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PROTEIN CONTENT IN THE PARENTAL DIET AFFECTS COLD TOLERANCE AND ANTIOXIDANT SYSTEM STATE IN THE OFFSPRING *DROSOPHILA*

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Dietary nutrients are the key determinants of the lifespan and metabolic health. The content of specific dietary compounds in the parental diet can epigenetically affect the physiological state of the offspring. Here, we studied how variable dietary protein content in the diet of parental generation affects antioxidant capacity of Drosophila melanogaster adult offspring. The dry yeast concentration ranging from 0.25% to 15% in the parental diet was the only variable in the experiments, whereas subsequent generation was kept on a diet of the same composition. We found, that flies fed with yeast-restricted (0.25%) diet produced F1 male flies with a higher cold tolerance and higher activity of the second-line antioxidant enzymes whereas in F1 females no effect of parental diet composition on the cold tolerance, catalase, GST, G6PDH, IDH activity and low thiols content was detected. The results suggest that nutrient-dependent changes of genes expression in the flies of paternal generation differently affect the stress response of males and females of the first-generation offspring.

K e y w o r d s: dietary protein content, Drosophila melanogaster, antioxidant enzymes, cold tolerance, parental generation, offspring.

Dietary conditions define the behavior of animals across many phyla. The fruit fly *Drosophila melanogaster* has been extensively used in metabolism and physiology studies [1] because of their ability to adapt and survive on a wide-variety of food sources. Moreover, there are many different recipes for a complex media described in the scientific literature. Dietary composition, the content of macronutrients, namely carbohydrates and protein, was demonstrated to determine life span in fruit flies [2-5]. Dietary yeast is consider to be a major source of essential nutrients including protein, carbohydrates, vitamins for the development of larvae and reproductive processes in adults *Drosophila* [6]. Hence, yeast concentration in the diet is the primary determinant of metabolic, physiological traits and longevity [7].

In recent years, the consequences of parental diet on the offspring physiology and metabolic traits have become a hot topic for the investigations [5, 8-10]. Ease of dietary manipulation, a short generation time, a high fecundity, a relatively short life expectancy, conserved metabolic signaling pathways, make *Drosophila* an excellent model organism to unravel mechanisms of non-genetic inheritance of dietary effect. Indeed, previous studies demonstrated, that parental nutrition may affect the body size, development, egg size, fertility, metabolism, life span of the next generation *Drosophila* [5, 8, 10]. In order to elucidate the molecular mechanisms, that can mediate the impact of parental diet on the offspring phenotypes, a variety of epigenetic mechanisms have been identified [11, 12]. The phrase "transgenerational epigenetic inheritance" was used by Deas and colleagues to indicate heritable alterations of phenotype which are not accompanied by a change in the DNA sequence [9]. Mammalian studies have suggested, that DNA methylation, histone modifica-

tions, and noncoding RNAs in sperm are the main epigenetic mechanisms involved in the parental effect in the transgenerational inheritance [13]. However, only low level of DNA cytosine methylation [14], modified histones and small RNAs [15, 16] was detected in *Drosophila*.

Recently, we have reported that lifespan, physiological traits and metabolism are affected by the dietary protein-to-carbohydrate ratio (P:C) of the prior adult generation [5]. However, how parental dietary conditions can be transmitted epigenetically to offspring in *Drosophila* remains still unknown. Oxidative stress is thought to be one of the main mechanisms, which mediate the effect of the nutrition on the organismal health. Little is known regarding the effects of protein content in the diet on the redox balance and oxidative stress markers. In this context, the effects of dietary protein content in the parental diet on the cold sensitivity, activities of antioxidant and associated enzymes, and oxidative stress markers were investigated. We hypothesized that the nutritional and the metabolic variations caused by manipulating dietary dry yeast in offspring [5] translate into altered cellular antioxidant system and results in changes in stress tolerance. We only manipulated the concentration of dry yeast (as a main source of protein) in the parental diet, but all offspring being placed on standard food. We found that low dry yeast concentration in the parental diet decreased F1 males resistance to cold stress. Parental dietary protein influences the activity of the first-line antioxidant enzymes, and the levels of protein and low molecular mass thiols in the offspring. We have also demonstrated, that lower concentrations of the dry yeast in the parental diet caused higher activity of the second-line antioxidant enzymes in F1 males. Taken together, our work highlights, that cellular antioxidant system is determined by dietary protein in the parent-of-origin manner and can influence males and females differently.

