Access schedules mediate the impact of high fat diet on ethanol intake in mice.

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Abstract

High fat diet (HFD)-induced obesity and alcoholism each individually promote insulin resistance and glucose intolerance, and together increase risk for Type II diabetes in the clinical setting. Conversely, animal studies, typically utilizing forced/continuous alcohol (EtOH) access, tend to show that EtOH intake decreases HFD-induced effects on insulin and glucose function, while HFD decreases EtOH intake. Intermittent access increases EtOH intake, but HFD effects on intermittent EtOH and resultant changes to metabolic function are not well characterized. The present studies sought to determine if HFD alters EtOH intake in male C57Bl/6J mice given differing two-bottle choice EtOH access schedules, and to determine the impact of HFD and scheduled EtOH intake on insulin sensitivity and glucose tolerance. In the first cohort, mice were given Unlimited Access EtOH (UAE)+HFD (n=15; HFD=60% calories from fat, 10% EtOH v/v, *ad libitum*) or UAE+Chow (n=15; standard mouse chow=10% calories from fat, *ad libitum*) for 6 weeks. UAE+HFD mice gained significantly more weight, had lower EtOH preference, consumed significantly less EtOH, and were insulin resistant and hyperglycemic compared with UAE+Chow mice. In the second cohort, mice were given Limited Access EtOH (LAE, 4 hrs/d; 3 d/wk)+HFD (n=5) or LAE+Chow (n=5) with increasing EtOH concentrations (10%, 15%, 20%). LAE+HFD mice gained more weight than LAE+Chow mice and had lower 10% EtOH preference, with no difference in total EtOH consumption at any EtOH concentration. LAE+HFD mice had seemingly normal insulin sensitivity but profound hyperglycemia and glucose intolerance. These results suggest that access schedules determine the impact of HFD on EtOH consumption and HFD+EtOH-induced metabolic dysfunction.

Introduction

Obesity and alcohol use disorders (AUDs) are two of the most common chronic conditions in the United States. Clinically, HFD-induced obesity is associated with insulin resistance, glucose intolerance, and increased risk for developing Type II diabetes (Haslam and James, 2005). Chronic EtOH drinking is also a risk factor for insulin dysfunction (Fan *et al*, 2006; Papachristou *et al*, 2006). Such clinical and epidemiologic findings suggest an overlap in the mechanisms by which HFD and EtOH exposure modulate insulin action and glucose homeostasis and that together, co-morbid chronic HFD and EtOH intake may increase risk for subsequent disease states such as Type II diabetes (Steiner *et al*, 2015). The majority of epidemiologic evidence, however, suggests that moderate EtOH intake has protective effects on insulin sensitivity (Traversy and Chaput, 2015), but this may only occur in non-obese patients (Yokoyama, 2011). Most preclinical studies also suggest that EtOH consumption mitigates HFD-induced metabolic dysfunction, which may be related to the moderate EtOH intake found in these models (Feng *et al*, 2012; Gelineau *et al*, 2017; Hong *et al*, 2009; Paulson *et al*, 2010). More recent epidemiologic studies, however, have brought these assumptions back up for debate (Griswold *et al*, 2018).

Numerous clinical studies show increased desire, cravings, and intake of high fat foods during and after drinking episodes (Breslow *et al*, 2013; Caton *et al*, 2004; Piazza-Gardner and Barry, 2014). A similar positive relationship for EtOH-induced increases in HFD intake has been shown in some animal models (Barson *et al*, 2009). While the inverse relationship of HFD exposure stimulating EtOH intake has also been suggested in some animal models (Carrillo *et al*, 2004), the majority of findings indicate that HFD exposure decreases EtOH consumption (Feng *et al*, 2012; Gelineau *et al*, 2017; Sirohi *et al*, 2017a, 2017b). The majority of previous studies, however, have examined EtOH intake in the face of HFD access without specific focus on metabolic function, or metabolic effects of EtOH and HFD without taking intake behaviors into account. While these studies and others (Guo *et al*, 2018) have greatly advanced our understanding of the impact that HFD and EtOH have on metabolic and end-organ function, these models typically do not replicate the escalation of drinking behaviors common to human AUDs. It is now well characterized that limited access scheduling increases EtOH intake in animal models in an escalating fashion akin to human AUD development (Melendez, 2011). The impact of HFD on this type of scheduled EtOH access, however, has not been examined. Therefore, the overall goals of these studies were to determine if HFD alters EtOH intake in mice consuming EtOH with limited or unlimited access schedules and how such access schedules modulate the interaction of HFD and EtOH on metabolic function in male C57Bl/6J mice. Our findings suggest that HFD reduces EtOH intake when EtOH is freely available but such effects are attenuated when scheduled access to EtOH is limited. Contrary to previous work, our study suggests that moderate intake of freely-available EtOH did not mitigate the impact of HFD on insulin sensitivity. Conversely, higher levels of EtOH intake in the limited access model improved insulin sensitivity, but worsened glucose tolerance, in HFD-fed mice, suggesting HFD and EtOH may interact to disrupt insulin action and glucose homeostasis by distinct mechanisms based on EtOH scheduling.

