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Article

Early life exercise training and inhibition of apolipoprotein B expression to improve age-related arrhythmias and prolong the average lifespan in Drosophila

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Abstract: Cardiovascular disease (CVD) places a heavy burden on older patients and the global healthcare system. A large body of evidence suggests that exercise training is essential in preventing and treating cardiovascular disease, but the underlying mechanisms are not well understood. Here, we used the Drosophila melanogaster animal model to study the effects of early-life exercise training (ELET) on the aging heart and lifespan. We found in flies that age-induced arrhythmias are conserved across different genetic backgrounds. The fat body is the primary source of circulating lipoproteins in flies. Inhibition of fat body apoLpp (the flies apoB homolog) demonstrated that low expression of apoLpp reduced the development of arrhythmias in aged flies but did not affect average lifespan. At the same time, ELET can also reduce the expression of apoLpp mRNA in aged flies and have a protective effect on the heart, which is similar to the inhibition of apoLpp mRNA. Although treatment of $apoLpp^{RNAi}$ and ELET alone had no significant effect on lifespan, the combination of $apoLpp^{RNAi}$ and ELET extended the average lifespan of flies. Therefore, we conclude that $apoLpp^{RNAi}$ and ELET are sufficient to resist age-induced arrhythmias, which may be related to the decreased expression of apoLpp mRNA, and that $apoLpp^{RNAi}$ and ELET have a combined effect on prolonging the average lifespan.

Keywords: Exercise training; arrhythmias; Drosophila; apolipoprotein B; aging

1. Introduction

Cardiovascular disease is the leading cause of death worldwide, and aging is crucial in developing cardiovascular disease [1]. The aging of the myocardium is often accompanied by significant electrophysiological changes that significantly increase the risk of arrhythmias in the elderly [2]. The aging of the cardiovascular system is interconnected with longevity through many pathophysiological mechanisms [3]. In fact, dyslipidemia, hyperglycemia, insulin resistance, and other cardiometabolic diseases share common pathological mechanisms with aging and longevity [3]. ApoB is a secreted glycoprotein with 16 N-linked oligosaccharides associated with the egg yolk protein vitellogenin, and its primary function is to carry lipids [4]. Dyslipidemia caused by aging is related to apolipoprotein B[5][6]. Excessive concentrations of apolipoprotein B in plasma are risk factors for various cardiovascular and metabolic diseases, such as obesity, diabetes, and atherosclerosis [7]. Conversely, inhibition of apoB can prevent obesity and reduce cardiovascular risk [8][9]. Furthermore, exercise is the cornerstone of life. Sedentary behavior can cause cardiovascular remodeling, obesity, and even sudden cardiac death, which threatens health[10][11][12]. However, there is considerable difficulty and complexity in studying cardiac aging due to long life spans and genetic redundancy in mammals. Therefore, we utilized the Drosophila model to design this experiment.

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Drosophila is a mature model animal, and its powerful genetic toolkit and short-lived characteristics are the best choices for studying aging [13]. Several exercise models have been developed in Drosophila that recapitulates the characteristics of exercise-generated adaptations that are remarkably similar to those of humans or mammals [14][15][16][17][18]. Insect fat body functions identical to the mammalian liver and adipose tissue and plays a crucial role in energy storage and utilization [19]. In Drosophila, the lipoprotein (Lpp) resembles mammalian apoB-containing lipoproteins. Lpp production in the fat body requires Mtp, and the Drosophila apoB homolog, apolipoprotein (apoLpp) [20]. ApoLpp is a member of the apoB family, conserved across the animal kingdom [20]. When Lpp is secreted from the fat body, it is subsequently recruited to the gut, where they are further loaded with lipids and transported to other tissues [20]. Previous studies have shown that inhibition of fat body apoLpp can substantially reduce whole-body lipid levels in flies fed a standard diet, highlighting the critical contribution of fat body apoLpp to whole-body lipid metabolism [21].

The broad benefits of exercise on cardiovascular aging have been recognized [22]. For example, regularly trained rats can prevent aging-induced impairment of mitochondrial function and mitochondria-mediated cardiomyocyte apoptosis [23]. Endurance exercise protects aged Drosophila from lipotoxic cardiomyopathy [24]. Despite mounting evidence that regular exercise has preventive and protective effects on cardiac aging, the underlying mechanisms remain poorly understood. ApoB is an important marker of cardiovascular events, and it may be involved in a critical part of healthy aging [25]. However, it is unclear whether the protective effect of ELET on the aging heart is related to apoB.

Using a Drosophila exercise model, we reveal the benefits of fat body apoLpp inhibition for age-related arrhythmias. Significantly, the protective effect of ELET on the aged heart may be related to the decreased expression of apoLpp. Another surprising finding is that ELET and fat body $apoLpp^{RNAi}$ have a combined effect on low prolongation of average lifespan in Drosophila.

2. Results

2.1. Age-related arrhythmias in different genetic backgrounds

Aging is inevitable and the greatest risk factor for cardiovascular disease [26]. The increased incidence of arrhythmias is associated with aging, most commonly from age-induced arrhythmias [27][28]. Drosophila from different genetic backgrounds is quite different [29]. To avoid errors caused by differences in gene background, we used three strains of W^{1118}, LSP_2-Gal4, and LSP_2-Gal4>W^{1118} as controls, and the results are shown in Fig. 1. We found that the heart rate of 5-week-old flies was significantly lower than that of 2-week-old flies in 3 different genetic backgrounds (Figure 1A). In contrast, the heart period and arrhythmia index were significantly higher than 2-week-old flies (Figure 1B–D). These results may be because aging reduces the spontaneous frequency and increases the arrhythmia index [30]. In addition, we also found that the arrhythmia index of 5-week-old flies in the W^{1118} background was significantly higher than that of LSP_2-Gal4 and LSP_2-Gal4>W^{1118} (Figure 1C). This may be due to the different susceptibility to arrhythmia in different genetic backgrounds [31]. Therefore, we believe that aging significantly reduces fly's spontaneous heart rate and increases the occurrence of arrhythmias, especially in W^{1118}.

