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Sex-dependent obesogenic effect of tetracycline on *Drosophila melanogaster* deteriorated by dysrhythmia

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ABSTRACT

Antibiotics have been identified as obesogens contributing to the prevalence of obesity. Moreover, their environmental toxicity shows sex dependence, which might also explain the sex-dependent obesity observed. Yet, the direct evidence for such a connection and the underlying mechanisms remain to be explored. In this study, the effects of tetracycline, which is a representative antibiotic found in both environmental and food samples, on *Drosophila melanogaster* were studied with consideration of both sex and circadian rhythms (represented by the eclosion rhythm). Results showed that in morning-eclosed adults, tetracycline significantly stimulated the body weight of females (AM females) at 0.1, 1.0, 10.0 and 100.0 μg/L, while tetracycline only stimulated the body weight of males (AM males) at 1.0 μg/L. In the afternoon-eclosed adults, tetracycline significantly stimulated the body weight of females (PM females) at 0.1, 1.0 and 100.0 μg/L, while it showed more significant stimulation in males (PM males) at all concentrations. Notably, the stimulation levels were the greatest in PM males among all the adults. The results showed the clear sex dependence of the obesogenic effects, which was diminished by dysrhythmia. Further biochemical assays and clustering analysis suggested that the sex- and rhythm-dependent obesogenic effects resulted from the bias toward lipogenesis against lipolysis. Moreover, they were closely related to the preference for the energy storage forms of lactate and glucose and also to the presence of excessive insulin, with the involvement of glucolipid metabolism. Such relationships indicated potential bridges between the obesogenic effects of pollutants and other diseases, e.g., cancer and diabetes.

Introduction

The nearly tripled prevalence of obesity since 1975 causes tremendous health risks and social burdens (WHO, 2018). Interestingly, more obese males than females are found in school-age children and adolescents, showing significant sex

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dependence (Zhang et al., 2018a). The obesity prevalence and corresponding sex dependence motivates a strong desire to understand its etiology. Recently, environmental pollutants have been reported to disturb lipid metabolism and therefore function as obesogens, contributing to the prevalence of obesity (Pan et al., 2021). However, sex dependence is seldom reported in studies on the obesogenic effects of environmental pollutants.

Recently, antibiotics have joined environmental obesogens because of their ubiquitous presence in the environment and food (Gu et al., 2021; Oberoi et al., 2019) and also their correlation with overweight and obesity (Bailey et al., 2014; Scott et al., 2016; Wada et al., 2020). Notably, they show sex dependence in many aspects, including prescription rates (Schröder et al., 2016; Smith et al., 2018), efficiency (Sánchez-Serrano, 2017), side-effects (Franconi et al., 2007), residues in the human body (Wang et al., 2015) and even their environmental toxicities toward reproduction and metabolism (Xin et al., 2021; Yu et al., 2019). Therefore, environmental antibiotics are assumed to show sex differences in their obesogenic effects. However, such assumption still needs direct evidence and mechanistic explanations.

The disturbance of lipid metabolism is known to be the direct reason for obesity. The obesogenic antibiotics indeed disturb the activities of key metabolic enzymes, e.g., acetyl CoA carboxylase (ACC, the rate-limiting enzyme in lipogenesis) and carnitine palmitoyl-transferase (CPT, the rate-limiting enzyme in lipolysis) (Li et al., 2020; Yu et al., 2020). Notably, sex differences in lipid metabolism can provide insight into the sex dependence of obesity. On the one hand, males usually store less fat content than females, and are more vulnerable to air-borne pollutants (e.g., $PM_{2.5}$), which are also environmental obesogens (Chen et al., 2020; de Bont et al., 2019). On the other hand, females have more demands on balancing energy metabolism and reproduction than males (Maggi and Torre, 2018), and therefore often show more changes in glucolipid metabolism in response to stressors (Torre et al., 2018). Despite contrary findings, the changes in lipid metabolism can provide essential information to explain the potential sex-dependent effects of antibiotics.

Interestingly, disruptions of circadian rhythms, i.e., dysrhythmia, are also well-associated with lipid metabolism and subsequent obesity (Engin, 2017). As expected, antibiotics disturb the circadian rhythms and stimulate the body weight of individuals with dysrhythmia compared to normal ones (Yu et al., 2020). Moreover, dysrhythmia-connected obesogenic effects are accompanied by significant disturbances of the key enzymes (e.g., ACC) and various energy storage forms (e.g., triacylglycerols, glucose, glycogen and lactate) in lipid and even glucolipid metabolism (Yu et al., 2020). Notably, the particular contribution of dysrhythmia to male obesity demonstrates its involvement in the sex-dependent obesity prevalence (Zhang et al., 2018b). Yet, whether antibiotics can provoke sex-dependent dysrhythmia remains unexplored.