Methods

Animals

Six-week old male C57Bl/6J mice were purchased from The Jackson Laboratory (stock # 000664). Upon arrival, mice were individually housed and given standard chow diet for a four-day acclimation period. Mice were weight matched and separated into four groups as described below. All mice were kept in a temperature and humidity controlled room on a 12-hour light/dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee at Penn State University College of Medicine (Hershey, PA).

EtOH Ramp

All mice underwent an EtOH-ramp initiation period prior to avoid potential confounds of EtOH taste aversion. The EtOH two-bottle choice ramp procedure consisted of home-cage 24-hour access to a bottle containing tap water and another bottle containing 3% EtOH for 48 hours, 7% EtOH for 72 hours, and 10% EtOH for 72 hours (all EtOH concentrations are vol/vol in tap water). Water and ethanol solution were administered via inverted 50 mL conical tubes (Fisher) and sealed with a rubber stopper (#5.5, Fisher) containing a 2-inch stainless-steel straight sipper tube (Ancare). Ethanol solution was made using ethyl alcohol (190 proof, PHARMCO-AAPER) diluted in tap water.

Unlimited access to EtOH with ad libitum diets (UAE model)

To examine the effects of continuous two-bottle choice EtOH drinking in the presence of *ad libitum* HFD (60% calories from fat, Bioserv F3282) or control diet (10% calories from fat, Bioserv F4031), mice were separated into an unlimited 24-hour access to EtOH group receiving either HFD (UAE+HFD; n=15) or control diet (UAE+Chow; n=15). Following the EtOH-ramp initiation period, UAE+HFD and UAE+Chow groups had home-cage 24-hour access to two-bottle choice of tap water and 10% EtOH (vol/vol in water) throughout the remainder of the experiment. Body mass, EtOH and water intake, and EtOH preference was assessed every 24 hours for six weeks. Summary of timeline provided in Fig 1.

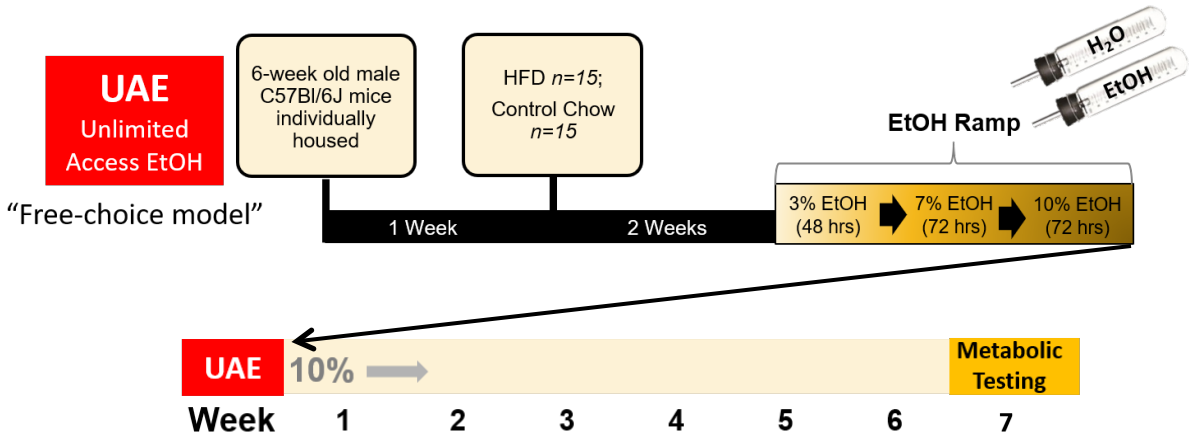

Fig 1. Summary of free-choice, unlimited access ethanol (UAE) timeline.

Limited access to EtOH with ad libitum diets (LAE model)

To examine the effects of limited two-bottle choice EtOH drinking in the presence of *ad libitum* HFD or control diet, mice were weight matched and randomly assigned to groups receiving either HFD (LAE+HFD; n=5) or control diet (LAE+Chow; n=5). Diets are the same as described above. Following the EtOH-ramp initiation period, LAE+HFD and LAE+Chow groups had home-cage to two-bottle choice of tap water and EtOH (vol/vol in water) limited to 4-hour access periods on Monday, Wednesday, and Friday beginning at 10am and ending at 2pm. These groups had access to 10% EtOH for three weeks, followed by 15% EtOH for two weeks, and 20% EtOH for two weeks. Body mass, EtOH and water intake, and EtOH preference was assessed after each drinking session.

