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DEVELOPMENTAL DIET DEFINES METABOLIC TRAITS IN LARVAE AND ADULT *DROSOPHILA*

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The influence of the developmental nutrition on adult metabolism and overall performance becomes a hot topic of modern evolutionary biology. We used fruit fly Drosophila melanogaster as a model and experimental nutrition media composed of different sucrose content (S) and dry yeast content (Y): 0S:2Y, 20S:2Y or 0S:5Y, 20S:5Y to show that the developmental nutrition conditions define metabolism in larvae and adults. The level of glucose, glycogen, triglycerids and total lipids in the larvae and flies body were measured with the diagnostic assay kits. We found that individuals developed on either low-yeast or high-sugar diet showed delayed developmental rate. When kept on the diets with high sucrose content the larvae and adult flies had lower weight and higher amount of lipids as energy reserves. Restriction of dry yeast content in the diet of larvae led to a decrease in glycogen storage and protein levels in larvae and adult flies. The results obtained indicate that the metabolic traits revealed in adult flies are the result of nutrition during development and may be associated with mechanisms of organisms adaptation to the developmental nutritional conditions.

K e y w o r d s: metabolic traits, development, nutrition, diet, glucose, glycogen, triglycerids, fruit fly.

Dietary conditions experienced early in life has long attracted attention in evolutionary biology. Developmental nutrition as the most critical factor profoundly affects health and physiology in adult life. The consequences of nutrition for animal growth, development and life history change with the developmental stage. Substantial evidence has demonstrated that both maternal over- or undernutrition increase the risk of metabolic disorders in offspring [1, 2]. However, the mechanisms are yet to be fully elucidated.

The fruit fly *Drosophila melanogaster* has been increasingly used as a model organism in nutrition research. Many different recipes of media for the *D. melanogaster* have been described in the scientific literature [3]. Modeling dietary nutrients affect the food intake, body composition, locomotor activity, intestinal barrier function, microbiota, cognition, fertility, aging, and lifespan of flies [4]. Maternal and developmental dietary conditions define the development of the embryo and also influence mature life stages in *Drosophila* [5]. Numerous studies in *Drosophila* have previously shown that developmental dietary conditions affect adult physiology and metabolism. Indeed, larvae nutrition influences body size and reproduction without affecting aging-related mortality [6]. Starvation during third instar larvae in *Drosophila* causes small body size, delayed eclosion, reduced fecundity and results in the production of adults with fewer ovarioles [5]. An impact of the type and amount of monosaccharides in the larval diet was shown for antioxidant system and body composition and metabolism in adult flies [7-10]. It was also analyzed some developmental, morpho-

logical, and physiological traits in response to larval exposure to either commercial diets: Formula 4-24 media or Jazz mix [11] or dietary nutrients content and significant impact of protein-to-carbohydrate ratio [12]. Nevertheless, it is therefore still open questions, which macronutrient (sucrose or yeast) in the developmental diet has the greatest impact on adult fly physiology; whether diet-induced effects on fly metabolic traits represent adaptive responses to the developmental diet.

In this study, we employed a complex experimental design with all combinations of four developmental diets that differed in the macronutrient composition. We aimed to explore (1) the impact of yeast (the source of amino acids, vitamins) and sugar concentration in the larvae diet on the metabolism of larvae and imago, (2) the coordination between larvae and imago metabolic traits, (3) establish whether investigated traits is affected by specific nutrient content or by macronutrient balance. We found that nutritional conditions experienced exclusively during development affect body weight, development rate and metabolism of both larvae and adults.

Material and Methods

Fly strain and husbandry. Canton-S (*D. melanogaster* Meigen) flies, obtained from the Bloomington Stock Center (Indiana University, USA), were used in the current study. Flies were maintained at 25°C, relative humidity of 60-70% and 12:12 h day/night cycle on a diet medium containing 7.5% (v/v) molasses, 5% (w/v) dry yeast, 6.1% (w/v) corn, 1.2% (w/v) agar, 0.3% (v/v) propionic acid and 0.18% (v/v) nipagin as anti-fungal and anti-bacterial agents [13].

Experimental procedures. Flies aged 3-7 days were subjected to 3-hour starvation with subsequent 15-hours eggs-laying on the medium composed of 5% sucrose and 2% agar. To prevent effects caused by larvae density, laid eggs were washed three times with distilled water, then concentrated in a volume of 1.5 ml and transferred into bottles containing 25 ml of experimental medium (150-250 eggs) [14]. Experimental media were composed of different sucrose content (S) and dry yeast content (Y): 0S:2Y, 20S:2Y or 0S:5Y, 20S:5Y. Larvae were allowed to develop, pupate and eclose. Eclosed flies were transferred on the fresh food of the same composition and held for the two days. Two-day-old flies were separated by sex and frozen in liquid nitrogen for further biochemical assays.

Development rate. The number of pupae formed from larvae fed experimental media was counted every 6/6/12 hours (at 9 am, 3 pm, 9 pm) until the end of pupation.

