide synthesis, similarly as there is no apparent coupling between AKH biosynthesis and its release into the hemolymph (Diederen et al., 2002). Further, the lower level of Drome-AKH in *Drosophila* larvae might be in accordance with the observation of Gáliková et al. (2015) that AKH signaling is dispensable for mobilization of nutrients until the end of *Drosophila* metamorphosis. On the other hand, the role of Drome-AKH in the control of lipid and carbohydrate metabolism in *Drosophila* larvae has been recorded previously (Lee and Park, 2004; Isabel et al., 2005; Nässel and Winther, 2010; Sajwan et al., 2015). Thus, it seems that the energy metabolism and its control is not completely clear in *Drosophila* larvae at present, and further research is necessary for better understanding.

In summary, this paper demonstrates changes in the metabolism of basic nutrients depending on the level of Drome-AKH and on starvation in *Akh* mutants of *Drosophila.* Lipids seem to be the main storage substances, and their level and metabolism is under AKH control. AKH abundance in the body results in lower levels of TGs and DGs than in controls, probably due to the more intensive metabolism. Lower level of glycogen in the *Drosophila* body and its partial control by AKH suggest its lesser role as the storage substance. On the other hand, glycogen apparently plays a critical role in maintaining free carbohydrate homeostasis. However, when glycogen is depleted, other sources are probably employed for free carbohydrate production. Levels of proteins and any changes in their levels under starvation do not appear to be under AKH control. Flies with AKH deficiency are more resistant, and flies with AKH abundance are more sensitive to starvation; females are more resistant than males to starvation regardless of the AKH level probably due to higher body mass and greater amounts of nutrients. The level of AKH in accordance with the level of all nutrients gradually decreases with increasing time of starvation.

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