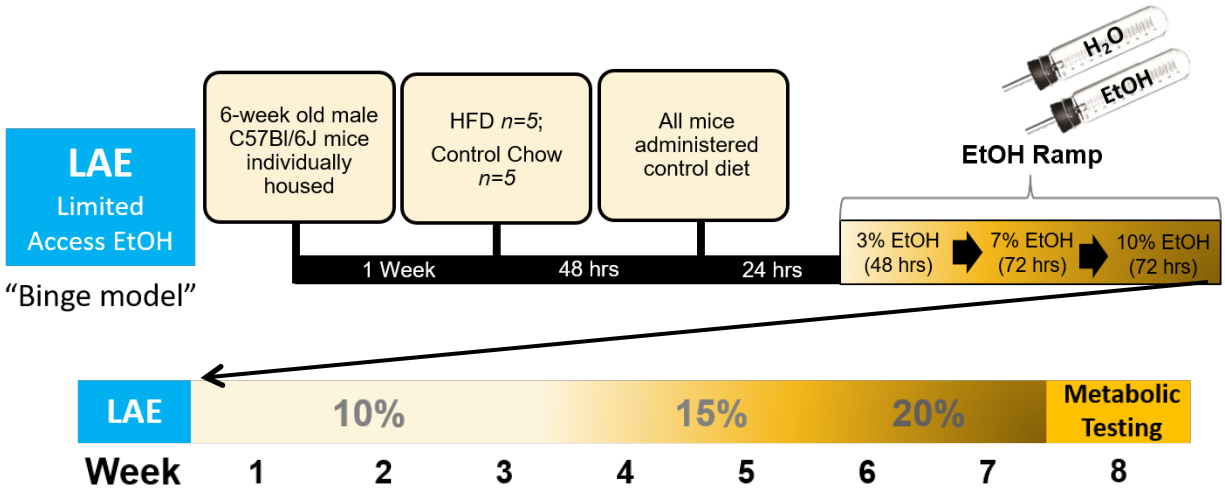

Fig 2. Summary of Limited Access Ethanol (LAE) timeline.

Metabolic Testing

Once the drinking studies concluded, mice underwent standardized insulin tolerance (ITT) and glucose tolerance (GTT) tests. For ITT, mice were fasted for four hours and then injected intraperitoneally with insulin (0.75 units/kg of regular U-100 insulin in 0.9% NaCl; Novolin). A tail vein blood sample was taken at baseline, 15, 30, 60, 90, and 120 minutes post-insulin injection to measure blood glucose with a glucometer (Prodigy AutoCode). For GTT, mice were fasted overnight and then injected intraperitoneally with dextrose (2 g/kg of 50% dextrose). Blood glucose was measured at baseline, 15, 30, 60, 90, and 120 minutes post-dextrose injection. Body composition (fat, lean, and fluid masses) were measured in conscious mice using a quantitative nuclear magnetic analyzer (Bruker Minispec). As controls, age and weight matched mice receiving HFD (n=15) and control diet (n=11) for similar periods but without EtOH exposure were used.

Statistics

Two-way ANOVA was used to compare the main effects of diets and number of drinking sessions on EtOH intake, and their interaction over the study time course. One-way ANOVA was used to compare end of study metrics comparing diet+EtOH groups to diet exposed control groups not receiving EtOH. Unpaired t-tests were used to compare end of study metrics between diet+EtOH groups. All data are represented as mean±standard error of the mean (SEM).

Results

We determined effects of *ad libitum* HFD or chow diet access on body mass and EtOH intake parameters in the UAE model, which had continuous free-access to 10% EtOH and water. Two-way ANOVA indicated UAE+HFD mice have significantly higher body mass than UAE+Chow mice over the course of the study (Diet: $F_{(1,28)}$=44.33, p<0.001; EtOH exposure sessions: $F_{(29,812)}$=224.5, p<0.001; Interaction: $F_{(29,812)}$=93.95, p<0.001; **Fig. 3A**). UAE+HFD mice consumed significantly less EtOH than UAE+Chow mice (Diet: $F_{(1,28)}$=27.38, p<0.001; EtOH exposure sessions: $F_{(25,700)}$=4.099, p<0.001; Interaction: $F_{(25,700)}$=0.8142, p=0.723; **Fig. 3B,C**) and had lower preference towards EtOH (Diet: $F_{(1,468)}$=35.71, p<0.001; EtOH exposure sessions: $F_{(25,468)}$=3.586, p<0.001; Interaction: $F_{(25,468)}$=0.6037; p=0.9362; **Fig. 3D**). These findings indicate *ad libitum* HFD access reduces EtOH intake in unlimited access "free-choice" model.

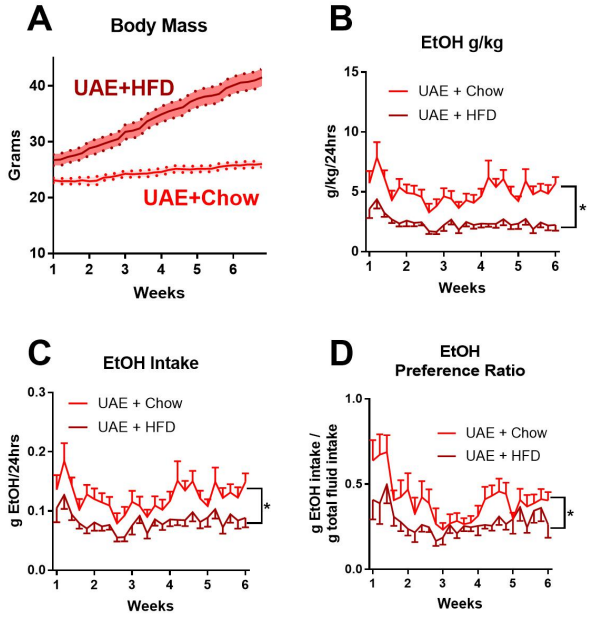

Fig 3: *Ad libitum* HFD access reduces EtOH intake in unlimited access "free-choice" UAE model. A) Time course of body mass changes by group during drinking period (n=15/group). Dark line indicates mean, shaded area with dots indicates range of standard error of the mean. **B-D)** HFD significantly reduces EtOH g/kg/24hrs, total EtOH g consumed/24hrs, and EtOH preference. Line indicates mean, error bars indicates standard error of mean. * indicates significant effect of diet as determined by two-way ANOVA; p<0.05.