Materials and Methods

Flies and experimental design. We used CantonS [*D. melanogaster* Meigen] flies received from the Bloomington Stock Center (Indiana University, USA). All flies were grown on the medium that contains 4% sucrose, 4% dry yeast, 1.2% agar and 0.18% nipagin as an anti-fungal agent. Flies were reared at 25°C and relative humidity of 60-70% on a 12 h day/night cycle. 5-days old flies were separated by sex and placed into demographic cages+ (200 flies per cage). A plastic vial filled with 5 ml of experimental food was attached to the cage. Food was changed every other day and dead flies were removed. Ancestral experimental mediums were composed of 4% of sucrose (S) (as a source of carbohydrate) and different concentrations of dry yeasts (as a source of protein and micronutrients) (Y): 0.25%, 4%, 10%, 15%; 1.2% of agar; 0.18% of nipagin. On the 25-th day of the experiment parental flies were allowed to egg laying. To prevent effects caused by larval density, laid eggs were washed three times with distilled water, then concentrated in a volume of 1.5 ml and transferred into plastic tubes containing 20 ml of experimental medium (100 ± 10 eggs) [17]. Collected eggs were given to develop on regular food (4S-4Y) and the day after eclosion, F1 flies were transferred on the fresh food (4S-4Y) and held for an additional 3 days for mating [5]. Then F1 offspring flies were sexed under light CO_2 anesthesia, selected for cold resistance test and frozen in the liquid nitrogen for biochemical measurements.

Cold sensitivity. Flies of F1 were subjected to cold exposure at 0°C for 1 minute. These conditions were sufficient to put all flies into chill coma without causing mortality. About 10 flies of each offspring cohort were placed in empty glass vials, which were put on ice in the isolated boxes (0°C). After cold treatment, flies from vials were quickly transferred into Petri dishes at room temperature (21-23°C), and chill coma recovery time (CCRT) [18] was measured.

Enzymatic activities. 3-day-old flies of F1 were crashed using a Potter–Elvejhem glass homogenizer (1:10 w:v) in cold 50 mM potassium phosphate buffer (pH = 7.5), supplemented with 0.5 mM EDTA and 1 mM PMSF (pH = 7.0). Centrifugation was performed at 16000 g for 15 min at 4°C using Eppendorf 5415R centrifuge (Eppendorf, Germany). The supernatants were collected and used for the determination of enzymatic activities and thiol content.

The activities of superoxide dismutase (SOD, EC 1.15.1.1) and catalase (EC 1.11.1.6) were measured as was described previously [19]. SOD activity was measured spectrophotometrically at 406 nm, by recording the inhibition of quercetin oxidation by superoxide anion. One unit of SOD activity was defined as the amount of soluble protein of superna-

Abbreviations: SOD, superoxide dismutase; ROS, reactive oxygen species; GST, glutathione-S-transferase; G6PDH, glucose-6-phosphate dehydrogenase; IDH, isocitrate dehydrogenase.

tant that inhibited the maximum rate of quercetin oxidation by 50%. Catalase activity was measured spectrophotometrically at 240 nm by recording the rate of hydrogen peroxide decomposition. Enzyme activity was calculated using extinction coefficient for hydrogen peroxide 39.4 $M^{-1}\cdot cm^{-1}$.

The activity of glutathione-S-transferase (GST, EC 2.5.1.18) was determined by measuring changes in absorbance of the conjugate formed between 1-chloro-2,4-dinitrobenzene and glutathione detected at 340 nm using molar absorption coefficient 9600 $M^{-1}\cdot cm^{-1}$.