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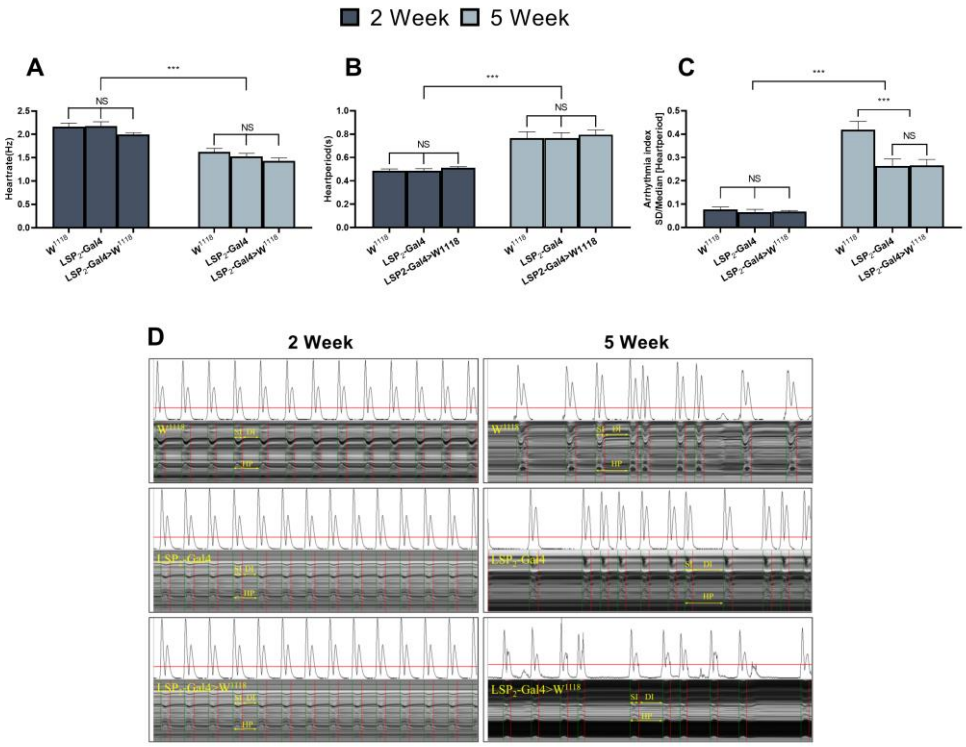

Figure 1. Arrhythmias in different genetic backgrounds. (A–C) Heart rate, cardiac cycle, and arrhythmia index of 2- and 5-week-old flies in the background of W^{1118}, LSP_2-Gal4, and LSP_2-Gal4>W^{1118}. N=30. The within-group comparisons were from one-way ANOVA, and the between-group comparisons were from student t-tests. Values are expressed as mean±SEM, *p<0.05, **p<0.01, ***p<0.001. (D) M-mode traces (8 s) prepared from high-speed movies of intact flies. The yellow arrows mark the SI, DI, HP, namely the systolic interval, the diastolic interval, and the heart period, respectively. This notation applies to all M-mode traces in this study unless otherwise stated.

2.2. Inhibition of fat body apoLpp mRNA expression rescues age-induced arrhythmias

Aging is often accompanied by decreased physical activity, weight gain, and metabolic disturbances [32]. Indeed, as shown in Figure 3, 5-week-old flies had a significant decrease in the climbing index and a significant increase in whole-body TG levels (Figure 2A, B). Furthermore, despite differences in body weight across genetic backgrounds, 5-week-old flies were significantly larger than 2-week-old flies (Figure 2C, E). We also found that the expression level of apoLpp mRNA was significantly elevated in the whole-body of 5-week-old flies (Figure 2D). A plausible explanation is that older flies have a low ability to scavenge lipids, leading to increased whole-body TG and body weight because these results are similar to humans and mammals [33].

We next asked whether fat body apoLpp plays a role in aging. We crossed LPS2-Gal4 with $apoLpp^{RNAi}$ to generate progeny flies with fat body apoLpp targeting KD to test this. In addition, we used LPS2-Gal4>W^{1118} flies as a control to exclude experimental errors caused by genetic background. In 2- and 5-week-old flies, inhibition of fat body apoLpp reduced the expression of whole body apoLpp mRNA by 43.5% and 78.2%, respectively (Figure 3D), indicating that fat body $apoLpp^{RNAi}$ was successful. We found that the knockdown of fat body apoLpp mRNA reversed the age-induced decrease in the climbing index and restored it to the same level as the control group (Figure 3A). Unexpectedly, inhibition of fat body apoLpp mRNA also increased the climbing index of 2-week-old flies beyond controls (Figure 3A). These results remind us of attention deficit hyperactivity disorder (ADHD). In ADHD patients, apoB concentrations are reduced, which may be associated with altered lipoprotein metabolism [34]. Furthermore, we also found that inhibition of fat body apoLpp reduced whole-body TG levels and body weight only in 5-week-old flies (Figure 3B, C). Although inhibition of fat body apoLpp reduced TG levels in 5-week-old

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flies, it was still higher than in controls (Figure 3B). The above results suggest that inhibition of fat body apoLpp is insufficient to counteract age-induced high whole-body TG levels. However, for body weight, inhibition of fat body apoLpp could restore body weight to the same level as the control group (Figure 3C). In general, the inhibition of fat body apoLpp can resist age-induced low exercise capacity and abnormal lipid metabolism to a certain extent.