At the end of the study, mice underwent metabolic testing. Metabolic data were compared to weight and age matched mice given HFD or chow diet without EtOH access. One-way ANOVA ($F_{(3,41)}$=59.96, p<0.001; **Fig. 4A**) indicated that body mass was elevated in HFD-EtOH naïve mice (44.9±0.7 g) and UAE+HFD exposed (43.4±2.5 g) animals when compared to chow-fed EtOH-naïve mice (28.8±0.6 g) or UAE+Chow mice (28.5±0.2). Likewise, one-way ANOVA showed EtOH consumption did not significantly alter HFD-induced changes to adiposity ($F_{(3,27)}$=71.1; p<0.001; **Fig. 4B**), lean mass ($F_{(3,27)}$=75.83, p<0.001; **Fig. 4C**), or fluid mass ($F_{(3,27)}$=57.93, p<0.001; **Fig. 4D**). These data indicate that EtOH consumption does not alter HFD-induced changes in body composition.

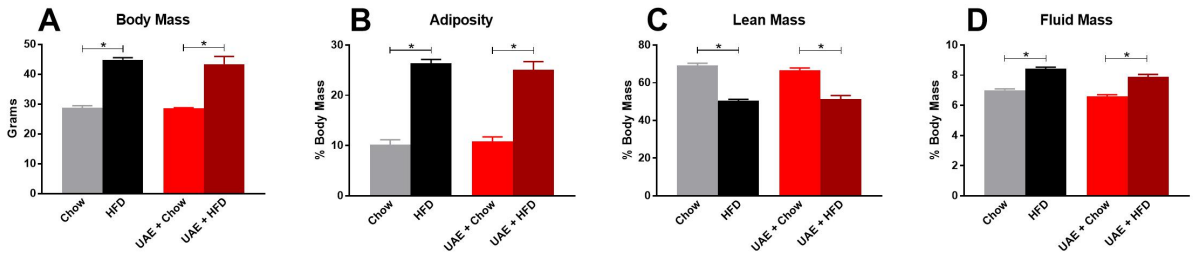

Fig 4: Free access EtOH consumption does not alter HFD-induced changes in body mass, adiposity, lean mass, or fluid mass. Body composition data are compared to chow- and HFD-fed EtOH naïve mice. **A)** Body mass of control mice and UAE mice prior to metabolic testing. Percent body mass of **B)** adiposity, **C)** lean mass, and **D)** fluid mass. * indicates significant difference between groups as determined by one-way ANOVA with Tukey's post hoc test; $p<0.05$.

HFD-induced increases in body mass and adiposity are well characterized to result in insulin resistance in mouse models, and previous research indicates that EtOH consumption may mitigate these effects. We therefore performed ITT in the UAE mice and compared these results to the same age and weight matched controls as utilized in Fig 4. One-way ANOVA followed by Tukey's post hoc analysis indicated a significant increase in 4-hour fasting glucose levels in HFD (186±9 mg/dl) and UAE+HFD (203±15 mg/dl), groups compared to chow alone (156±6 mg/dl) ($F_{(3,41)}$=2.97, p=0.043; **Fig. 5A**). UAE+Chow mice (182±12 mg/dl) were not significantly different from any other group. Figure **5B** shows the time course of change in blood glucose levels following intraperitoneal insulin injection; data are normalized to baseline to account for differences in fasting glucose levels among groups. The ITT area under the curve indicates reduced insulin sensitivity in EtOH naïve HFD-fed mice (-1748±1024 glucose mg/dl*min) compared to chow-fed mice (-5903± 662.4 glucose mg/dl*min), and EtOH consumption did not alter HFD-induced insulin resistance in the UAE-HFD mice (-1282± 1091 glucose mg/dl*min) ($F_{(3,44)}$=3.71, p=0.018; **Fig. 3C**). UAE+Chow mice were not significantly different from chow control mice. Contrary to many previous findings, these results indicate that free-access EtOH consumption does not improve insulin sensitivity in HFD exposed mice.