The purpose of this study was to confirm the obesogenic effects of tetracycline with focus on sex dependence and the influence of dysrhythmia. Tetracycline is representative of antibiotics that can be detected in both environmental and food samples (Gu et al., 2021). The test organism was *Drosophila melanogaster*, which is a model for studying the obesogenic potentials of environmental pollutants to human beings, and also facilitates investigations on the influences of sex and rhythm as well as the underlying mechanisms (Yu et al., 2020). Increases in body weight were employed to demonstrate the potential to provoke overgrowth and obesity. The present results confirmed that the obesogenic potentials of tetracycline were more significant in females with normal rhythm and in males with dysrhythmia. The present findings also provided potential hints to explain the connection between the obesogenic effects of environmental pollutants and other health problems, including cancer and diabetes.

1. Materials and methods

1.1. Chemicals

The tetracycline used was in the form of tetracycline hydrochloride (TCH, CAR RN: 64-75-5). Its stock solution was prepared with sterilized distilled water with a concentration of 10 mg/L which was verified via UPLC-MS/MS according to an earlier report (Liu et al., 2018). The stock solution was stored at 4°C in the dark.

1.2. Preparation of animals

Wild type *D. melanogaster* (Oregon R, OR) were gifted from the College of Life Science and Technology, Tongji University. Briefly, the fruit flies were cultured on solid medium containing 135.0 g brown sugar, 7.0 agar powder, 85.0 g corn flour, 8.0 g yeast and 4.0 mL propionic acid in 1.0 L distilled water (Yu et al., 2020). The flies were cultivated at 25 °C, with 60% humidity and a 12 hr.:12 hr. photoperiod. They were temporarily anesthetized by CO_2 for sex identification, regular transfers and random grouping (Shen et al., 2018). According to routine observation, each fertile female produced an average of 25 eggs within the first 24 hr after mating.

1.3. Tetracycline exposure

There were 5 groups, including one control group and four TCH treatment groups with concentrations of 0.1, 1.0, 10.0, and 100 μg/L. These concentrations were selected to represent environmental levels of tetracycline (Gu et al., 2021). Briefly, the stock solutions of TCH were diluted in sterilized distilled water to prepare a series of working solutions with concentrations of 1000, 100, and 10 μg/L. Then, 1.0 mL of the stock and working solutions were added into 99.0 mL of warm growth medium, which was mixed thoroughly and aliquoted into individual glass culture tubes (Shen et al., 2018). There were at least 10 replicate culture tubes per group. In the morning (8:00 am), virgin female and naïve male flies (5:5) were randomly selected and transferred into the culture tubes, and the time was marked as T0 (Yu et al., 2020). At T24 (i.e., 24 hr. since T0), all parent flies were removed and the newly laid eggs were left in the culture medium to continue the exposure.

1.4. *Eclosion rhythm and development and corresponding sampling*

After T72, 20 third-instar larvae that appeared on the surface of the tube were randomly collected from each group for subsequent measurement on lipid droplets (LDs). After T96, the cumulative numbers of pupae were recorded every 12 hr. (Yu et al., 2020). Notably, the fruit flies tend to eclose into adults around dawn each day (Myers et al., 2003). After T192, the cumulative numbers of adult flies were recorded every 12 hr. The adults eclosed from dawn to 8:00 am were marked as AM adults, while those between 10:00 am and 20:00 pm were marked as PM adults. The adults from each glass culture tube were separated by sex and randomly collected into 1.5 mL centrifuge tubes (Eppendorf) with at least six individuals in each. There were 50 centrifuge tubes in total in every group. All samples were stored at -80°C until assayed.

1.5. *Apical obesogenic consequences*

The body weights of individual flies were weighed with a microbalance (Sartorius BT125D, Sartorius, Germany). Then, the images of the individual flies were collected with a dissection microscope (Olympus) and microscopic camera (Olympus DP 73, U-TV0.5XC-3, Olympus, Japan). The images were used to measure the body length, body width, thickness, perimeter and area by Photoshop CS5 (Yu et al., 2020).

1.6. *Biochemical assays*

The AM and PM adults were homogenized in ice cold PBS in an ice bath. After centrifugation at 4°C, aliquots were taken of the supernatants to measure the following biochemicals. Total protein (TP) was quantified by a BCA Protein Assay Kit (Thermo Fisher Scientific, USA) and functioned as the denominator in quantifying other biochemical indices to balance the biomass variation (Tennessen et al., 2014). Metabolic substances including triglycerides, glucose, glycogen, pyruvate, lactate and cholesterol were measured by commercial ELISA kits according to the instructions (Yu et al., 2020). Further assays were performed on acetyl-CoA carboxylase (ACC), acyl-CoA oxidase (ACO), acyl-CoA synthetase (ACS), carnitine palmitoyl transferase (CPT), fatty acid desaturase (FAD), fatty acid synthase (FAS), fatty acid transport proteins (FATP), glucokinase (GCK), glycerol-3-phosphateacyl transferases (GPAT), lipase and insulin (INS). These assays were also carried out with ELISA kits (Yu et al., 2020). They were separated into three categories, where (1) GPAT, FAS and FAD facilitate lipogenesis; (2) lipase, FATP, ACS, CPT, ACO and ACC facilitate lipolysis; and (3) GCK and INS are involved in glucolipid metabolism.