Next, an assessment of the fly's cardiac function found that inhibition of fat body apoLpp did not affect heart rate in 2- and 5-week-old flies (Figure 3E). Still, it decreased the heart period in 5-week-old flies, although it did not recover to the same level as the control group (Figure 3F). Similarly, inhibition of fat body apoLpp did not affect the arrhythmia index in 2-week-old flies but restored arrhythmias in 5-week-old flies to the same level as controls (Figure 3G, H). These data above suggest that inhibition of fat body apoLpp can reverse age-induced arrhythmias, which may be mediated by improvements in lipid metabolism.

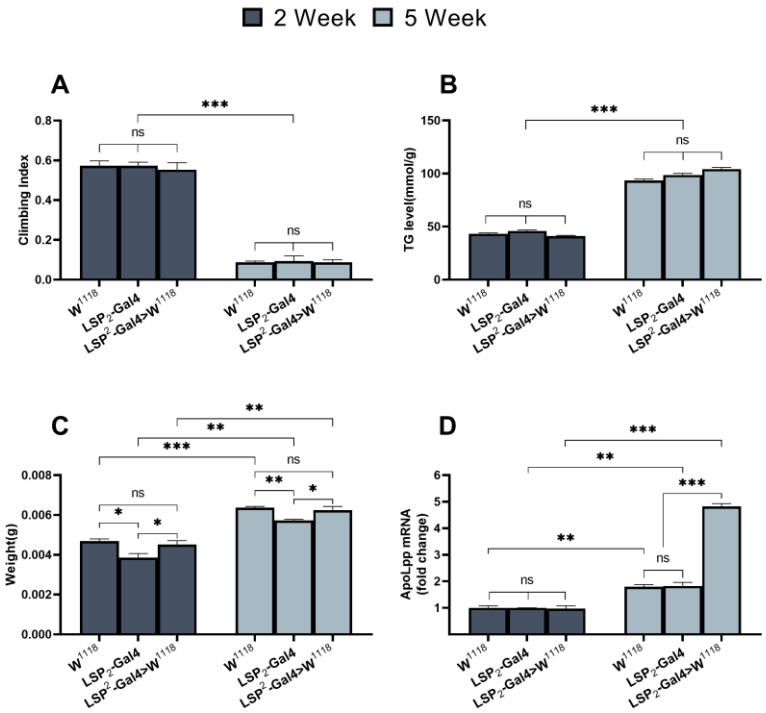

Figure 2. Climbing and lipid metabolism in aging flies with different genetic backgrounds. (A) Climbing index of 2- and 5-week-old flies. N=50, see the "Materials and methods" section to calculate the climbing index. (B) Whole-body TG levels in 2- and 5-week-old flies. N=5. Error bars represent three independent replicates. (C) Body weight of 2- and 5-week-old flies. N=5, values are expressed as the body weight of 5 flies and measured in triplicate. (D) Whole-body apoLpp mRNA expression levels in 2- and 5-week-old flies. GAPDH was used to normalize these values, N=10. All within-group comparisons were from one-way ANOVA, and between-group comparisons were from student t-tests. All values except body weight are expressed as mean±SEM, *p<0.05, **p<0.01, ***p<0.001.

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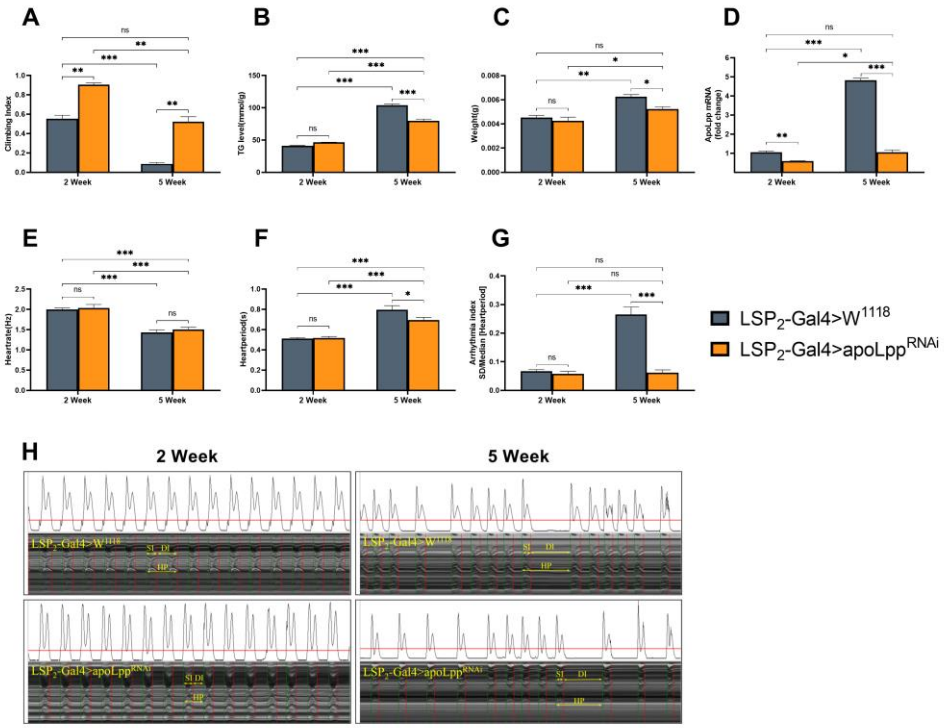

Figure 3. Effects of fat body apoLpp^{RNAi} on arrhythmia and lipid metabolism. (A) Climbing index of fat body apoLpp^{RNAi} in 2- and 5-week-old flies. N=50, see the "Materials and methods" section to calculate the climbing index. (B) Whole-body TG levels in 2- and 5-week-old flies by apoLpp^{RNAi} in the fat body. N=5. Error bars represent three independent replicates. (C) Body weight of 2- and 5-week-old flies with apoLpp^{RNAi} in the fat body. N=5, values are expressed as the body weight of 5 flies and measured in triplicate. (D) Whole-body apoLpp mRNA expression levels in 2- and 5-week-old flies for fat body apoLpp^{RNAi}. GAPDH was used to normalize these values, N=10. (E–G) Heart rate, cardiac cycle, and arrhythmia index of 2- and 5-week-old flies with fat body apoLpp^{RNAi}. N=30. (H) M-mode traces (8 s) prepared from high-speed movies of intact flies. All P-values are from student t-tests, all values are expressed as mean±SEM, *p<0.05, **p<0.01, ***p<0.001.