Larvae and flies weight. We assessed the wet larval weight and total dry body weight of adult flies. For measurement body weight 20 flies were weighted using WTW 2 balance (Techniprot, Pruszkow, Poland) and transferred to a 0.5 ml plastic vial with the cut bottom. The weighted flies were dried for two days at 60°C with the subsequent weighting for the dry body mass. To determine wet larvae weight the WTW 2 balance was also used.

Hemolymph extraction. Larvae hemolymph was extracted by making a small cuticular tear just in front of the caudal spiracles. Anesthetized flies were pierced in the thoracic segment of the body. Next, either larvae or flies were placed in a plastic tube (0.5 ml with a hole on the bottom) placed in a larger tube (1.5 ml) with further centrifugation (7000 g, 8 min, 21°C). Collected hemolymph was treated with 0.154% dithiothreitol. The hemolymph was deproteinized by heat treatment at 70°C for 5 min with followed centrifugation (16 000 g, 15 min, 4°C). Supernatants were used to measure circulating glucose.

Glucose and glycogen assay. Pre-weighted bodies were homogenized in 50 mM sodium phosphate buffer, centrifuged (16 000 g, 15 min) and used for determination of body glucose and glycogen levels [15]. Extracted hemolymph was used to measure circulating glucose levels. Measurements were performed using a glucose assay kit (Liquick Cor-Glucose diagnostic kit, Cormay, Poland) following manufacturer instructions. Glycogen was converted into glucose by incubation with amyloglucosidase from *Aspergillus niger* (25°C, 4 h) (Sigma-Aldrich, Chemie GmbH Germany). For triglycerides (TAG) determination flies were weighed, homogenized in 200 mM PBST (phosphate buffered saline containing 0.05% Triton X100), boiled and centrifuged (13 000 g, 10 min) [13, 16]. The resulting supernatants were used for TAG assay with the Liquick Cor-TG diagnostic kit (Cormay, Poland).

Total lipids assay. Total lipid content was determined as was described previously [17] using diagnostic kit BIO-TEST TL180 (Erba Lachema s.r.o., Czech Republic). Flies were homogenized in ice-cold ethanol 96% in a ratio 1:20 (fly weight:ethanol volume). The homogenates were supplemented with an equal volume of chloroform and centrifuged (8 000 g, 2 min, 21°C). Next, 40 µl of su-

pernatants were maintained on the ice until complete evaporation of the solution followed by hydrolysis of the residue by 200 µl of H_2SO_4 (95%) with subsequent incubation under 95°C for 20 min. After cooling, 200 µl of diagnostic reagent was added. A standard calibration curve with different oleic acid concentrations was built to calculate total lipid content. The absorption of the samples was determined using Specoll-211 at 540 nm.

Total protein assay. The concentration of total protein was measured according to the Bradford method [18]. The amount of protein was determined in supernatants with Coomassie Brilliant G-250 dye at 595 nm using bovine serum albumin as a standard.

Statistical analysis. Data are presented as mean ± SEM. Data were analyzed using Holm-Sidak's (H-S) test. Differences between groups were considered statistically significant when $P < 0.05$. Log-rank test was used to analyze development curves. Graphing and statistical analysis were performed by using GraphPad Prism.

Results

Drosophila larvae pupated slower under high sucrose and low protein conditions. We first tested whether larvae dietary conditions influence the developmental rate in *Drosophil*a. In the current study, sucrose as a dietary component significantly decreases pupation rate. Pupation rate was lower under 20S-2Y as compared to 0S-2Y (log-rank test, $P < 0.0001$; $\chi^2 = 2315$). Similarly, we found decelerated pupation under 20S-5Y as compared to 0S-5Y (log-rank test, $P < 0.0001$; $\chi^2 = 951.4$). Flies reared on the medium with low dry yeast concentration displayed developmental delay. Indeed, we observed retardation in development on 0S-2Y as compared to 0S-5Y (log-rank test, $P < 0.0001$; $\chi^2 = 1063$). Slower development was also found in larvae reared on 20S-2Y as compared to 20S-5Y (log-rank test, $P < 0.0001$; $\chi^2 = 392.4$).

Dietary conditions experienced during development profoundly affect larvae body weight. Larvae reared on high sucrose diet displayed lower body weight. Under 0S-2Y diet larvae weight was about 1.5 mg. Consumption of a diet 20S-2Y led to 38% lower larvae weight as compared to 0S-2Y diet (Fig. 1, *B*; H-S test, $P = 0.0002$). Similarly, 15% lower larvae weight was observed under 20S-5Y as compared to 0S-5Y (Fig. 1, *B*; H-S test, $P = 0.0012$). Larvae body weight was not affected by dry yeast concentration in the diet.