1.7. *Data presentation and statistical analysis*

The data in the four TCH treatment groups are presented as the percentage of the control (POC) or the fold change compared to the control, and therefore data in the control group were uniformly normalized to 100% or 1.0 (Yu et al., 2020). For each indicator, there were at least 10 replicates in each group. First, the data were checked for normality via the Shapiro-Wilk test in Origin 9.0 (Origin Lab Corp., USA). Then, one-way or two-way ANOVA with Tukey's post hoc test at 0.05 level ($p < 0.05$) was used to analyze the statistical differences for data with more than three groups. A *t* test was used to analyze the statistical differences between pairs of groups (e.g., comparisons between females and males, and between AM and PM adults) for data at the 0.05 level ($p < 0.05$). Principal component analysis (PCA) followed by clustering analysis was employed to explore potential connections among the indicators (Shi et al., 2021).

2. Results and discussion

2.1. *Sex-dependent obesogenic effects of tetracycline deteriorated by dysrhythmia*

The effects of TCH on the body weight of larvae and adults were measured (Fig. 1). In larvae, TCH did not significantly influence the body weight at any concentration. In the AM adults, TCH significantly stimulated the body weight in females at all concentrations ($p < 0.05$), while it only stimulated the body weight in males at 1.0 μg/L ($p < 0.05$). In the PM adults, TCH significantly stimulated the body weight in females at 0.1, 1.0 and 100.0 μg/L ($p < 0.05$), while it showed more significant stimulation in males at all concentrations ($p < 0.05$). Notably, the stimulation levels in females were similar between AM and PM adults, while those in males were significantly greater in PM than AM adults. The results showed clear sex dependence for the obesogenic effects of TCH, which was diminished by dysrhythmia. Another noteworthy point was that TCH stimulated the body weights of all adults at 1.0 μg/L, showing a dependence on the exposure concentration.

The changes in body weight were well-related to development, which was indicated by pupation (Fig. 2) and eclosion (Fig. 3). In the aspect of pupation, TCH at 1.0 μg/L significantly promoted the pupation rate at earlier time points despite no differences being found in the pupation weights (Fig. 2). In the aspect of eclosion, TCH significantly stimulated the female eclosion percentage at 1.0 μg/L, and the male one at 1.0 and 10.0 μg/L (Fig. 3a and c). The stimulating effects on the eclosion were well-connected to the stimulation of body weight in female adults at 1.0 μg/L and male ones at 1.0 and 10.0 μg/L (Fig. 1). Moreover, TCH reduced female eclosion but stimulated the male one in the morning (Fig. 3b and d). The results showed opposite disturbances to the rhythm in both sexes, implying potential interactions between sex and rhythm.

The results in the present study clearly demonstrated the sex dependence of the obesogenic effects of tetracycline. In the AM adults, tetracycline promoted more obesogenic effects in females than in males. This sex dependence is different from the report of less female than male obesity in school-age children and adolescents (Zhang et al., 2018a). Such difference can be related to the influence of age. It has been found that female obesity is indeed less in adults ranging from 20 to 74 years old (Hou et al., 2013), while it overtakes male obesity in adults over 45 (Yuan et al., 2016). The present study raised a noteworthy point, that the sex-dependent obesogenic effects of antibiotics depended on age. Such sex- and age-dependent obesogenic effects are also observed for phthalates, which is another representative environmental

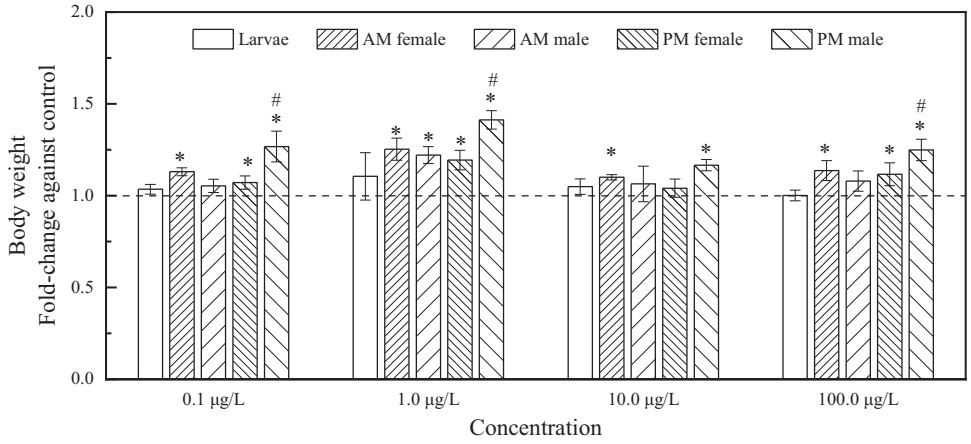

Fig. 1 – Effects of tetracycline hydrochloride (TCH) on body weights of *D. melanogaster* larvae and adults. The values represent fold changes compared to the control (normalized to 1.0). Error bars indicate standard deviation (SD); * indicates significant difference of TCH treatment from sex- and rhythm-matched controls by *t*-test, $p < 0.05$; # indicates significant difference of PM adults from sex-matched AM ones by *t*-test, $p < 0.05$.