2.3. Flies fat body apoLpp^{RNAi} does not extend lifespan

First, we compared the lifespan of flies in the W^{1118}, LSP$_2$-Gal4, and LSP$_2$-Gal4>W^{1118} backgrounds. We found that flies' mean and maximal lifespans in the LSP$_2$-Gal4 background were significantly lower than in the other two backgrounds (Figure 4A). We speculate that this significant difference may be due to different genetic backgrounds, but the reasons for this result remain unclear. To interrogate the effect of inhibition of fat body apoLpp mRNA on lifespan, we crossed LSP$_2$-Gal4 with apoLpp^{RNAi} flies and used LSP$_2$-Gal4>W^{1118} as a control. We found that flies' fat body apoLpp^{RNAi} did not prolong the mean lifespan (Figure 4B).

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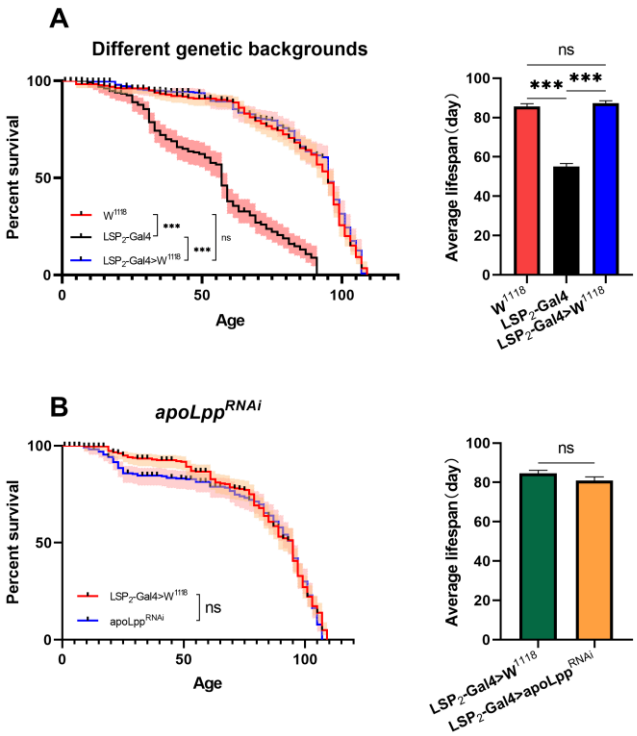

Figure 4. Effects of fat body apoLpp^{RNAi} on lifespan. (A) Lifespan of different genetic backgrounds. On the left is the flies' survival rate (%), and on the right is the average lifespan of flies. The sample sizes of W^{1118}, LSP$_2$-Gal4, and LSP$_2$-Gal4>W^{1118} were 202, 190, and 205. (B) Effects of fat body apoLpp^{RNAi} on lifespan. On the left is the flies' survival rate (%), and on the right is the average lifespan of flies. The sample sizes of LSP$_2$-Gal4>W^{1118} and LSP$_2$-Gal4>apoLpp^{RNAi} were 205 and 195. P-values for all survival curves were obtained from the log-rank test. For mean life, P-values were obtained from student t-tests, and all values were expressed as mean±SEM. *p<0.05, **p<0.01, ***p<0.001.

2.4. ELET saves age-induced arrhythmias but does not prolong average lifespan

There is currently solid scientific evidence to support the cardiovascular benefits of regular exercise [22]. However, lack of exercise is a common phenomenon in today's society. In the elderly, the safety margin of exercise dose decreases with age, which predisposes them to sports injury [35]. Therefore, to circumvent this problem, it will be essential to know whether the effects of ELET can be retained in later life. We performed an ELET intervention in flies using gravity-negative geotaxis. In the W^{1118} strain, the results showed that ELET-treated 2-week-old and 5-week-old flies had no significant difference in heart rate and heart period compared with their respective controls (Figure 5A, B) but reduced arrhythmias index (Figure 5C). In the LSP$_2$-Gal4 strain, ELET-treated 2- and 5-week-old flies had no significant differences in heart rate and heart period compared with their respective controls (Figure 5D, E); only in 5-week-old flies arrhythmic indices were reduced (Figure 5F). In contrast, in the LSP$_2$-Gal4>W^{1118} line, ELET-treated 2- and 5-week-old flies had increased heart rate and decreased heart period compared with their respective control groups (Figure 5G, H); but reduced arrhythmia index only in 5-week-old flies (Figure 5I). Thus, for heart rate and heart period, the reduction in heart rate and the increase in heart period in aged flies were not reversed by ELET, except in the LSP$_2$-Gal4>W^{1118} strain. Interestingly, although ELET's effect on reducing arrhythmia index in young flies was unstable across different genetic backgrounds, it was stable in older flies. Therefore, we believe that ELET can effectively reduce the arrhythmia index in aged flies under these three different genetic backgrounds, and the effect of ELET is stable (Figure S1). In addition, ELET treatment was more sensitive to LSP$_2$-Gal4>W^{1118} because aged flies of the LSP$_2$-Gal4>W^{1118} strain responded positively to ELET in heart rate,

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heart period, and arrhythmia indices. In addition, we also examined the lifespan of flies. The results showed that ELET did not prolong maximum or average lifespan (Figure 5J–L). Although we found shorter lifespans in flies in the LSP$_2$-Gal4 background, ELET still failed to extend their average lifespan (Figure 5K).