The activities of glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), isocitrate dehydrogenase (IDH, EC 1.1.1.49) were measured using Specoll-211 as was described in [20]. Rates of NADPH production were determined by measuring the change in absorbance at 340 nm. To calculate enzyme activities, an absorption coefficient for NADPH 6220 $M^{-1}\cdot cm^{-1}$ was used.

Oxidative stress markers. The content of free thiols was measured by the Ellman's method using DTNB (5,5-dithiobis (2-nitrobenzoic acid)) at 412 nm, as was described previously [2]. The content of protein thiols (P-SH) groups was calculated as the difference between total and low molecular mass thiols. For the determination of low molecular mass thiols (L-SH), the supernatants were mixed with TCA (trichloroacetic acid), centrifuged (10 000 g for 15 min at 21°C) to precipitate protein. Resulted supernatants were used for L-SH assay. The protein thiol (P-SH) content was calculated by subtracting the L-SH concentration from total thiol concentration. Protein thiol levels were expressed as nanomoles of SH-groups per milligram of soluble protein. The content of the protein thiols was expressed in mmol per 1 mg of protein and low molecular weight per mg of wet weight.

Statistical analysis and graphical representation. Experimental data are presented as mean ± SEM and $P < 0.05$ is considered as significantly different. Statistical analysis was performed using "Prism" (GraphPad Software, Inc.). Tukey's multiply comparison test has been used to compare all investigated parameters. All graphs were generated in "GraphpadPrism7".

Results

Parental dietary protein affects offspring cold sensitivity. To study the effect of parental dietary protein on cold tolerance of the offspring, flies of parental generation were fed by diets with different dry yeast concentrations for 25 days, and their offspring were developed on the standard food. The time needed to recover from chill coma was measured after exposure to 0°C during 1 min and referred as chill coma recovery time (CCRT) [18]. We found that F1 males from parents, which consumed the medium with the lowest yeast concentration (0.25% Y), were more susceptible to cold stress as compared to the other groups (Fig. 1). Indeed, these F1 males recovered 40-50% later than the other F1 males (Tukey's test, $P < 0.02$). However, cold resistance of the F1 females was not affected by parental dietary protein content (Fig. 1).

Activities of the first-line antioxidant enzymes in the offspring depend on protein content in the parental diet. The first line defense antioxidant enzymes basically include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). They are important for neutralization of the reactive oxygen species (ROS) that is generated under normal metabolism, particularly through the mitochondrial energy production pathway, and impair cellular function. We found, that functioning of the first line of the antioxidant defense system depends on ancestral protein concentration in the diet. Parents, that consumed diet with 4% Y, generated males with 55% lower SOD activity as compared to 10% Y (Fig. 2, *A*; Tukey's test, $P = 0.002$). About 43% lower SOD activity was detected in females from parents, which consumed 15% Y as compared to those on 0.25% Y (Fig. 2A; Tukey's test, $P = 0.049$). Hence, SOD activity depends on protein content in the ancestral diet in the sex-specific manner. Moreover, we found 20% lower catalase activity in F1 males from parents reared on 0.25% Y as compared to 4% Y (Fig. 2, *B*; Tukey's test, $P = 0.014$). Catalase activity in F1 females was not affected by yeast concentration in the parental diet.

Activities of the second-line antioxidant enzymes in the offspring are affected by protein content in the parental diet. To further investigate the role of parental nutrition on the antioxidant system of the offspring, we measured the activities of the second-line antioxidant enzymes. The antioxidant system depends on the production of NADPH, that acts as a donor of reductive potential to glutathione and thioredoxins for proper function of ROS-detoxifying enzymes [21]. Mammalian cells possess a few enzymes able to produce NADPH and, among them are glucose-6-phosphate dehydrogenase (G6PDH) and isocitrate dehydrogenase (IDH). Glutathione-S-transferases (GST) is also involved in detoxification