Larvae metabolites pool is affected by dietary nutrients. Sucrose concentration in the developmental diet influence the level of circulating glucose in larvae. The concentration of glucose in hemolymph was 17% higher in 20S-2Y consuming cohorts as compared to 0S-2Y (Fig. 2, *A*; H-S test, $P = 0.036$). Similarly, development on the 20S-5Y diet induced a tendency to higher circulating glucose. Larvae displayed two-fold higher hemolymph glucose level as compared to 0S-5Y (Fig. 2, *A*; H-S test, $P = 0.0004$). Glycogen level was dependent on sucrose concentra-

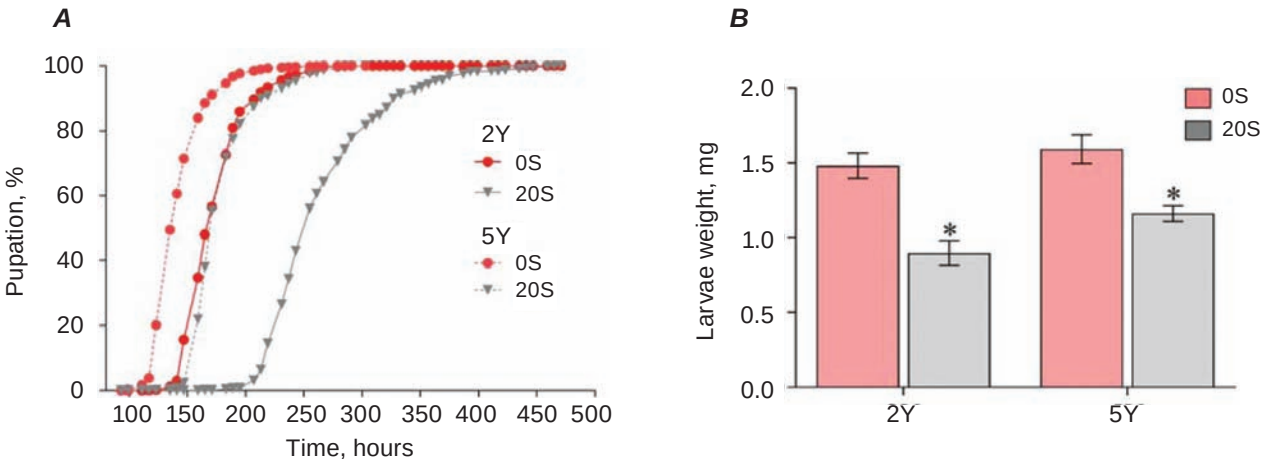

*Fig. 1. Developmental rate (**A**) and larvae weight (**B**) of Drosophila reared on media composed of either 2% or 5% of yeast content (Y) and sucrose (S) at concentrations: 0% and 20%. Graphs show the percentage of larvae that pupated over time. Data are mean ± SEM, n = 6. Group comparisons were performed using Holm-Sidak's test. Asterisk indicates a significant difference between groups with P < 0.05*

tion in the larvae diet. Larvae reared on 20S-2Y diet had 40% lower glycogen level as compared to 0S-2Y (Fig. 2, *C*; H-S test, $P = 0.0026$). Moreover, we found 49% lower glycogen content in larvae reared by 20S-5Y as compared to 0S-5Y (Fig. 2, *C*; H-S test, $P = 0.0019$). Total lipids (TL) were higher under high sucrose diets. Indeed, larvae showed 2.3-fold higher TL content when fed by 20S-2Y diet as compared to 0S-2Y (Fig. 2, *E*; H-S test, $P < 0.0001$). Similarly, development on the 20S-5Y diet led to a 1.7-fold higher TL level as compared to 0S-5Y (Fig. 2, *E*; H-S test, $P= 0.0008$). The level of protein in the larvae body was not affected by dietary sucrose, however, significantly depended on yeast concentration in the diet. We found a 12.5-fold higher protein level in larvae reared on the diets with 5% of dry yeast concentration as compared to 2% (Fig. 2, *A*; H-S test, $P < 0.0001$). Larvae body glucose and triglycerides levels were not affected by sucrose or yeast concentration in the diet (Fig. 2, *B*, *D*).

Effects of developmental diet on carbohydrate metabolism in adult Drosophila. *Drosophila* larvae were reared on fly food prepared at various concentrations of either sucrose or yeast (for details see Materials and methods) to study the effects of larvae diet on adult metabolism. Flies that emerged from larvae reared on various dietary conditions were aged for two days and various metabolic parameters were analyzed in these adult flies. Interestingly, both male and female flies that developed on 20S-2Y displayed a higher level of hemolymph glucose as compared to 0S-2Y (Fig. 3, *A*, *B*; H-S test, $P < 0.0001$). However, under 5% of yeast in the diets we did not observe any significant impact of sucrose content on hemolymph glucose level (Fig. 3, *A*, *B*). Higher body glucose level (by 26%) was observed in males developed on 0S-5Y as compared to 0S-2Y (Fig. 3, *C*; H-S test, $p = 0.0183$). Body glucose in females was not affected by nutritional conditions (Fig. 3, *D*). Glycogen level was significantly reduced in flies of both sexes developed on 0S-2Y as compared to 0S-5Y (Fig. 3, *E*, *F*; H-S test, $P < 0.0004$). We also observed enhanced glycogen stores in males fed by 20S-2Y as compared to 0S-2Y and 20S-5Y (Fig. 3, *E*; H-S test, $P < 0.0004$). Consumption of 20S-5Y diet at larvae stage led to lower glycogen content in