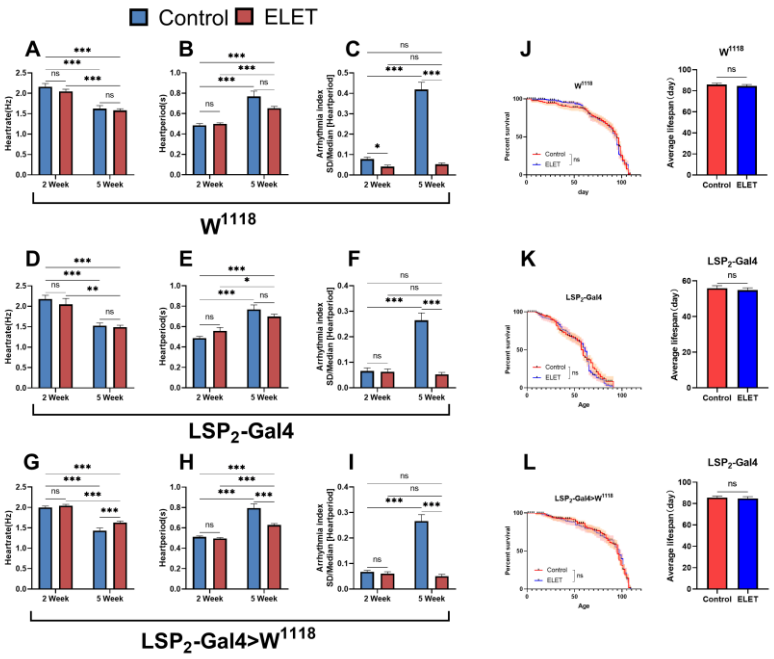

Figure 5. Effects of ELET on arrhythmias and lifespan. (A–C) Heart rate, heart period, and arrhythmia indices in ELET-treated 2- and 5-week-old flies in the W^{1118} genetic background. N=30. (D–F) Heart rate, heart period, and arrhythmia index in ELET-treated 2- and 5-week-old flies in the LSP$_2$-Gal4 genetic background. N=30. (G–I) Heart rate, heart period, and arrhythmia index in ELET-treated flies in the LSP$_2$-Gal4>W^{1118} genetic background. N=30. (J) The lifespan of ELET-treated 2- and 5-week-old flies in the W^{1118} genetic background. On the left is the fly's survival rate (%), and on the right is the average lifespan of flies. The sample sizes for Control and ELET were 202 and 210. (K) The lifespan of ELET-treated flies in the LSP$_2$-Gal4 genetic background. On the left is the fly's survival rate (%), and on the right is the average lifespan of flies. The sample sizes for Control and ELET are 190 and 193. (L) The lifespan of ELET-treated flies in the LSP$_2$-Gal4>W^{1118} genetic background. On the left is the fly's survival rate (%), and on the right is the average lifespan of flies. The sample sizes for Control and ELET were 205 and 181. P-values for all survival curves were obtained from the log-rank test. All P-values except survival curves were from student t-tests, and all values are expressed as mean±SEM. *p<0.05, **p<0.01, ***p<0.001.

2.5. ELET inhibits whole-body apoLpp mRNA accumulation in flies and improves age-induced abnormal lipid metabolism

Considering that TG levels are very dependent on genetic background [36], we simultaneously examined the effects of ELET on TG, body weight, and apoLpp mRNA in three genetic backgrounds. The results showed that for TG, ELET only reduced TG levels in 5-week-old flies but had no significant effect on 2-week-old flies (Figure 6A, D, G). Differently, in the W^{1118} background, ELET reduced the age-induced elevation of whole-body TG levels and was comparable to that of the 2-week-old control group (Figure 6A). In contrast, in the LSP$_2$-Gal4 and LSP$_2$-Gal4>W^{1118} backgrounds, ELET did not restore whole-body TG in 5-week-old flies to the same level as in the 2-week-old controls (Figure 6D, G). These results indicate that although ELET reduces the whole-body TG of aged flies to different extents under different backgrounds, it can suggest that ELET has an inhibitory effect on triglyceride accumulation in old flies. Regardless of the background, only aging significantly increased body weight, while ELET had no reduced effect (Figure 6B, E, H). In addition, we also found that ELET significantly reduced the expression of apoLpp mRNA in the whole body of 2- and 5-week-old flies under three different backgrounds

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(Figure 6C, F, I). Interestingly, although ELET reduced whole-body apoLpp mRNA expression in 2-week-old flies in three different backgrounds, it did not reduce whole-body TG levels (Figure 6A, C, D, F, G, 6I). This may be due to the inherently low whole-body TG levels in 2-week-old flies, resulting in a reduced sensitivity of ELET to whole-body TG. In general, these results support that ELET can reduce the expression level of apoLpp mRNA whole-body of old flies and improve lipid metabolism.