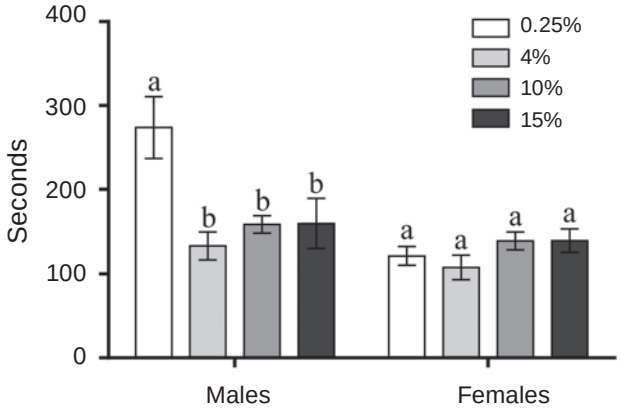

Fig. 1. Sensitivity toward cold stress of the offspring flies from parents reared on diets with different dry yeast concentrations. Points and bars represent mean values and standard error of means of 10 different replicates for each sex/diet. Groups were compared by the Tukey's test: a – indicates the highest values among all tested groups; b – significant difference from a with P < 0.05

of cytotoxic products, that formed during oxidative stress conditions. Therefore, we investigated the effect of yeast content in the parental diet on the activities of GST and NADP-dependent G6PDH and IDH in the offspring flies. We found, that GST activity in F1 males increased in the following order: 20% Y < 10% Y and 1% Y < 4% Y (Fig. 3, *A*; Tukey's test, $P < 0.02$). F1 males generated by parental flies that consumed 0.25% Y and 4% Y in the diets displayed approximately 25% higher G6PDH activity as compared to the 10% Y and 15% Y in the diet (Fig. 3, *B*; Tukey's test, $P < 0.007$). Interestingly, F1 males generated by parents reared on low protein diets, including 4% Y also displayed significantly higher IDH activity as compared to protein enriched diet – 15% Y (Fig. 3, *C*; Tukey's test, $P = 0.045$).

However, ancestral dietary protein content had no impact on the investigated second-line antioxidant enzymes activities – GST and NADP-dependent G6PDH and IDH in F1 females (Fig. 3, *A*, *B*, *C*).

Thiol content in the offspring is affected in sex-dependent manner. Thiol-containing compounds are among the major antioxidants and they play important role in the defense against ROS. Hence, protein and low-molecular weight thiol group levels are an indirect measurement of oxidative stress biomarkers. We determined whether protein content in the parental diet influences the oxidative stress markers in the F1 offspring reared on the standard diet. Interestingly, females generated by parents reared on the highest dry yeast concentration in the diet (15% Y), displayed decreased P-SH content as compared to all the rest experimental diets (by 20% as compared to 0.25% Y; by 16% compared to 4% Y in the diet, and by 37% compared to 10% in the diet) (Fig. 4, *A*; Tukey's test, $P < 0.05$). Consumption of the 0.25% Y diet by parental flies decreased L-SH content of their male offspring flies by approximately 60% as compared to all the rest experimental diets (Fig. 4, *B*; Tukey's test, $P < 0.04$). However, yeast concentration in the parental diet had no impact on L-SH level in the F1 females. Therefore, ancestral dietary protein determines offspring stress markers in sex dependent manner.