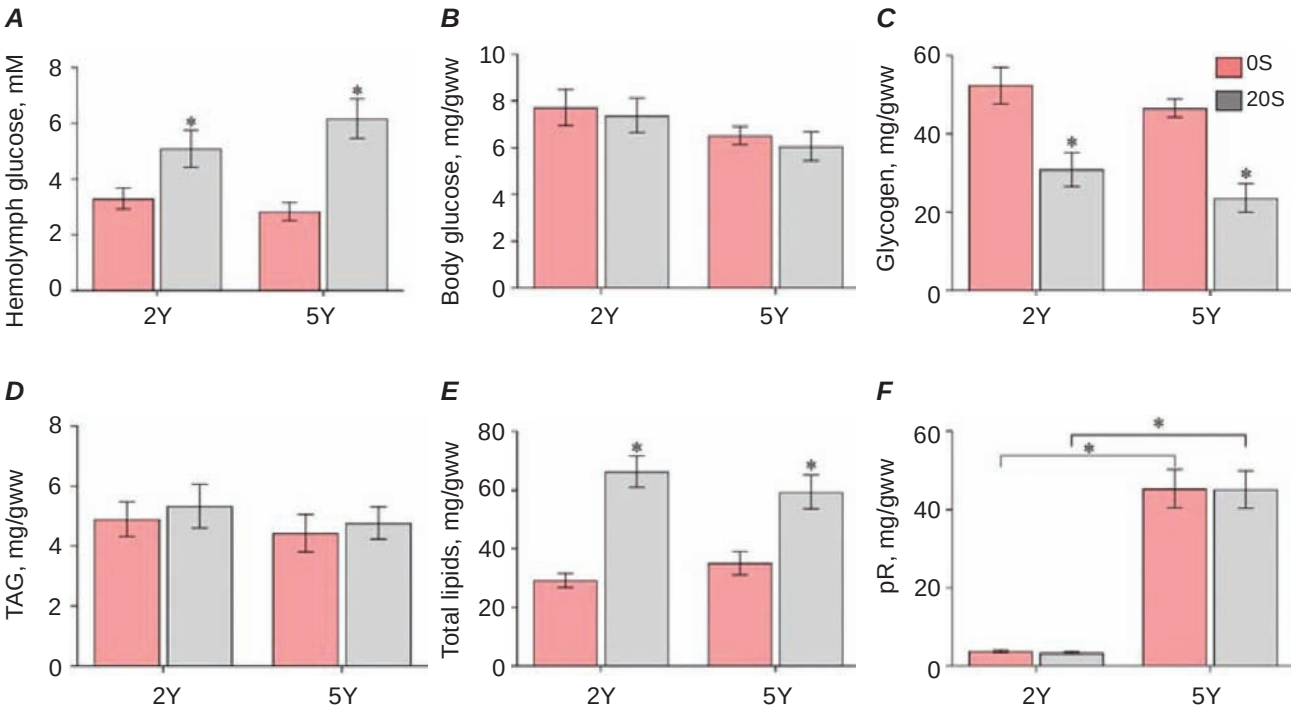

*Fig. 2. The levels of hemolymph glucose (**A**), body glucose (**B**), glycogen (**C**), triglycerides (**D**), total lipids (**E**) and protein (**F**) in Drosophila larvae that were reared on media composed of either 2% or 5% of dry yeast content and sucrose at concentrations 0% and 20%. Results represent the mean ± SEM of 4-6 replicates per group. Group comparisons were performed using Holm-Sidak's test. Asterisk indicates a significant difference between groups with $P < 0.05$*