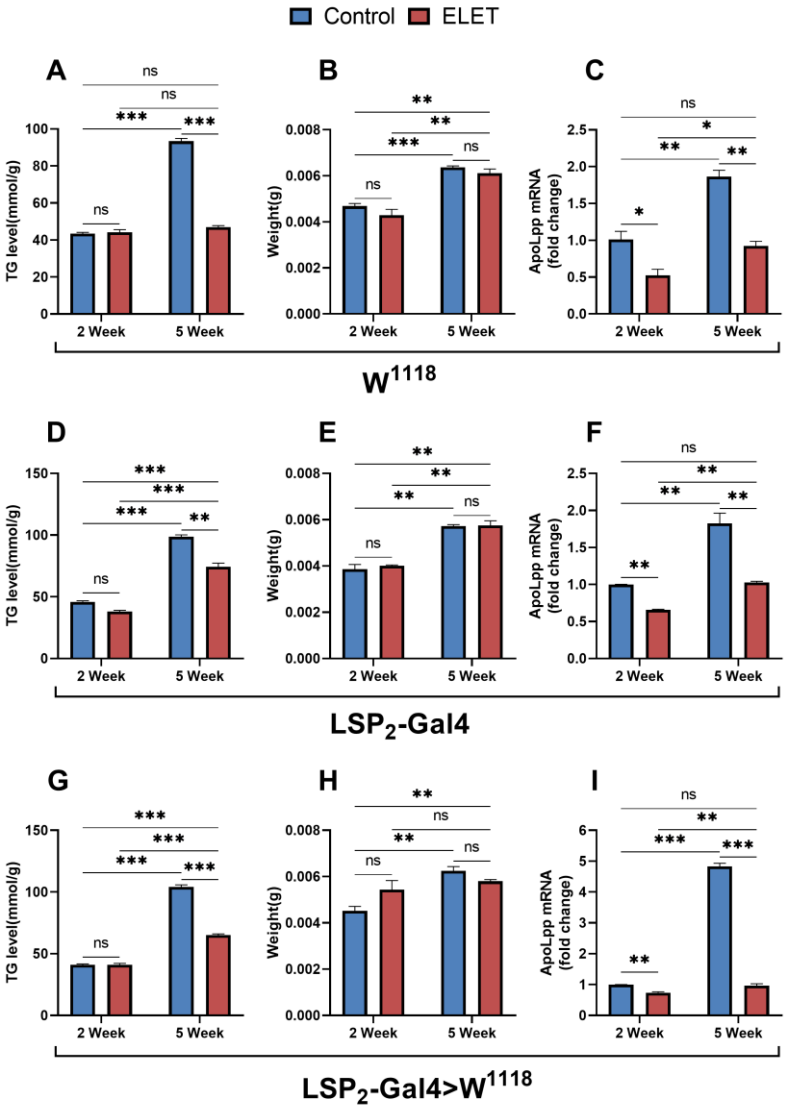

Figure 6. Effects of ELEL on lipid metabolism in flies under different genetic backgrounds. (A–C) Whole-body TG levels, body weight, and whole-body apoLpp mRNA levels in ELET-treated 2- and 5-week-old flies in the W^{1118} genetic background. (D–F) Whole-body TG levels, body weight, and whole-body apoLpp mRNA levels in ELET-treated 2- and 5-week-old flies in the LSP_2-Gal4 genetic background. (G–I) Whole-body TG levels, body weight, and whole-body apoLpp mRNA levels in ELET-treated 2- and 5-week-old flies in the LSP_2-Gal4>W^{1118} genetic background. To detect whole-body TG, the sample size of all flies was 5, and measured in triplicate. For body weight, values are expressed as the body weight of 5 flies and measured in triplicate. GAPDH was used to normalize these values for whole-body apoLpp mRNA expression levels in flies, and N=10. All values are expressed as mean±SEM, and all P-values are from student t-tests. *p<0.05, **p<0.01, ***p<0.001.

2.6. ELET combined with fat body apoLpp^{RNAi} improve age-induced arrhythmias and prolongs the average lifespan

The studies above suggest that although ELET and fat body apoLpp^{RNAi} do not prolong the average lifespan of flies, they can improve age-induced arrhythmias. But, ELET-

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binding fat body apoLpp^{RNAi} on lifespan and age-induced arrhythmias in flies is unclear. To elucidate whether the combined effect of ELET and fat body apoLpp^{RNAi} could have a more profound impact, we performed ELET on flies with fat body apoLpp^{RNAi}. The results showed that in 2-week-old flies, apoLpp^{RNAi}+ELET had no significant difference on arrhythmia index (Figure 7A), and it was also not significantly different from LSP$_2$-Gal4>apoLpp^{RNAi} and LSP$_2$-Gal4>W^{1118}+ELET (Figure 7A). In 5-week-old flies, although apoLpp^{RNAi}+ELET significantly reduced the arrhythmia index, it was also not significantly different from LSP$_2$-Gal4>apoLpp^{RNAi} and LSP$_2$-Gal4>W^{1118}+ELET (Figure 7A). These data suggest that although apoLpp^{RNAi}+ELET reduced arrhythmia index in aged flies, the combined effect did not confer additional benefits on arrhythmia. Next, we counted the lifespan of the flies. LSP$_2$-Gal4>W^{1118} with the same genetic background was used as a control group. The results showed that apoLpp^{RNAi}+ELET extended the average lifespan of flies by about 7.84% (Figure 7B). In general, these results suggest that the combined effect of apoLpp^{RNAi} and ELET has no additional benefit in reducing arrhythmia index in aged flies. Still, it extends the average lifespan of flies.