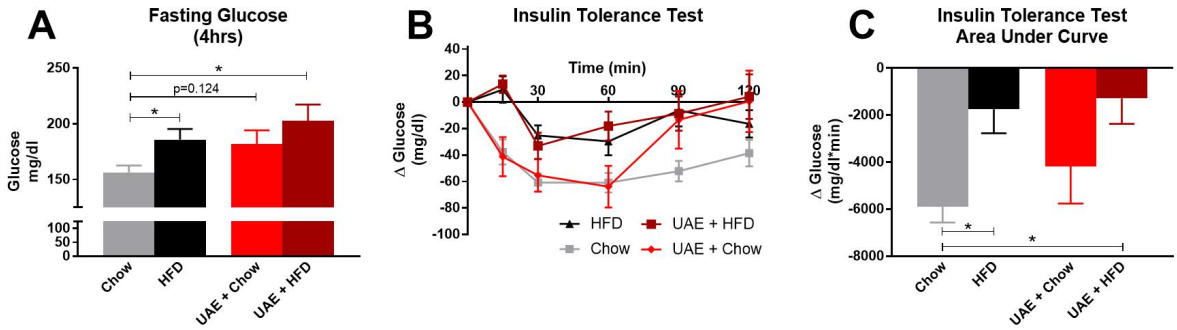

Fig 5. Moderate EtOH consumption does not alter HFD-induced insulin resistance. A) 4-hour fasting glucose levels prior to insulin tolerance test. **B)** Change in blood glucose levels over time following insulin injection; data are normalized to 0 at baseline. **C)** Area under the curve for change in blood glucose levels during insulin tolerance test. One-way ANOVA; *p<0.05

The above findings indicate that HFD decreases EtOH intake when provided in an unlimited "free-access" procedure. Previous research indicates limiting EtOH access in an every-other-day intermittent access model increases EtOH intake in rodents compared to continuous access (Melendez, 2011). Whether HFD might alter intermittent EtOH intake has not been well examined. Therefore, we placed weight-matched mice in a limited access EtOH procedure (LAE; 4hr/day, Monday, Wednesday, Friday every week, see Fig 2) and gave mice *ad libitum* HFD or chow. Two-way ANOVA indicated LAE+HFD mice gained significantly more body mass than LAE+Chow mice over the course of the study (Diet: $F_{(1,8)}$=11.92, p=0.009; EtOH exposure sessions: $F_{(34,272)}$=95.85, p<0.001; Interaction: $F_{(34,272)}$=35.68, p<0.001; **Fig. 6A**). LAE+HFD mice had significantly lower EtOH g/kg levels than the LAE+Chow group (**Fig. 6B**), but this may not reflect significant changes in intake since the HFD-fed mice had significantly elevated body mass on days indicated as having lower intake. Two-way ANOVA indicated a significant effect of exposure session on g/EtOH consumed (EtOH exposure sessions: $F_{(20,168)}$=46.99, p<0.001) with a trend for a significant effect of diet on g/EtOH consumed (Diet: $F_{(1,168)}$=3.878, p=0.051) and a significant interaction between the two variables (Interaction: $F_{(20,168)}$=1.737, p=0.032; **Fig. 6C**). Although there was a trend for a reduction of EtOH intake between the diet conditions, cumulative g/EtOH consumed over the course of the study was not significantly different between LAE+HFD vs LAE+Chow mice (2.17±0.16 vs 2.42±0.20 g/EtOH, respectively, t=0.9747, df=8, p=0.3583). LAE+Chow mice had a higher preference towards EtOH compared to LAE+HFD mice (Diet: $F_{(1,8)}$=9.322, p=0.016; EtOH exposure sessions: $F_{(20,160)}$=2.941, p<0.001; Interaction: $F_{(20,160)}$=1.218, p=0.246 **Fig. 6D**), but post-hoc analysis indicated these differences predominately occurred during the 10% EtOH intake period.

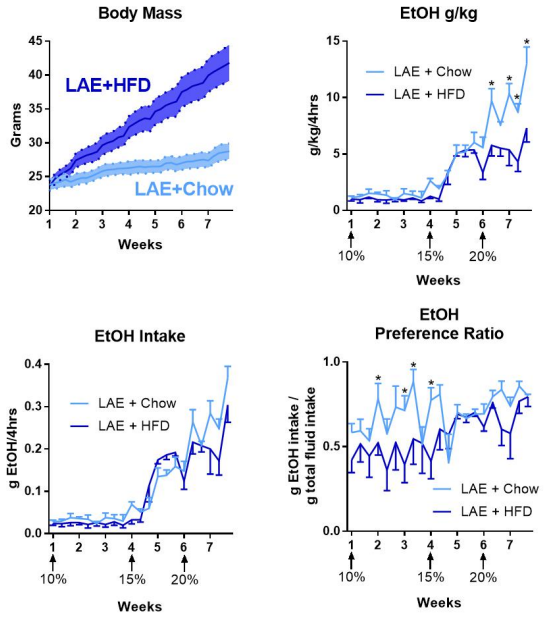

Fig 6: EtOH intake is not affected by diet in the limited access "LAE" model. A) Time course of body mass changes by group during drinking period (n=5/group). **B-D)** HFD has no significant effect on the amount of EtOH consumed during limited access, but modestly affects preference. * indicates significant effect of diet as determined by two-way ANOVA; p<0.05.