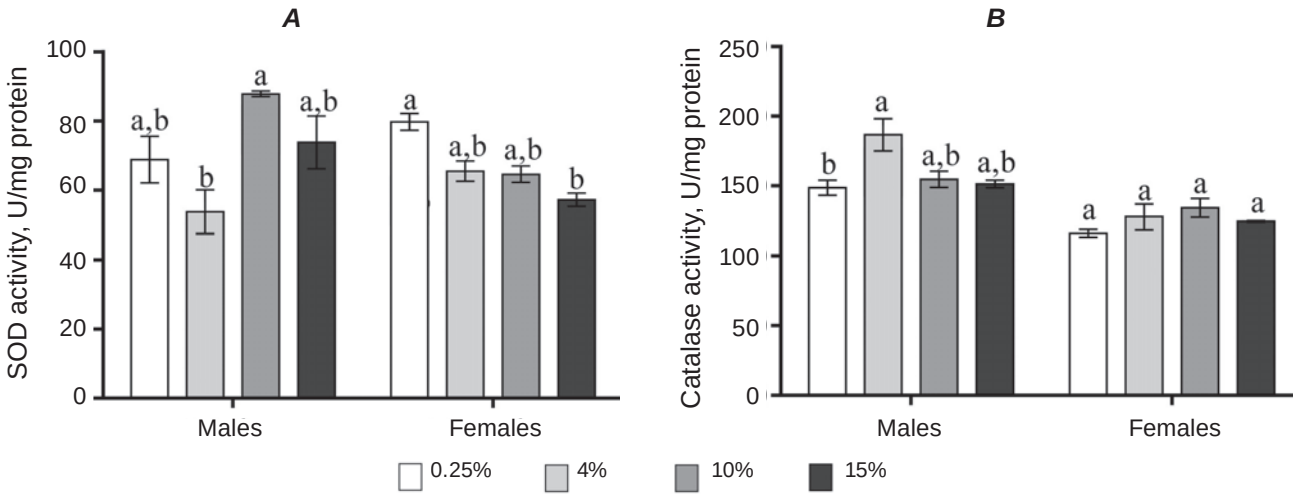

*Fig. 2. SOD (**A**) and catalase (**B**) activities in the offspring flies from parents reared on diets with different dry yeast concentrations. Results are shown as mean ± SEM, n = 4. Values were compared by the Tukey's test: a – indicates the highest mean among all tested groups; b – indicates a significant difference from a with P < 0.05*