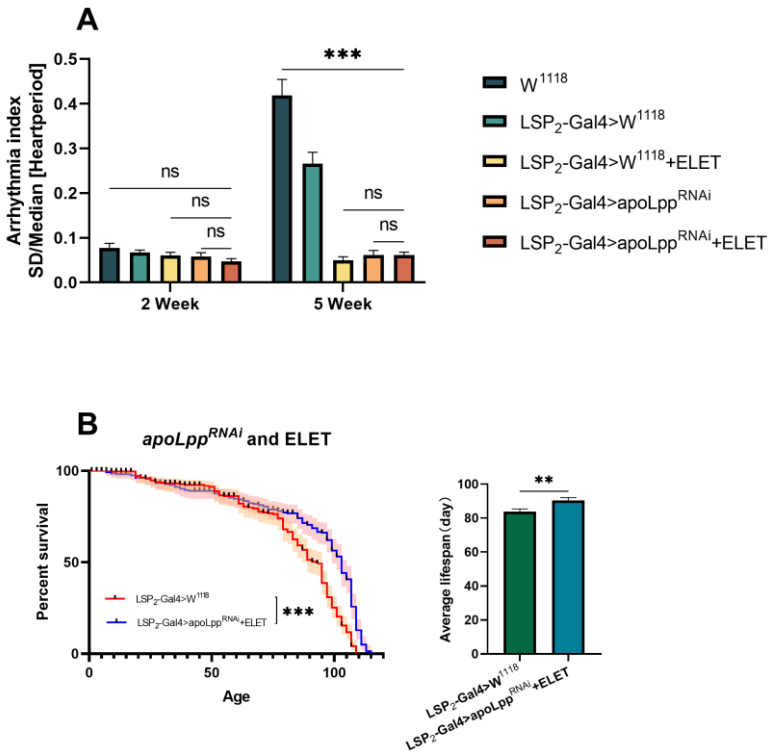

Figure 7. Effects of ELET combined with fat body apoLpp^{RNAi} on lifespan and age-induced arrhythmias in flies. (A) Arrhythmia index in flies under co-action of ELET with fat body apoLpp^{RNAi}. N=30. Two-way ANOVA was used for LSP$_2$-Gal4>W^{1118}, LSP$_2$-Gal4>W^{1118}+ELET, LSP$_2$-Gal4>apoLpp^{RNAi}, and LSP$_2$-Gal4>apoLpp^{RNAi}+ELET followed by post hoc tests using Bonferroni correction. The P-values for W^{1118} and LSP$_2$-Gal4>W^{1118}+ELET were from student t-tests. (B) The lifespan of flies under co-action of ELET with fat body apoLpp^{RNAi}. On the left is the flies' survival rate (%), and on the right is the average lifespan of flies. The sample sizes of LSP$_2$-Gal4>W^{1118} and LSP$_2$-Gal4>apoLpp^{RNAi}+ELET were 205 and 183, respectively. The P-values for survival curves were obtained from the log-rank test, and the P-values for mean lifespan were obtained from student t-tests. *p<0.05, **p<0.01, ***p<0.001.

3. Discussion

Aging is a significant cause of cardiovascular disease, including cardiac arrhythmias, fibrillation, and coronary atherosclerosis. The polygenic and multiorgan nature of the aging-induced cardiovascular disease makes it difficult to determine the relative contribution of each of these diseases. We used a Drosophila model to simulate aging-induced arrhythmias to elucidate the underlying mechanisms. Because the Drosophila model is a

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simpler system, it can help us examine these complex interactions. We exploited the mature genetic toolkit and short-lived characteristics of Drosophila to study the crosstalk between apolipoprotein B-mediated lipid metabolism and aging-induced arrhythmias. We found that arrhythmias in aged flies may be associated with elevated apolipoprotein B. Inhibition of apoLpp mRNA, a homolog of apolipoprotein B, and early exercise training could reduce the occurrence of age-related arrhythmia risk and promote healthy aging. We provide evidence that old flies exhibit lower spontaneous heart rates and develop arrhythmias, with similar features across different genetic backgrounds. In addition, aging also shows increased TG and body weight and decreased climbing ability, which has identical features to age-related abnormal lipid metabolism [43][44][45].

Since apolipoprotein B is a marker of cardiovascular disease risk and a key molecule in lipid metabolism, we investigated the effect of altered fat body apolipoprotein B gene expression on arrhythmias under aging conditions. We found that inhibition of apoLpp mRNA expression in the fat body of aged flies reduced the development of age-related arrhythmias. In addition, inhibition of fat body apoLpp mRNA also reduced whole-body TG levels and body weight in old flies and improved climbing ability. Recently, studies have begun to reduce apolipoprotein B as an emerging therapy to prevent cardiovascular disease [9][46]. For example, inhibition of liver apoB mRNA in mice reduced apolipoprotein B concentrations and reduced atherosclerosis [47]. Furthermore, deletion of PCSK9 in mice resulted in decreased lipid and apoB levels and atherosclerotic LDL reduction, and reduced atherosclerosis [48]. Therefore, inhibiting the expression of apolipoprotein B may be an essential mechanism to reduce age-induced arrhythmias. In addition, we also examined the effect of inhibiting the expression of apoLpp mRNA on lifespan. We found that flies with different genetic backgrounds had large differences in lifespan. Although it is unclear how this difference is caused, fat body apoLpp^{RNAi} did not affect flies' lifespan compared with flies of the same genetic background.

To determine the contribution of exercise to the aging phenotype, we used a Drosophila exercise apparatus to simulate exercise [18]. Flies begin exercise training 24 hours after eclosion, which we call early life exercise training or ELET. The results of ELET showed that ELET mainly reduced whole-body TG levels in aged flies but had no effect on body weight. A previous study in rats showed that trained older rats had enlarged hearts and improved cardiac function compared with sedentary older rats [49]. Here, we found that ELET reduced the arrhythmia index in aged flies. The above shows that not only does exercise training in later life provide cardiac benefits, but exercise training early in life can also preserve its benefits into later life. Although studies have shown that maintaining a certain limit of physical activity can reduce the risk of death and achieve the purpose of prolonging life [50], we found that ELET did not prolong the average lifespan of flies, which may be related to different exercise methods. In addition, ELET also reduced the expression of apoLpp mRNA in the whole-body of flies and improved lipid metabolism, and This has similar results to a study in obese mice [51]. Therefore, we believe that ELET reduces age-induced arrhythmias associated with reduced whole-body apoLpp mRNA expression.