Mice then underwent metabolic testing. Results from LAE mice were compared to the same EtOH-naïve HFD and Chow control mice utilized in the previous experiment. One-way ANOVA showed body mass was elevated in both EtOH naïve+HFD (44.9±0.7) and LAE+HFD mice

(43.3±2.8) when compared to LAE+Chow (28.8±1.2) or chow-fed EtOH naïve mice (28.8±0.6) ($F_{(3,32)}$=69.26, p<0.001; **Fig. 7A**). LAE+Chow mice were not significantly different from Chow-fed EtOH naïve control mice. One-way ANOVA also indicated that EtOH consumption did not significantly alter adiposity ($F_{(3,37)}$=132.9, p<0.001; **Fig. 7B**), lean mass ($F_{(3,37)}$=126.1, p<0.001; **Fig. 7C**), or fluid mass ($F_{(3,37)}$=104.6, p<0.001; **Fig. 7D**) within HFD and chow fed groups.

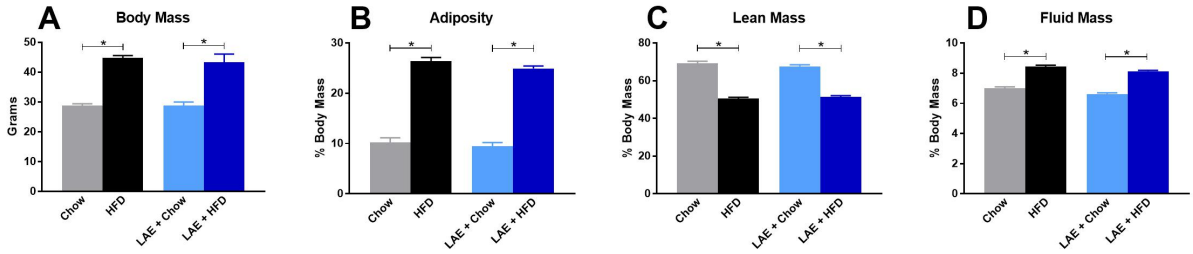

Fig 7. Limited Access EtOH (LAE) consumption does not alter HFD-induced changes to body mass, adiposity, lean mass, or fluid mass. Body composition data are compared to chow- and HFD-fed EtOH naïve mice. **A)** Body mass of control mice and LAE mice prior to metabolic testing. Percent body mass of **B)** adiposity, **C)** lean mass, and **D)** fluid mass. One-way ANOVA; *p<0.05

Further, one-way ANOVA ($F_{(3,32)}$=7.147, p<0.001; **Fig. 8A**) followed by Tukey's post hoc test indicated 4-hour fasting glucose was elevated in the EtOH-naïve HFD (186±9) and LAE+HFD (176±9) mice compared to EtOH-naïve Chow mice (156±6) with the highest elevation found in the LAE+Chow group (228±11). One-way ANOVA ($F_{(3,30)}$=4.759, p=0.008; **Fig. 8B,C**) indicated that insulin sensitivity was reduced in the EtOH-naïve HFD group (-1748±1024) compared to EtOH-naïve Chow mice (-5903±662.4), with no significant difference between LAE+Chow mice (-4228±1079) or LAE+HFD mice (-6630±1277) compared to EtOH-naïve Chow mice. ITT in EtOH-naïve HFD mice was significantly lower than LAE+HFD mice, suggesting EtOH intake improves insulin sensitivity in HFD-fed mice in this model. Given the disparate changes in fasting glucose vs ITT in LAE+HFD mice, we next performed GTT to determine the impact of HFD and EtOH on the ability to dissipate changes in blood glucose in response to a glucose load. One-way ANOVA indicated that 12-hour fasting glucose levels were elevated in HFD compared to chow-fed mice ($F_{(3,32)}$=13.52, p<0.001; **Fig. 8D)**, but EtOH intake did not alter HFD-induced changes as LAE+HFD (205±10) was not statistically different from EtOH-naive HFD mice (196±6). Although there were differences in 4-hour fasting glucose between chow groups, 12-hour fasting glucose was not different in LAE+Chow (125±8, mg/dl glucose) vs EtOH-Naïve Chow mice (144±12 mg/dl). One-way ANOVA ($F_{(3,32)}$=10.38, p<0.001; **Fig. 8E,F**) indicated a significant increase in the GTT area under the curve in LAE+HFD mice (44921±2829 delta-glucose mg/dl*min) compared to all other groups (EtOH-naïve HFD: 29344±2542; EtOH-naive Chow: 23036±2255; LAE-Chow: 18818±2578). Together, these findings suggest that LAE+HFD mice exhibit glucose intolerance, which may be distinct in mechanism from the metabolic disturbances seen in the UAE+HFD model.