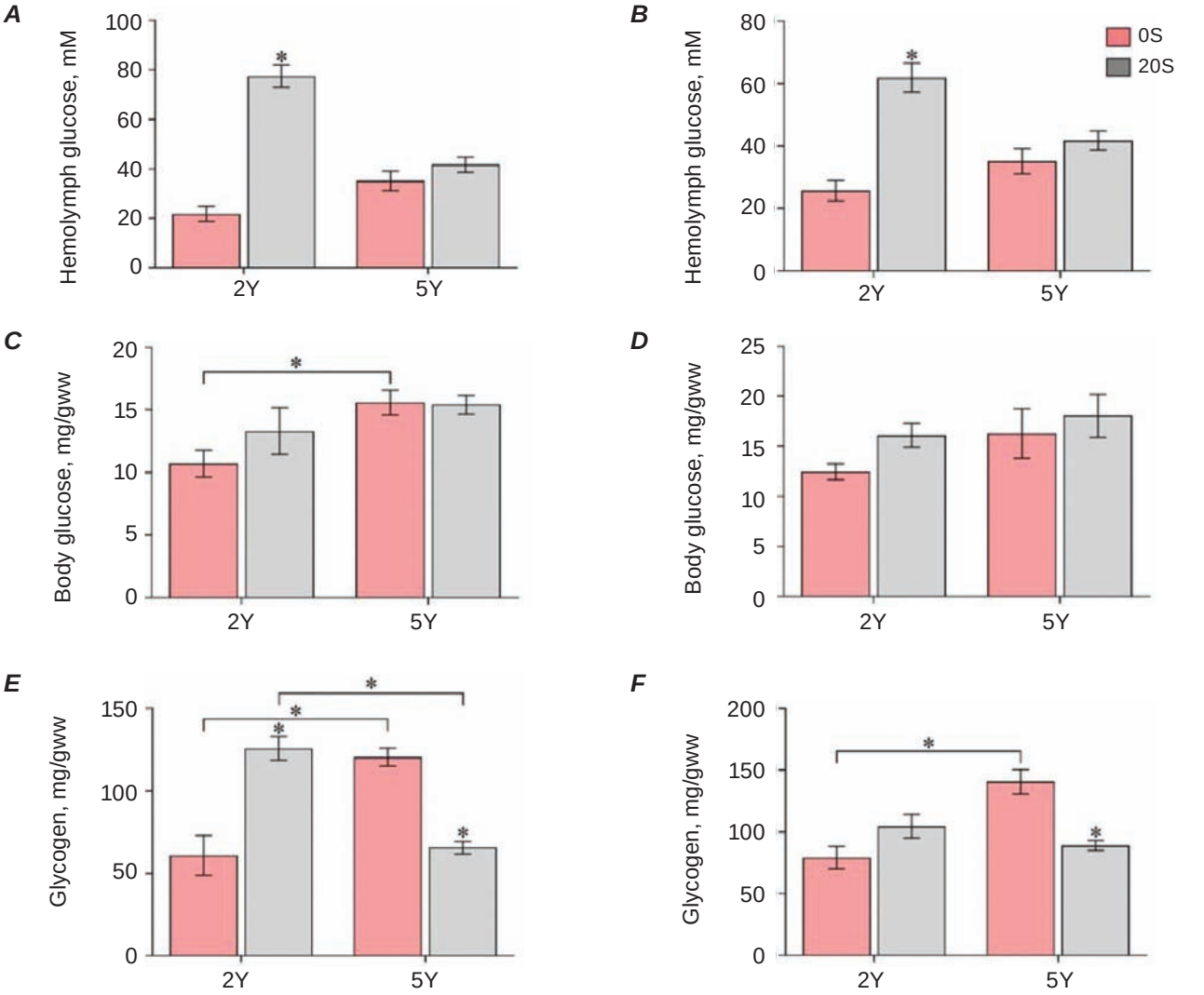

*Fig. 3. Levels of hemolymph glucose (**A** – males; **B** – females), body glucose (**C** – males; **D** – females) and glycogen (**E** – males; **F** – females) in adult Drosophila that were developed on food composed of either 2% or 5% of dry yeast content and sucrose at concentrations 0% and 20%. Results represent the mean ± SEM of 4-6 replicates per group. Group comparisons were performed using Holm-Sidak's test. Asterisk indicates a significant difference between groups with $P < 0.05$*

flies of both sexes as compared to 0S-5Y (Fig. 3, *E*, *F*; H-S test,$P = 0.0004$).

Effects of developmental diet on body weight, lipid and protein metabolism in adult Drosophila. We found that fly body weight, TAG and TL levels are more sensitive to sucrose concentration in the diet. Development on the sugar enriched diets significantly decreased the body weight of both males and females (Fig. 4, *A*, *B*; H-S test, $P < 0.005$). However, consumption of the 20S-2Y diet led to a 1.6-2-fold higher TAG level in flies of both sexes as compared to the 0S-2Y diet (Fig. 4, *C*, D; H-S test, $P < 0.009$). Additionally, 42% higher TAG content was found in males developed on a 20S-5Y diet as compared to 0S-5Y (Fig. 4, *C*; H-S test, $P = 0.0088$). High-sugar diet increased the amount of TL content. Indeed, TL level was approximately two-fold higher in flies of both sexes that developed on either 20S-2Y or 20S-5Y as compared to 0S-2Y or 0S-5Y, respectively (Fig. 4, *E*, *F*; H-S test, $P < 0.0006$).

Sugar level in the developmental diet on showed no effect on protein content. The flies of both sexes that had developed on the yeast-poor diet with 2% yeast had 10-12-fold lower protein content as compared to 5% irrespective of adult nutrition (Fig. 4, *G*, *H*; H-S test, $P < 0.0001$).