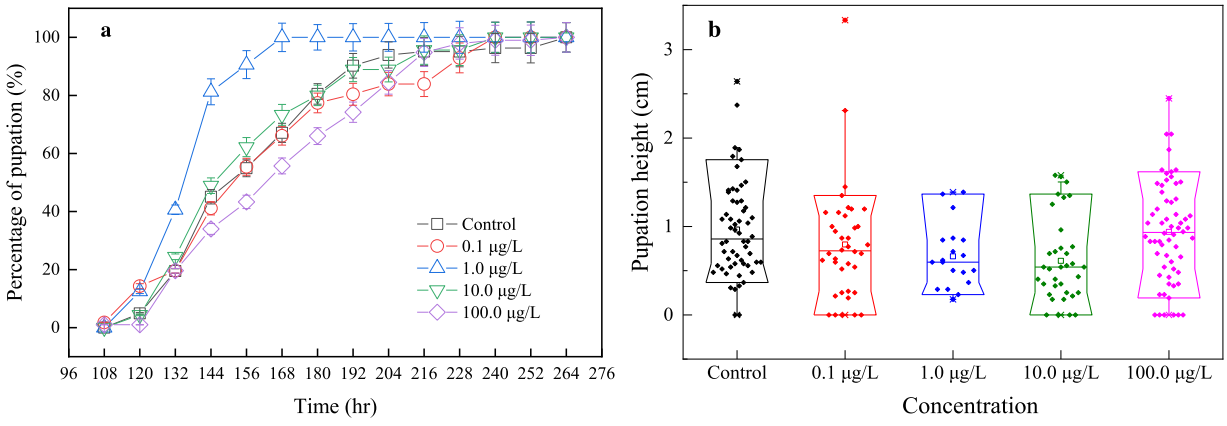

Fig. 2 – The effects of tetracycline hydrochloride (TCH) on pupation rate (a) and height (b) of *D. melanogaster* pupae.

obesogen group (Buser et al., 2014). Yet, the exact reasons for the sex- and age-dependent obesogenic effects still need further investigation.

The present results also implied interactions between sex and rhythm in the obesogenic effects of tetracycline. In the PM adults, tetracycline promoted significantly smaller obesogenic effects in females than in males. The overall results for both AM and PM adults indicated that males with normal rhythm were more resistant to the obesogenic effects, while the males with dysrhythmia were more susceptible to the effects. On the one hand, the results showed greater influence of rhythm on the health of males than females, and the reasons still need further investigation. On the other hand, the results were consistent with the contribution of dysrhythmia to male obesity in both school-aged children (Zhang et al., 2018b) and also adults over 60 years old (Zhou et al., 2020). That is to say, the sex- and rhythm-dependent obesogenic effects were also related to age. Moreover, dysrhythmia caused by tetracycline is also observed for other pharmaceuticals, including antibiotics (sulfamethoxazole, ciprofloxacin, ampicillin and rifamycin), harmine, paroxetine and sodium nitroprusside, etc. (Cui et al., 2020; Fakoorziba et al., 2012; Liu et al., 2019; Lozinsky et al., 2012; Yu et al., 2020). The antibiotics are already confirmed as obesogens, and the obesogenic potentials of other pharmaceuticals should be considered in their future prescription or in assessment of environmental hazards.

It should be noted that both sex difference (and corresponding sexual dimorphism) and circadian rhythm are important for the adaptability of organisms to environmental stressors (Palanza et al., 2021). The influences of tetracycline on both aspects demonstrated the potential for environmental antibiotics to influence the ecological balance. Such potential is further amplified by the long-term exposure and toxicities of antibiotics, which can last over generations on the one hand (Li et al., 2020; Yu et al., 2017; Zhang et al., 2020), and also the transfer of obesity over generations on the other hand (Costa-Font and Jofre-Bonet, 2020). Therefore, the health impacts of obesogenic antibiotics, especially the long-term outcomes underlying the sex- and rhythm-dependent mechanisms, should be further explored in future studies.