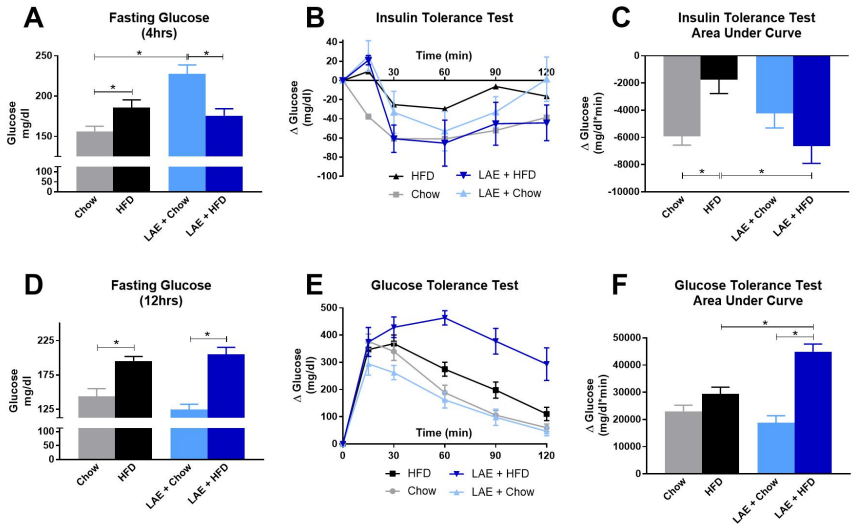

Fig 8. LAE improves insulin sensitivity but worsens glucose tolerance in HFD-fed mice. A) 4-hour fasting glucose levels prior to insulin tolerance test. **B)** Change in blood glucose levels over time following insulin injection; data are normalized to 0 at baseline. **C)** Area under the curve for change in blood glucose levels during insulin tolerance test. **D)** 12-hour fasting glucose levels prior to glucose tolerance test. **E)** Change in blood glucose levels over time following dextrose injection; data are normalized to 0 at baseline. **F)** Area under the curve for changes in blood glucose levels during glucose tolerance test. One-way ANOVA; *p<0.05

Discussion

The current findings indicate that *ad libitum* HFD access only altered EtOH intake when EtOH intake was *ad libitum* (UAE+HFD mice). *Ad libitum* HFD however, did not alter EtOH intake when EtOH access was limited utilizing a paradigm previously shown to escalate EtOH intake in rodents (LAE+HFD). These findings suggest that scheduling access is an important factor in determining the role of HFD on EtOH intake, a factor that has often been overlooked in previous studies on HFD and EtOH interactions. Although UAE+HFD mice had lower EtOH intake, this moderate level of EtOH intake did not alter body mass, adiposity, lean mass, fluid mass, or insulin sensitivity compared to mice given HFD for the same amount of time without EtOH. LAE+HFD mice had similar levels of EtOH intake compared to LAE+Chow mice, and this higher level of EtOH intake did not alter body mass, adiposity, lean mass, or fluid mass, but did improve insulin sensitivity compared to HFD control mice. Surprisingly, even in the face of improved insulin sensitivity, glucose tolerance was severely impacted in LAE+HFD mice, with glucose disturbances greater than that seen in HFD control mice. Together, these findings indicate that EtOH access schedules are critical in mediating the impact of HFD on EtOH intake and that patterned EtOH intake in the face of HFD can produce metabolic disturbances via a distinct mechanism than that seen in free-access EtOH and HFD consumption.

Our finding that *ad libitum* HFD access decreases *ad libitum* EtOH intake (UAE+HFD group) is a common finding in the literature in both male and female rodents (Feng *et al*, 2012; Gelineau *et al*, 2017; Sirohi *et al*, 2017a, 2017b). This may be due to a number of factors. One possibility is that rodents find HFD a rewarding food choice and prefer this to potential rewarding effects of EtOH. Indeed, animals exposed to HFD initially undergo a hyperphagic response (Hariri and Thibault, 2017), suggesting this diet has some rewarding value leading to escalation of intake. Further research supports this hypothesis by showing that HFD exposure can alter dopamine activity in the nucleus accumbens (Fordahl *et al*, 2016; Rada *et al*, 2012), ventral tegmental area (Valdivia *et al*, 2015), and alter neuronal signaling in other key brain regions mediating reward processing (Barson *et al*, 2012; Sharma *et al*, 2013; Valdivia *et al*, 2014). The current findings that UAE+HFD mice have a lower EtOH preference score than UAE+Chow mice further support this hypothesis. However, we only examined 10% EtOH intake in the UAE cohort and it will be important to examine the preference and intake of EtOH at higher concentrations such as was performed in the LAE cohort. Another possibility is that EtOH metabolism and/or clearance may have been altered in UAE+HFD mice due to changes in adiposity and lean mass (Feldstein, 1978; Reed and Kalant, 1977). C57Bl/6J mice typically consume enough EtOH to reach pharmacologically relevant BEC levels in relatively short access periods (~ two hours) (Becker and Lopez, 2004). C57Bl/6J mice have been shown to have numerous drinking bouts when EtOH is provided over extended time periods (Risinger *et al*, 1998). Therefore, although not directly examined here, it is plausible that the UAE+Chow mice may have had numerous bouts of drinking over the 24 hr access period. If UAE+HFD mice had similar initial bouts but prolonged EtOH clearance/metabolism due to changes in adiposity or lean mass, then they may not seek EtOH in subsequent bouts to maintain pharmacologically relevant BECs, thus lowering their total EtOH consumption over the 24 hr access period. This possibility will be fully addressed in future studies.