We present data showing that both ELET and fat body apoLpp^{RNAi} can reduce age-related arrhythmias and reduce whole-body TG levels in aged flies. However, ELET and fat body apoLpp^{RNAi} treatment alone did not prolong the average lifespan of flies. Therefore, we also examined whether the combination of ELET and fat body apoLpp^{RNAi} could prolong the average lifespan of flies. The results showed that the combination of ELET and fat body apoLpp^{RNAi} extended the average lifespan of flies. However, this combined effect had no additional benefit for age-related arrhythmias. In a word, our data support that ELET and fat body apoLpp^{RNAi} reduce arrhythmias in old flies and that the combination of the two prolongs the average lifespan of flies.

4. Materials and Methods

4.1. Fly Stocks and Maintenance

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All lines were obtained from the Bloomington Drosophila Stock Center: W^{1118} (BL3605), LSP_2-Gal4 (BL6357), $apoLpp^{RNAi}$ (BL33388). All flies were maintained at 25℃, 50% humidity, and a 12-hour light-dark cycle using standard SYA (Saccharomyces cerevisiae agar) food. Unless otherwise stated, all flies used in the experiments were female virgin flies.

4.2. Exercise Training

The Drosophila exercise training device is designed according to Tower power and Swing boat [14][37]. As previously, flies were stimulated to actively walk upwards by flipping the vial for exercise [38]. In this study, the flies in the ELET group were placed in the exercise training device within 24 hours after eclosion, working out 2.5 hours a day, 5 days a week with two days off, for a total of two weeks. We call this program Early Life Exercise Training or ELET.

4.3. RT-PCR

Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions, and 10 μg of total RNA was synthesized from total RNA using Superscript II reverse transcriptase (Invitrogen) using oligonucleotides (dT). qPCR amplification reactions were performed in triplicate by mixing 1 μl of RT product with 10 μl of SYBR qPCR master mix (TaKaRa) containing the appropriate PCR primers. Thermal cycling and fluorescence monitoring were performed in an ABI7300 (Applied Biosystems, USA) using the following PCR conditions: (30 s at 95°C, 5 s at 95°C, 30 s at 60°C) × 40. Normalized with Gapdh. The primers used are as follows:

Gapdh F：5'-GCGTCACCTGAAGATCCCAT-3'
R：5'-GAAGTGGTTCGCCTGGAAGA-3'
apoLpp F：5'-AATTCGCGGATGGTCTGTGT-3'
R：5'-GCCCCTTAGGGATAGCCTTT-3'

4.4. Semi-intact Drosophila Heart Preparation and Heartbeat Analysis

Flies were anesthetized using Fly Nap, the head and ventral thorax were rapidly removed, and oxygenated artificial hemolymph (AH) was injected, followed by removal of the ventral abdominal cuticle and all internal organs to expose the ventral canal [39][40]. A 30-s digital movie of high-speed heartbeats was captured using an EM-CCD high-speed camera at 120–140 fps and recorded using HCImage software (Hamamatsu, Japan). Heart rate, cardiac cycle, arrhythmia index, etc., were precisely quantified using semi-automated optical heartbeat analysis software (available from SOHA, Ocorr, and Bodmer) [39].

4.5. Climbing assay

The negative geotaxis climbing ability test was adapted from a previous method [41]. The climbing device was composed of five 20 cm long glass tubes with an inner diameter of 2.8 cm (sponges were placed at the ends of the tubes to prevent escape but allow air exchange). The sponge plugs at each end of the long glass tube are 2 cm each, allowing 16 cm of climbing space for the flies. The long glass tube is equally divided into 1, 2, 3, and 4 quadrants from bottom to top, and each quadrant is 4 cm. Allow flies to acclimate to the vial for 30 min before assessing negative geotaxis. Negative geotaxis was triggered by tapping the climbing device in rapid succession to drop the flies to the bottom of the bottle. The location of the flies was captured in digital images taken at the end of 10 s after eliciting the behavior. This process was repeated 3 times. 20 flies per tube. The photos were placed in Photoshop for analysis of the climbing index. Climbing index = the number of flies in the fourth quadrant / the total number of flies in the glass bottle.

4.6. Triglyceride and weight assay

Triglycerides: take 15 flies, and 1 mL of extract was added and homogenized on ice. Then centrifuge at 12,000 rpm for 10 min at 4°C, and take the supernatant for testing. The

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TG content was determined by measuring the absorbance at 510 nm using the Triglyceride (TG) Content Test Kit (mlbio, #ml076637, China) following the manufacturer's instructions. Body weight: flies were weighed using an electronic microbalance (Uni bloc, AUW220D, Japan), recorded every 5 flies, and performed 3 biological replicates.

4.7. Lifespan assays

Lifespan assays were mainly performed as described [42]. Flies were reared at a controlled larval density. Flies from one mating were CO2 anesthetized, sex-sorted, and transferred to vials (20 flies/vial). Dead flies were counted every 2 days. 10 replicates (= 200 flies) were used for each condition. Survival curves were drawn using GraphPad Prism 6.

4.8. Statistical analysis

Analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 21.0 (SPSS Inc., Chicago, IL, United States) for Windows and graphed using GraphPad Prism 6. Comparisons between different ages were performed using student t-tests. Comparisons between different groups of the same age were performed using student t-tests (comparison between 2 groups) or one-way ANOVA (comparison among 3 groups). An LSD post hoc test always followed one-way ANOVA. Two-way ANOVA was used to analyze the combined effect of ELET and apoLpp^{RNAi}, followed by post hoc testing with Bonferroni correction. P-values for survival curves are derived from log-rank. The statistical significance level was set at $p < 0.05$. Data are presented as mean ± SEM unless otherwise stated.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: M-mode traces (8 s) prepared from high-speed movies of intact flies of 2- and 5-week-old flies. File S1: Raw data.

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