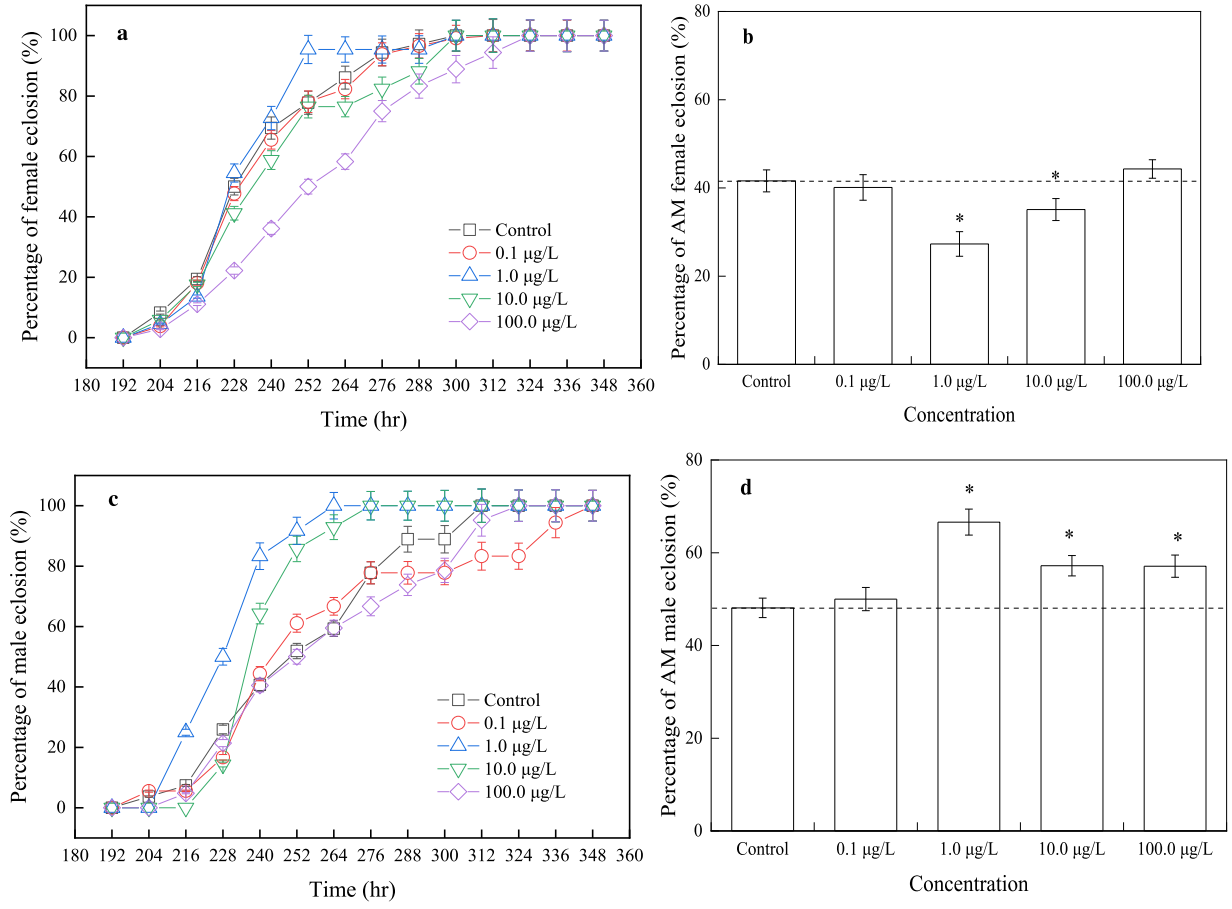

Fig. 3 – The effects of tetracycline hydrochloride (TCH) on the female eclosion percentage (a), the percentage of morning female eclosion (AM female) to the total eclosion (b), male eclosion percentage (c) and the percentage of morning male eclosion (AM male) to the total eclosion (d). * indicates significant difference of TCH treatment from sex-matched controls by t-test, $p < 0.05$.

2.2. *Disturbances of lipid metabolism underlying the obesogenic effects of tetracycline*

Biochemical assays were performed in the 1.0 μg/L TCH group to explore potential mechanisms. Triacylglycerols (TAG), glucose, pyruvate, glycogen, lactate and cholesterol were chosen as representative energy storage forms. Results in larvae showed that TCH inhibited the contents of glucose, pyruvate and glycogen ($p < 0.05$), while it stimulated that of cholesterol ($p < 0.05$) without significant influences on TAG and lactate (Fig. 4). In the AM adults, TCH showed inhibiting effects on TAG in males, pyruvate in females, and glycogen in both sexes (Fig. 4). Moreover, TCH provoked stimulation of glucose and lactate in both sexes, and the glucose stimulation was greater in males. In the PM adults, TCH inhibited TAG and cholesterol in both sexes, and the inhibiting effect on cholesterol was less in males. Meanwhile, TCH stimulated glucose, pyruvate and lactate in both sexes, and the stimulating effects were collectively greater in males. The overall results demonstrated that both sex and rhythm significantly influenced the effects of TCH on energy storage forms.