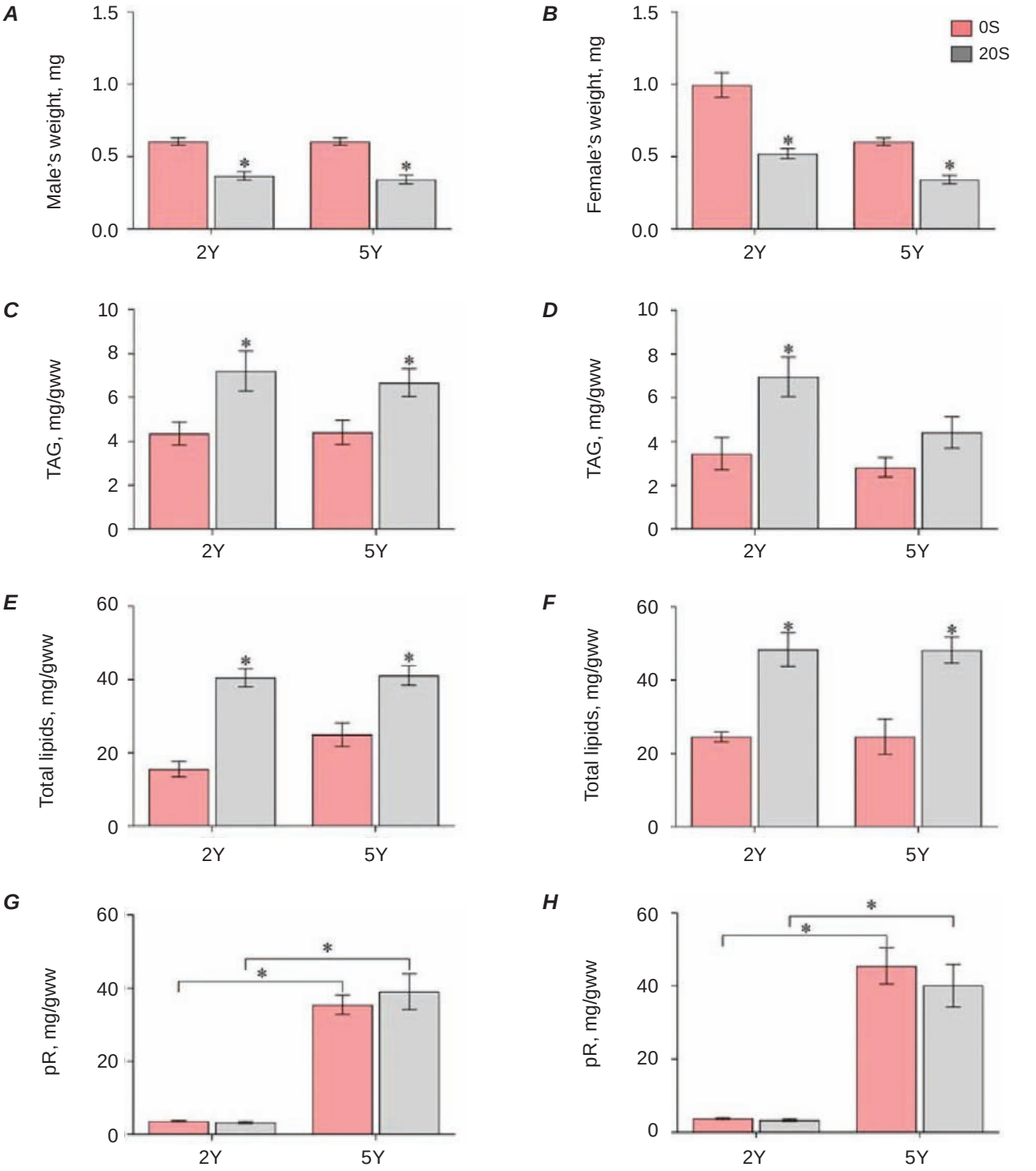

*Fig. 4. Dry body weight of (**A** – males; **B** – females) and the level of triglycerides (**C** – males; **D** – females), total lipids (**E** – males; **F** – females) and protein (**G** – males; **H** – females) in two-day-old flies Drosophila, developed on food composed of either 2% or 5% of dry yeast content and sucrose at concentrations 0% and 20%. Results represent the mean ± SEM of 4-6 replicates per group. Group comparisons were performed using Holm-Sidak's test. Asterisk indicates a significant difference between groups with $P < 0.05$*

Discussion

Macronutrients have a significant impact on life-history traits, such as disease vulnerability, reproduction, longevity and stress resistance in *Drosophila* [19, 20]. Proteins and carbohydrates are the most considerable macronutrients that have an equivalent caloric value. Proteins provide amino acids that are mostly used as building blocks for cells, tissues, organs, enzymes and other regulatory proteins. Carbohydrates are the nutrients mostly used as an energy source for the biosynthetic processes; carbohydrate excess is converted into fats [21]. Changes in the content of specific nutrient lead to alteration the relative balance between macronutrients in the diet that substantially affect lifespan and functional senescence in fruit flies [22].