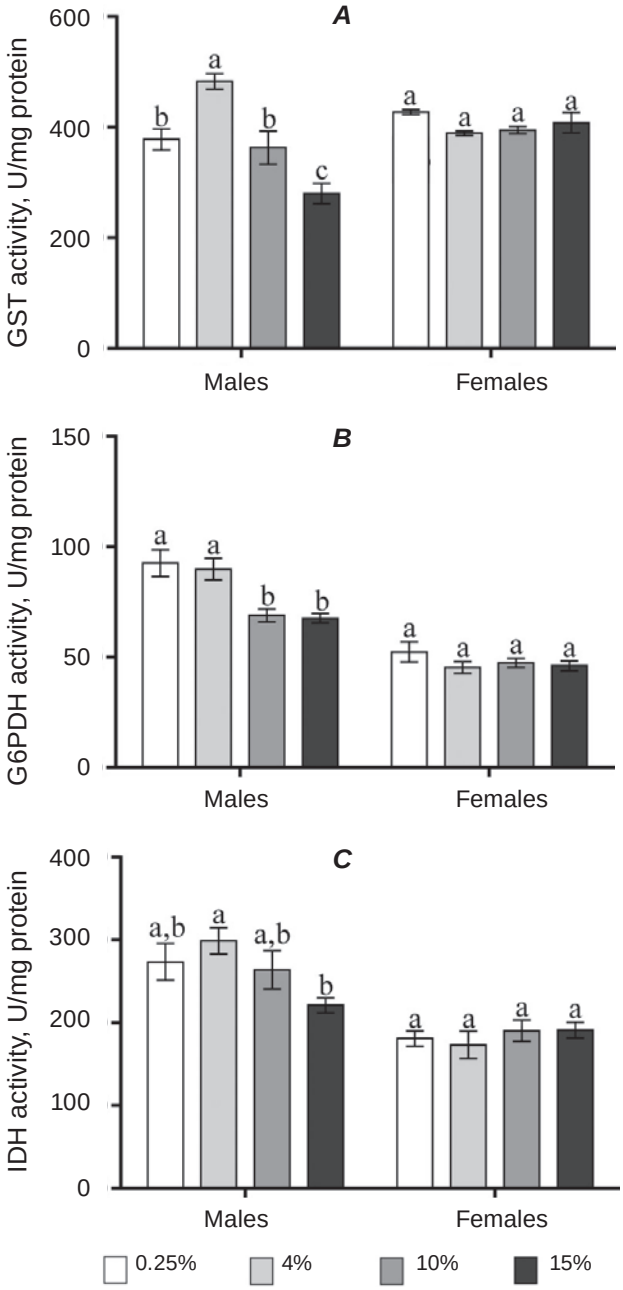

*Fig. 3. Effect of dry yeast content in the parental diet on the activities of GST (**A**) and NADP-dependent G6PDH (**B**), IDH (**C**) in the offspring flies. Results are shown as mean ± SEM, n = 4. Values were compared by the Tukey's test: a – indicates the highest mean among all tested groups; b – indicates a significant difference from a with P < 0.05*

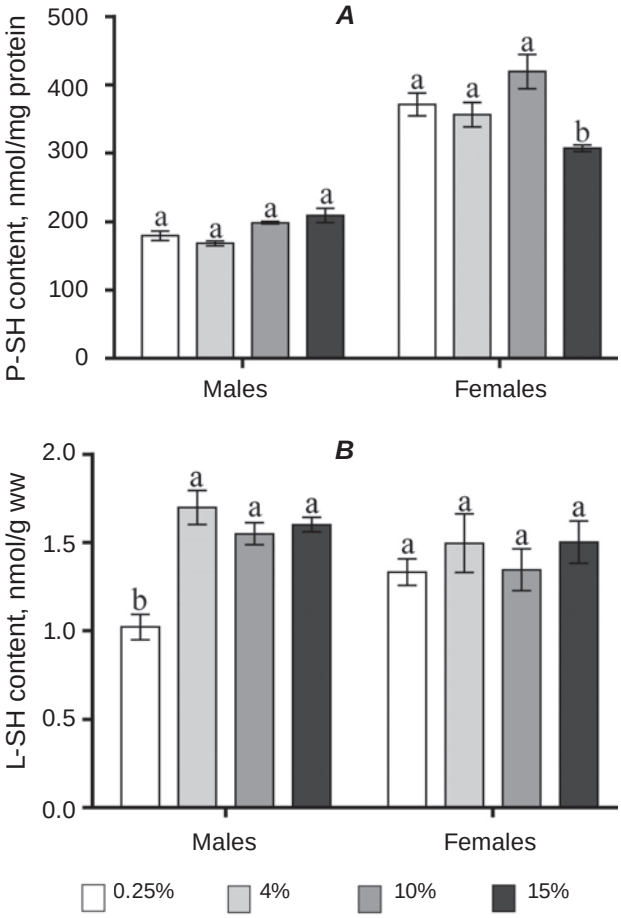

*Fig. 4. Contents of protein- (**A**) and low-molecular-mass (**B**) thiol-containing compounds in 3-day-old offspring D. melanogaster, which parents consumed diets with different dry yeast concentrations. Results are shown as mean ± SEM, n = 4. Values were compared by the Tukey's test: a – indicates the highest mean among all tested groups; b – indicates a significant difference from a with P < 0.05*

Discussion

The purpose of the present study was to investigate the influence of protein content of the parental diet on the cold tolerance and oxidative stress markers in the offspring *Drosophila*. Previously, a number of studies have observed, that parental dietary conditions influence offspring traits and lifespan [5, 10]. In the current study, we investigated the influence of an ancestral dietary yeast on adult oxidative stress markers to better understanding transgenerational inheritance of metabolic state. Parental flies were exposed to an altered dry yeast diet (as a source of protein and vitamins) for a single generation and transmitted effects were measured in unexposed offspring of the subsequent generation.