Further analysis was performed on the key enzymes and biochemicals that facilitate lipogenesis, lipolysis and glucolipid metabolism. In larvae, TCH stimulated ACC, FATP and INS ($p < 0.05$, Fig. 5) related to lipogenesis, lipolysis and glucolipid metabolism, respectively. Meanwhile, TCH inhibited FAS, GCK, ACO and CPT in larvae ($p < 0.05$). In the AM adults, TCH stimulated ACC, ACO, GCK and INS in both sexes, and the stimulating effect on INS was less in males. Meanwhile, it inhibited lipase, FATP, ACS and CPT in both sexes, and the inhibition of lipase and CPT was greater in males. Moreover, TCH inhibited GPAT, FAS and FAD, which facilitate lipogenesis in females, while the inhibition changed to non-influence and even stimulation in males. In the PM adults, TCH stimulated GPAT, ACC, lipase, ACO, GCK and INS in both sexes. The stimulation of GPAT, ACC, GCK and INS was greater in males, while that of lipase and ACO was less in males. Meanwhile, TCH inhibited CPT in both sexes with greater inhibition in males. Moreover, TCH showed stimulation of FAS in males and FAD and FATP in females, without significant influence on the other sex. Again, the results clearly demonstrated the influences of both sex and rhythm in the effects of TCH on metabolism.

PCA and subsequent cluster analysis demonstrated the closeness among indicators by clustering positions or distance, where the more closely clustered (or shorter distance apart) the indicators were, the closer positive connection they would have. Regarding sex and rhythm, the PM males showed

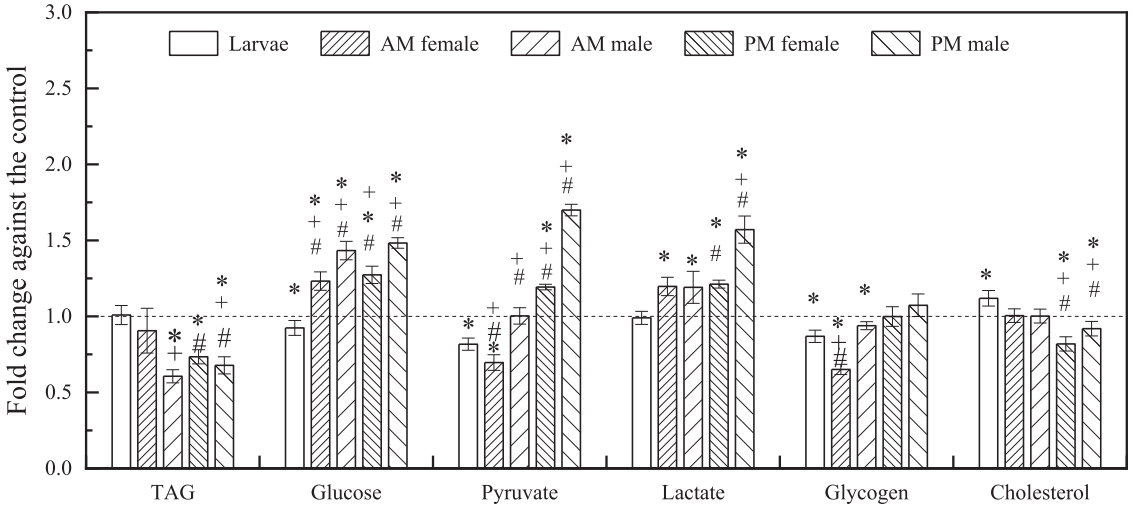

Fig. 4 – Effects of tetracycline hydrochloride (TCH, 1.0 μg/L) on energy storage forms in *D. melanogaster*. The values represent fold changes compared to the control (normalized to 1.0). Error bars indicate standard deviation (SD). TAG, triacylglycerols; * indicates significant difference of TCH treatment from sex- and rhythm-matched controls by *t*-test, $p < 0.05$; + indicates significant differences of females from the males within AM or PM adults by *t*-test, $p < 0.05$; # indicates significant difference of PM adults from the sex-matched AM ones by *t*-test, $p < 0.05$.