In this study, we combined high or low sucrose content with either high or low yeast to investigate the impact of both macronutrients on various life-history traits expressed at larvae and adult stages in *Drosophila*. Furthermore, our results indicated that the development rate responds significantly to the ratio of proteins-to-carbohydrates (P:C). Indeed, we observed a similar trend in development between larvae fed by either 0S-2Y or 20S-5Y, suggesting that change in P:C ratio has primary importance to the *Drosophila* development rather than single nutrient concentration. We observed acceleration of development rate under high P:C ratio, and delay of development rate under low P:C ratio. Previous studies demonstrated the effects of P:C ratios of developmental diets on egg-to-adult viability and starvation resistance, however, had no lasting effects on *Drosophila* lifespan [23]. Based on our data, low-yeast or high-sucrose diets retard developmental rate. Indeed, previous studies demonstrated a decreased speed of larvae development under a high-sugar diet and increased pupa mortality [15, 24]. Our results are in good agreement with the previous data that demonstrated increased pupation time as an effect of low protein concentration in the diet [25]. Moreover, delayed development is associated with increased pupation height, higher egg-to-adult development duration and adult lifespan extension under a low-protein diet [25]. However, low-protein high-carbohydrate promotes long life in fruit flies [26, 27]. It is worth noting that low-protein high-carbohydrate diets are associated with a reduced mitochondrial number [28] that is consistent with the inactivation of target of rapamycin (TOR) pathway. Juvenile hormone (JH) and 20-hydroxyecdysone (20-HE) are involved in the regulation of developmental processes in insects [29, 30]. The release of JH and 20-HE was shown to be regulated by nutrients via nutrient-sensing signaling pathways including insulin and TOR [31, 32]. Moreover, high-sugar diet decreases the size of both larvae and adult due to insulin resistance [33]. Consistent with these previous results, we found that both larvae and adults displayed lower body weight when fed high sucrose diets regardless of yeast content.

Flies fed a high-sucrose diet displayed health deterioration that is associated with obesity-related metabolic phenotype [24] that, in turn, led to lifespan shortening. Indeed, we observed significantly higher hemolymph glucose levels in both larvae and adult flies under a high-sucrose developmental diet. However, high sucrose content which is accompanied with high yeast content in the diet had no impact on hemolymph glucose level as compared to sucrose-free diet. Hence, the glucose level is affected primarily by the high carbohydrate-to-protein ratio. It was previously shown that larvae ingested more carbohydrates on a high-sucrose diet despite consuming less food and obtaining less protein derived from yeast [15]. On the low-sucrose diet larvae consumed more food causing yeast overeating [15]. Carbohydrate restriction that is accompanied by yeast overfeeding during larvae stages resulted in oxidative stress that is associated with increased superoxide dismutase (SOD) activity, higher levels of oxidized lipids and proteins [9].

Glucose excess gets stored in the fat body as glycogen or, with the help of insulin, is converted into fatty acids stored as fat in adipose tissue [34]. High dietary sugar leads to the enhanced expression of glycogen synthase (GlyS). Moreover, GlyS depletion from the larvae fat body causes development retardation under a high-sugar diet [34, 35]. The enhanced relative expression of glycogen phosphorylase, which is involved in glycogenolysis – glycogen breakdown, was demonstrated under a high-sugar diet [24]. It is known that glycogen metabolism and lipid biosynthesis are interrelated processes. It was shown that inhibition of lipid biosynthesis results in enhanced glycogen levels in larvae [35]. Indeed, we also observed lower glycogen content in individuals with higher TAG and total lipid content.

Similar to previous studies, our data show that consumption of the diet with high sucrose amount maximized the triglycerides and total lipid contents in both larvae and adults [36]. Feeding with a high-

sugar diet leads to significantly larger lipid droplets of fat bodies than those in the low-sugar group [36]. When dietary protein is scarce the insects consume larger amounts of calories that will store as fat and lead to obesity phenotype [37]. Moreover, larvae diet influenced the expression of brummer and lipid storage droplet-2 genes that are involved in fat mobilization in adult flies [38, 39]. Larvae are more sensitive to the toxic effect of high sugar concentration in the diet as compared to adults [40]. Furthermore, exposure to malnutrition at juvenile stages has more effects on the life history traits as compared to adults [41].

Development on the diet with high sucrose content results in elevated dilp3 relative expression [15] that suggests about the involvement of insulin/insulin-like growth factor (IGF-1) signaling (IIS) in mediating the link between developmental diet and adult health. Nutrients regulate IIS by controlling the expression of insulin-like peptides (DILPs) [42, 43]. Restriction of nutrients that is associated with IIS inhibition improves healthspan and extends lifespan in fruit fly [44]. Opposing effects of dietary sugar and yeast suggest the involvement of distinct mechanisms in the regulation of physiology by macronutrients. Target of rapamycin pathway is involved in sending the intracellular and extracellular levels of macronutrients including amino acids [20].

Conclusion. We found that carbohydrate content in the developmental diet has a more significant influence on larvae and adult metabolism as compared to dietary yeast. We observed similar trends in the studied traits between larvae and adults. Forced development, higher circulating glucose, an increased pool of TAG and total lipid and reduced body weight indicate that high sucrose content and decreased protein-to-carbohydrate ratio provide unfavorable developmental conditions (Fig. 5). We also showed the contribution of macronutrient balance to development; acceleration of developmental rate was found under high P:C ratio, and delay of development rate observed under low P:C ratio. Given the strong evolutionary conservation of nutrient-sensing system, our results provide a mechanistic framework that focuses on the diet with high carbohydrate content or low P:C ratio as a means of impairing