The mechanisms underlying the effects of dietary protein on the regulation of ageing have been widely studied. Recent study demonstrated, that restricted protein consumption improved longevity and metabolic health in *Drosophil*a [22]. Nutritional in-

terventions, such as a protein restriction was shown to suppress activity of mTORC1, that is associated with the induction of autophagy and improvement of insulin resistance to promote heathspan [23]. Autophagy may protect cells against various age-related stress conditions, including oxidative stress [24]. Moreover, restriction of protein content in the diet without strong caloric restriction decreases mitochondrial ROS production in the rat liver [25]. Branched-chain amino acids (BCAA) in the human diet result in oxidative stress in peripheral blood mononuclear cells via the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, nuclear factor-κB (NF-κB) and mTORC1 [26]. Hence, protein content in the diet influences oxidative damage and antioxidant system in organisms. However, how an antioxidant defense status affected by dietary protein can be transmitted through generation is still unexplored.

It is widely known, that dietary conditions have a strong impact on various life-history traits, including resistance to thermal stress. Consumption diets with higher concentration of live yeasts as a source for protein enhance resistant to cold [7]. Interestingly, we found, that low dry yeast concentration in the parental diet, which potentially may be results in lower cold tolerance, could be transmitted through one generation. Indeed, we observed longer chill coma recovery time in F1 males from parent reared on low protein diet. While, chill coma recovery is accompanied with elevation of metabolic rate [27], that, in turn, lead to enhancement in ROS levels [28]. High protein diet may contribute to the induction of heat shock genes expression [29]. These events result in increased synthesis of heat shock proteins, mostly Hsp22-28 and Hsp70 [30]. Thus, high protein ratio in the diet is related to thermal stress resistance. Our study indicated, that effect of low protein parental diet on cold tolerance could remain stable across generations.

An optimal redox state maintenance is essential to cellular survival. Intracellular defense systems that protect cells from ROS-induced damage include antioxidant enzymes. The main first-line antioxidant enzymes are SOD and catalase; SOD convert $O_2^{\bullet-}$ to H_2O_2; catalase catalyze the conversion of H_2O_2 into oxygen and water. The second-line antioxidant enzymes including GST, G6PDH, and the NADP-dependent form of IDH also play role in antioxidant defense processes against oxidative stress, which include reactive species scavenging. They assist the first-line antioxidant enzymes to inactivate ROS by supplying reductive equivalents in a form of GSH and NADPH [21]. In the current study, we investigated the effects of parental dietary yeast concentration on the activities of antioxidant and associated enzymes in their offspring. Slightly elevated activities of antioxidant enzymes including SOD in F1 males from parents reared on 10% Y as compared to 4% Y and in F1 females, generated by parents which consumed 0.25% Y as compared to 15% Y may suggested about small increase in ROS level, that may induce mild oxidative stress. Moreover, parental flies, which consumed diets with 4% Y generated males with enhanced activity of catalase (as compared to 0.25% Y), GST (as compared to 0.25% Y, 10% Y, 15% Y), G6PDH (as compared to 10% Y, 15% Y), IDH (as compared to 15% Y). It was previously found, that caloric restriction leads to increased activity of SOD in males and higher activity of G6PDH and IDH [31].

The level of thiol-group-containing compounds is an indirect marker of oxidative stress, because it indicates amount of reduced groups in proteins, peptides or amino acids [32]. Males and females of the F1 generation differed in the level of protein and low molecular mass thiols affected by parental dietary protein content in the present study. Decreased level of P-SH was detected in F1 females generated by parents reared on the diet with 15% Y, however was unaffected in F1 males. Interestingly, lower pool of L-SH was observed in F1 males originated from parents consumed 0.25% Y, but was unaffected in females. Decreased thiols level could be as a result of thiol consumption in reaction to the presence of ROS. The level of thiol-containing compounds may be determined at the level of gene expression, that could be depended on sex.