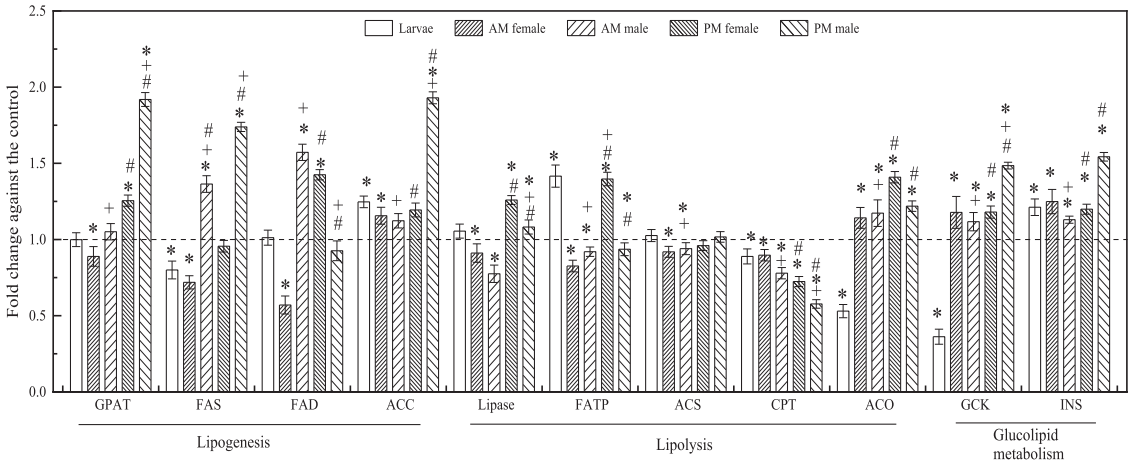

Fig. 5 – Effects of tetracycline hydrochloride (TCH, 1.0 μg/L) on key enzymes and biochemicals in lipid and glucolipid metabolism in *D. melanogaster*. GPAT (glycerol-3-phosphateacyl transferase), FAS (fatty acid synthase) and FAD (fatty acid desaturase) facilitate fat accumulation; Lipase, fatty acid transport proteins (FATP), acyl-CoA synthetase (ACS), carnitine palmitoyl transferase (CPT), acyl-CoA oxidase (ACO) and acetyl-CoA carboxylase (ACC) promote fat consumption; Glucokinase (GCK) and insulin (INS) are involved in glucolipid metabolism. The values shown in the graph represent fold change compared to control (normalized to 1.0) with the error bars representing standard deviation (SD); * indicates significant difference of TCH treatment from controls by *t*-test, $p < 0.05$; + indicates significant differences of females from the males within AM or PM adults by *t*-test, $p < 0.05$; # indicates significant difference of PM adults from the sex-matched AM ones by *t*-test, $p < 0.05$.

significant clustering position or distance from others (Fig. 6), further supporting the influence of sex and rhythm. Regarding the indicators, the stimulating effect on body weights was positively attributed to lactate and glucose, regarding the energy storage form, and those of INS, ACC, GPAT and FAS regarding glucolipid metabolism and lipogenesis. Meanwhile, the results also showed distant and probably negative attributions from cholesterol in the energy storage form and from CPT, ACS and FATP regarding lipolysis. That is to say, the obesogenic effects as reflected in increases in body weight resulted from the following three aspects: (1) the alteration of energy storage forms to lactate and glucose, (2) excessive insulin, which targeted glucolipid metabolism, and (3) the bias toward lipogenesis against lipolysis.

Regarding the first aspect, increases in lactate have been commonly observed in obese subjects and are well-connected with metabolic syndrome (e.g., enhanced glycolysis and a disordered Krebs cycle) (Jones et al., 2019; Liu et al., 2021). More-