Extending the hypothesis that HFD has a higher relative reward value than EtOH, LAE+HFD mice had significantly lower EtOH preference compared to controls at 10% EtOH. These mice also had similar preference to controls at 15% and 20% EtOH. This finding may suggest that the rewarding value of 10% EtOH was lower in HFD exposed mice across the two models, but that this may be overcome at higher EtOH doses. Overall though, 10% EtOH consumption was low in the LAE model but increased significantly at higher concentrations, suggesting that the overall reward value of 10% EtOH may have been low when given in a 4 hr access period. Caution must also be used when comparing the UAE and LAE model given the differences in access time to EtOH (24 vs 4 hrs, continuously vs 3 times/week). Importantly, the LAE model does suggest that HFD does not alter EtOH intake when EtOH access is limited, regardless of EtOH reward value. This is an important consideration given the previous preclinical literature indicating a reduction in EtOH intake by HFD but numerous clinical findings indicate that EtOH stimulates HFD intake and vice versa(Breslow *et al*, 2013; Caton *et al*, 2004; Feng *et al*, 2012; Gelineau *et al*, 2017; Piazza-Gardner and Barry, 2014; Sirohi *et al*, 2017a, 2017b). Therefore, utilizing a limited access model in rodents at >15% EtOH may better recapitulate the impact of HFD on EtOH intake in humans.

Extensive previous research has shown moderate EtOH consumption improves insulin sensitivity in both clinical (Traversy and Chaput, 2015) and preclinical settings (Hong *et al*, 2009). Therefore, it was surprising that UAE+HFD mice, which consumed a moderate amount of EtOH on a per day basis, had pronounced insulin resistance. This level of insulin resistance was similar in magnitude to HFD mice without EtOH intake history. The level of HFD-induced

insulin resistance in control HFD mice here is similar to our previous research (Loloi *et al*, 2018; Williams *et al*, 2016). Consistent with a lack of insulin sensitivity, UAE+HFD mice had elevated 4hr fasting glucose to a similar degree as the modest hyperglycemia observed in HFD mice. The reason for the lack of replication between our current study and previous research indicating ethanol consumption improves insulin sensitivity in high fat diet exposed animals is unclear, but previous research does indicate that twice daily intra-gastric EtOH exposure was more beneficial to improve insulin sensitivity in HFD-fed rats than continuous free access intake, even when total daily EtOH dosage (5g/kg) was accounted (Feng *et al*, 2012). This was due to differences in peak plasma EtOH concentrations of the different modes of exposure. Since daily EtOH intake in the UAE+HFD mice was generally between 2-3g/kg per day, this level of EtOH intake may not have been high enough to produce beneficial effects on insulin sensitivity.

In contrast to UAE+HFD mice, LAE+HFD mice had improved insulin sensitivity compared to HFD controls. This could be due, at least in part, to the higher EtOH intake levels in the 4 hr period in the LAE mice (~5g/kg per day over the last three weeks of the study) compared to 24 hr intake levels in the in UAE mice. While insulin sensitivity was normalized in LAE+HFD mice, glucose homeostasis was drastically disturbed. Mice in the LAE+HFD group had significantly elevated 12hr fasting glucose and were highly glucose intolerant. Previous findings also indicate that EtOH can greatly impact glucose metabolism (Steiner *et al*, 2015). Together, these findings suggest normal whole-body insulin receptor function but an inability of the animals to clear glucose, which may be due to impairments in several factors including intestinal glucose absorption, endogenous pancreatic insulin secretion, glucose effectiveness, and counter-regulatory responses. Supporting this concept, previous research indicates that chronic EtOH consumption can potentiate pancreatic beta-cell dysfunction (Kim, 2015). Future studies will be needed to directly test the role of these factors, including endogenous insulin production, in the glucose disturbances seen in the LAE+HFD model.

Overall, the two models of combined EtOH and HFD consumption described here point to little benefit of EtOH in the face of metabolic dysfunction. The concept that moderate EtOH drinking has beneficial health effects has come under increased scrutiny in the past year (Griswold *et al*, 2018) and brings back into debate the potential interactive role of EtOH and HFD in the development of Type II diabetes. Indeed, given the well established roles of EtOH and HFD as individual risk factors for the development of metabolic disturbances and the increasing understanding that chronic EtOH and HFD have similar effects on peripheral and central signaling mechanisms, it is surprising that clinical evidence points to moderate EtOH consumption as a mitigating factor in HFD-induced metabolic disturbances. The findings here suggest that there are many factors that may influence how EtOH and HFD interact to promote or mitigate metabolic disturbances, such as frequency and duration of EtOH access. It should be noted that several studies report a U- or J-shaped relationship between EtOH and insulin function (Kiechl *et al*, 1996; Lazarus *et al*, 1997; Villegas *et al*, 2004), or that beneficial effects of EtOH may only seen in those individuals without obesity or insulin resistance (Yokoyama, 2011). Such findings further suggesting the need to better examine the interactions of EtOH and HFD on insulin and glucose function both clinically and pre-clinically while controlling for time course, duration, and frequency of both EtOH and HFD exposures.

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