The impact of parental diet on offspring metabolism, physiology and lifespan was demonstrated previously [5, 10]. Indeed, F1 females from mothers that ate high protein/low sugar were heavier, with more protein, glycogen, triglycerides in their hemolymph, and that laid more eggs as compared to low protein/high-sugar diet [10]. Low parental protein:carbohydrate (P:C) ratio increased feeding rate in progeny. An increase in the P:C ratio from 0.03 to 0.65 decreased the levels of body glucose and trehalose, as well as circulating hemolymph glucose and trehalose in the offspring. Offspring also accumulated less triglycerides but more glycogen when parents were fed a low P:C diet [5]. Schwasinger-

Schmidt and colleagues showed altered metabolism in F1 flies from starvation-resistant parental flies [33]. These studies provided strong support for parental diet influencing some offspring life history traits. In addition, paternal diet regulates chromatin-defined genes in the germline and offspring, that could be potential mechanisms intergenerational control of physiological and metabolic traits [34]. Offspring phenotype was shown to be evolutionarily encoded directly into the chromatin state of relevant loci and alter an expression of genes important to both cytosolic and mitochondrial metabolism [34]. Interestingly, target genes involved in the regulation of many key metabolic pathways, including glycolysis, TCA cycle, mitochondrial OxPhos, chitin, and polysaccharide metabolism [34]. However, the effects of ancestral diet depend of sex and genotype [10, 36]. Indeed, we did not observe any significant impact of parental protein content in the diet in the activities of antioxidant enzymes in F1 females. Further work is required to investigate sex-specific effects of molecular mechanisms underlying metabolic programming.

Conclusion. The main findings of this study were: 1) low dry yeast concentration in the parental diet decreased males offspring resistance to cold stress; 2) parental dietary protein influence the activity of the first-line antioxidant enzymes, and the levels of protein and low molecular mass thiol groups in the offspring; 3) lower concentrations of the dry yeast in the parental diet caused higher activity of the second-line antioxidant enzymes in F1 males.

In current study, we showed, that metabolic alterations in the offspring caused by changes in parental dietary conditions [5] are associated with shift in the functioning of an antioxidant system. Low yeast amounts (0.25% Y and 4% Y) in the parental diet induced oxidative stress in offspring males only *Drosophila*. This is due to the fact that females and males *D. melanogaster* are characterized by some metabolic differences. Indeed, it was previously found that the type of diet has a more pronounced effect on antioxidant system of males as compared to females [2, 36]. This fact can explain the more explicit reaction of antioxidant system in males reared on the diet with different yeast concentrations.

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

ВМІСТ ПРОТЕЇНІВ У ДІЄТІ БАТЬКІВ ВПЛИВАЄ НА СТІЙКІСТЬ ДО ХОЛОДУ ТА СТАН АНТИОКСИДАНТНОЇ СИСТЕМИ У НАЩАДКІВ *DROSOPHILA*

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Поживні речовини у складі харчового раціону впливають на тривалість життя та здоров'я організму. Вміст компонентів дієти батьків може епігенетично позначатися на фізіологічному стані нащадків. Ми вивчали, як вміст протеїнів у харчовому раціоні батьків мух *Drosophila melanogaster* впливав на стійкість до холодового стресу та стан антиоксидантної системи у дорослих нащадків. Батьківське покоління дрозофіл утримували на дієтах із чотирма варіаціями кількості дріжджів у межах від 0,25 до 15% та сталим вмістом сахарози, а наступне покоління годували дієтою однакового складу. Показано, що мухи, які споживали дієту з обмеженим вмістом (0,25%) дріжджів, давали нащадків самців F1 із більшою стійкістю до холоду та вищою активністю ензимів другої лінії антиоксидантного захисту, тоді як у самок F1 не було виявлено впливу складу харчового раціону батьків на стійкість до холоду та активність каталази, GST, G6PDH, IDH та рівень тіолів. Одержані результати дозволяють припустити, що залежна від доступності поживних речовин зміна експресії генів у батьківському поколінні може по-різному впливати на стресову відповідь самців та самок першого покоління нащадків.

Ключові слова: вміст протеїнів у дієті, *Drosophila melanogaster*, толерантність до холоду, антиоксидантні ензими, батьківське покоління, потомство.

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