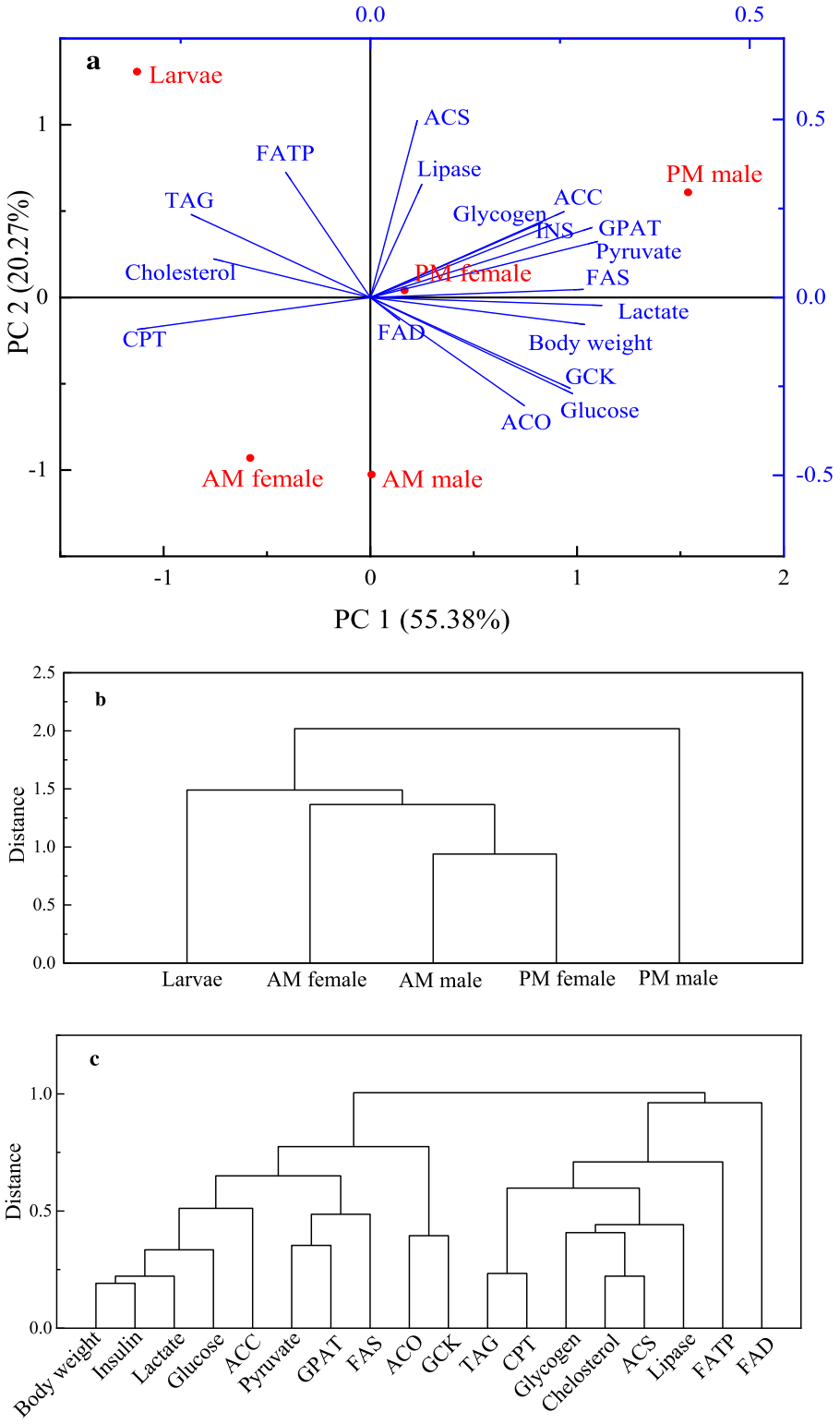

Fig. 6 – Principal component analysis (a) and clustering analysis (b and c) on the effects of tetracycline hydrochloride (TCH, 1.0 μg/L) on apical and biochemical indices in *D. melanogaster*.

over, the increases in lactate also provided on essential bridge between obesity and other diseases, e.g., cancer (Kalezic et al., 2020). However, stimulation of lactate is not observed with sulfamethoxazole (SMX, another obesogenic antibiotic) (Yu et al., 2020). Such contrary findings call for more investigation to show whether lactate increases could be used as a marker in screening obesogenic pollutants.

In the second aspect, excessive insulin (or insulin resistance) is usually accompanied by obesity (Ahmed et al., 2021), and even shows sex dependence (Jayanthi and Srini-

vasan, 2019). Moreover, insulin resistance is also related to the aforementioned increases in lactate (Lovejoy et al., 1992). Insulin resistance or the involvement of insulin-regulating pathways are also observed for SMX (another obesogenic antibiotic) (Li et al., 2020; Yu et al., 2020) and PFASs (Li et al., 2021). Such findings further support the connection between obesity and other diseases (e.g., diabetes), as well as the contributions of environmental pollutants to a vast number of health problems.

Regarding the third aspect, an obesogenic outcome is the result of a lack of balance between lipogenesis and lipolysis, instead of independent increases in lipogenesis or decreases in lipolysis. Such bias is also observed in the obesogenic effects of SMX (Li et al., 2020; Yu et al., 2020). However, such bias between lipogenesis and lipolysis did not show particular patterns among antibiotics. Such results indicated the different modes of action among antibiotics. For example, SMX (sulfonamide antibiotic) targets the growth and reproduction of both Gram-positive and negative bacteria, while TCH (tetracycline antibiotic) eliminates Gram-positive and negative bacteria and also intracellular bacterial pathogens. Such variation would result in different impacts on the microbiota that are essential in energy metabolism, e.g., lactate and fatty acid synthesis (Rodriguez-Castaño et al., 2017). Notably, it was reported that the microbiota are also closely related to the circadian rhythm (Thaiss et al., 2016). Moreover, disturbances in the microbiota, e.g., higher ratios of Firmicutes to Bacteroides, were indeed reported in obese patients (Ley et al., 2006). As expected, the influence of antibiotics (e.g., SMX) on microbiota and also the influence on the host rhythm were found to underlie obesogenic effects (Yu et al., 2020). However, the contribution of the microbiota to the sex-dependent variations remains to be answered in future studies.

Notably, *Drosophila* larva live inside the medium and directly ingest the medium as food, which serves as the exposure route. This situation is different from human beings, who have much bigger body sizes, more complex exposure routes and more sophisticated metabolism than these flies. Therefore, extrapolation of the findings of the present study to human beings still needs further investigation.

3. Conclusions

Tetracycline significantly stimulated body weight in AM females at all concentrations, while it only showed stimulation in AM males at 1.0 μg/L. Meanwhile, tetracycline significantly stimulated the body weight in PM females at 0.1, 1.0 and 100.0 μg/L, with more significant stimulation in PM males at all concentrations. Notably, the stimulation levels were similar between AM and PM females, while those in PM males were significantly greater than AM ones. Further biochemical assays and clustering analysis showed that the sex- and rhythm-dependent obesogenic effects resulted from the bias toward lipogenesis against lipolysis. Moreover, they also showed close relationships with cancer and diabetes due to the energy storage form preference for lactate and insulin resistance.

Acknowledgments

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