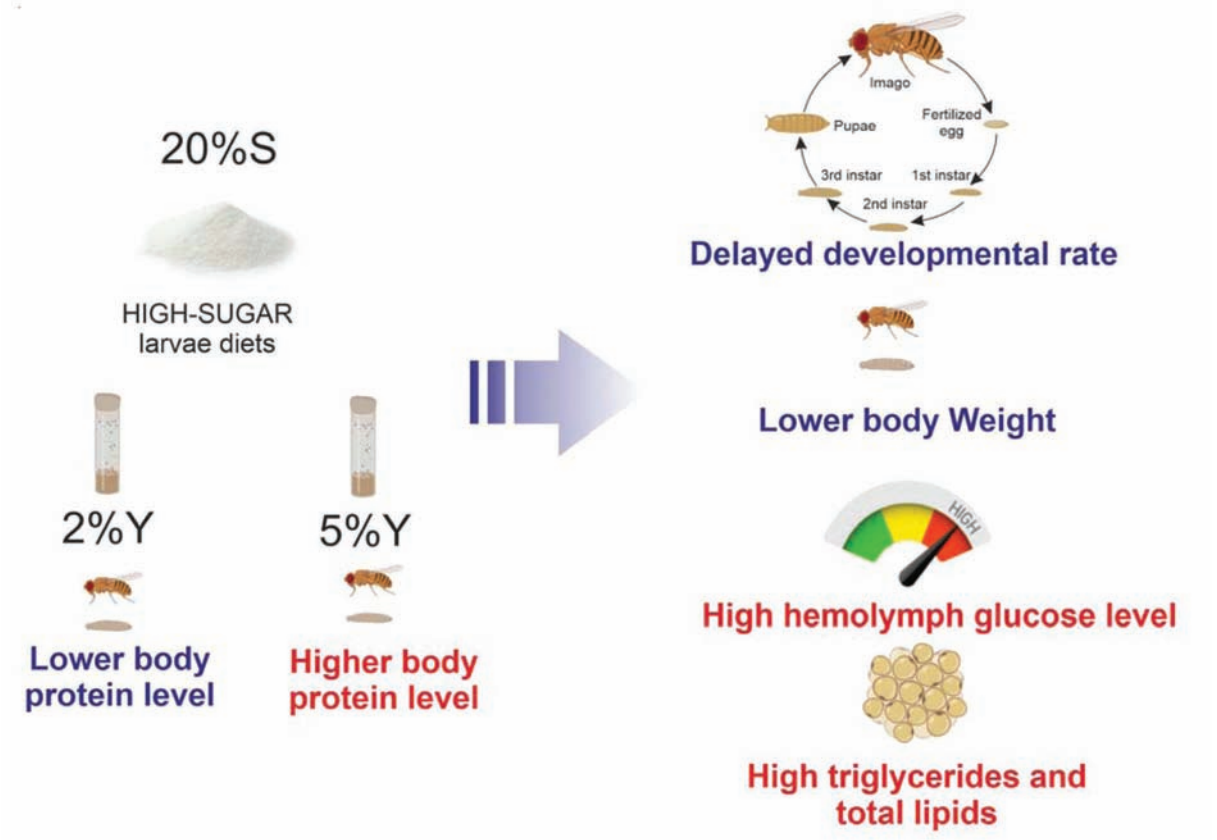

Fig. 5. Effects of high-sugar developmental diets on physiological and metabolic traits of larvae and imago Drosophila

organismal health, which may be further translated into preclinical studies with subsequent practical interventions. However, the specific mechanism requires further exploration for better understanding these complex phenotypes in humans.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

ДІЄТА ПІД ЧАС РОЗВИТКУ ВПЛИВАЄ НА МЕТАБОЛІЧНІ ПОКАЗНИКИ ЛИЧИНОК ТА ДОРОСЛИХ МУХ *DROSOPHILA*

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Залежність метаболізму та фізіологічного стану дорослого організму від харчування під час його розвитку стає гарячою темою сучасної еволюційної біології. Ми використали плодову мушку *Drosophila melanogaster* та живильне середовище з різним вмістом сахарози (S) і сухих дріжджів (Y): 0S:2Y, 20S:2Y або 0S:5Y, 20S:5Y, щоб показати, що умови харчування під час розвитку впливають на обмін речовин у личинок та імаго. Рівень глюкози, глікогену, тригліцеридів та загальних ліпідів в організмі личинок та дорослих мух вимірювали за допомогою діагностичних наборів. Виявлено затримку розвитку дрозофіл, які розвивалися на дієтах із низьким вмістом дріжджів або з високим вмістом сахарози. Показано, що утримання личинок на харчовому раціоні з високим вмістом сахарози спричиняло зниження маси та збільшення енергетичних запасів (кількості ліпідів) у личинок та дорослих мух. Обмеження вмісту сухих дріжджів у дієті личинок призводило до зниження накопичення глікогену та рівня протеїнів у личинок та дорослих мух. Виявлені особливості метаболізму дорослих мух є результатом харчування під час розвитку і можуть бути пов'язані з механізмами адаптації організму до умов живлення.

Ключові слова: метаболічні показники, розвиток, харчування, дієта, глюкоза, глікоген, тригліцериди, плодова мушка.

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