Impact of Repeated Heavy Ethanol Intoxication During Early Adolescence on Adult Anxiety-Like, Exploratory, Risk Assessment, and Drinking Behaviors in Mice

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Abstract
Drinking beyond binge levels, to the point of passing out, is common among youth and associated with both acute and long-term harm. We evaluated the effects of repeated ethanol-induced heavy intoxication during early adolescence on behavioral outcomes in early adulthood. Male and female C57BL/6J mice (5 weeks old) underwent four Loss of Righting Reflex (LORR) tests over two weeks, induced by intraperitoneal (i.p.) injections of 3.2 g/kg ethanol (intoxicated group) or saline (control group). A subset of intoxicated mice had blood collected on days 1 and 4 for blood ethanol concentration analysis, confirming heavy intoxication (~289 mg/dL). At 9 weeks of age, animals were tested in the Light-Dark Box (LDB) and Elevated Plus Maze (EPM) immediately after receiving either 1.2 g/kg ethanol or saline. In the LDB, adolescent ethanol intoxication led to increased latency to enter the light compartment, although pre-test ethanol did not affect anxiety-like behaviors. Intoxicated females showed enhanced exploratory activity and males had reduced risk assessment behaviors. In the EPM, the effects of pre-test ethanol were more pronounced than adolescence intoxication, producing an anxiolytic effect accompanied by increased exploration and a reduction in risk assessment behaviors. Finally, a separate cohort was tested for ethanol drinking using a two-bottle choice Intermittent Overnight Drinking (IOD) protocol in adulthood. Adolescent ethanol intoxication induced higher ethanol drinking in females. Repeated adolescent intoxication reduced risk assessment while increasing exploratory behavior, indicating riskier decision-making, and selectively elevated ethanol consumption in females.
Keywords: alcohol, sex difference, plus-maze, light-dark box, two-bottle choice, C57Bl/6 mice.

Highlights

- Adolescent ethanol heavy intoxication leads to sex-specific adult behavioral changes.
- In the LDB, adolescent ethanol increased exploration and reduced risk assessment.
- In the EPM, adolescent ethanol did not alter acute ethanol effects.
- Early ethanol exposure increased ethanol intake in adult females.
- Adolescent intoxication may impair risk-based decision-making in adulthood.

1. Introduction

Heavy episodic ethanol intoxication increases the risk of injuries and other harmful situations, as well as represents a risk factor for developing alcohol use disorders (AUD) (Conegundes et al., 2020; American Psychiatric Association, 2022). In Brazil, 5% of adolescents (12-17 years) and 20.5% of young adults (18-24 years) reported at least one episode of binge drinking in the last 30 days (Bastos et al., 2017). According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA) at the National Institutes of Health (NIH), binge drinking is defined as consuming four to five standard drinks within a two hours. However, episodes of heavy drinking can exceed these amounts. High-intensity drinking is characterized as consuming two or three times the standard binge threshold: Level II (8-10 drinks) and Level III (12-15 drinks) (Patrick and Azar, 2018). It is known that heavy drinking impairs motor control and cognitive functions, while high-intensity intoxication (blood ethanol concentration - BEC - around 200-300 mg/dL) can lead to a reduction of consciousness and even cause sedation (Wetherill and Fromme, 2016). Adolescents and young adults have been commonly drinking to blackout, which is characterized by an intoxication level that causes partial (fragmentary) or total *(en bloc)* anterograde amnesia, which can develop to a passing out state (sedative, sleep-like state) (Miller et al., 2019; Yuen et al., 2021).

C57BL/6 mice are commonly used as animal models for studying alcohol intoxication due to their high levels of voluntary ethanol consumption. However, animals do not typically drink to the point of loss of consciousness (passing out). Therefore, non-contingent (experimenter-administered) peripheral ethanol administration is necessary to achieve high levels of intoxication. To model high-intensity intoxication, BEC must reach elevated levels and be accompanied by behavioral signs such as pronounced motor impairment and amnesia, which are typically observed at doses above 3.0 g/kg (Ponomarev and Crabbe, 2002). At these doses, mice might lose their righting reflex (LORR), a well-established indicator of deep intoxication. Notably, even lower doses, such as 2.4 g/kg, have been shown to produce amnestic effects, indicating the impact on memory (Sanday et al., 2013). Thus, the LORR model is appropriate for studying alcoholic blackouts and passing outs.

Adolescents are particularly vulnerable to the neurotoxic effects of ethanol, which can interfere with brain maturation and disrupt behavior, especially inhibitory control (Spear, 2018; Lees et al., 2020). The harmful effects of ethanol during the developmental period may persist into adulthood, resulting in long-lasting behavioral impairments (Jones et al., 2018; López-Caneda et al., 2014). Accumulating evidence suggests that adolescent ethanol intoxication can impair cognitive functions, alter anxiety-like behaviors, and lead to either tolerance to the drug's aversive effects or increased sensitivity to its rewarding properties (Crews *et al.*, 2016; Ruan *et al.*, 2019; Courtney *et al.*, 2020).

Markedly, studies investigating early adolescent ethanol exposure effects on anxiety-related behaviors and risk assessment in adulthood remain inconclusive, and not all studies replicate increases in ethanol intake following adolescent exposure (Towner and Varlinskaya, 2020). Additionally, most of the literature on the effects of adolescent ethanol exposure on adult behavior has focused exclusively on male animals, leaving it unclear

whether females exhibit similar behavioral responses (Jury et al., 2017a). In the present study, we investigated how repeated heavy ethanol intoxication during early adolescence affects anxiety-like, risk assessment, and exploratory behaviors, as well as voluntary ethanol consumption in adulthood in both male and female C57BL/6 mice.

2. Methods

2.1. Animals

This study was approved by the Comissão de Ética no Uso de Animais (CEUA) of the Universidade Federal de São Paulo (Unifesp) (#2423170220). The experiments were in accordance with the Conselho Nacional de Controle de Experimentação Animal (CONCEA). Male and female C57BL/6 mice were obtained from Centro de Desenvolvimento de Modelos Experimentais para Biologia e Medicina (CEDEME). Animals arrived at the fourth week of age and were group-housed in automatically ventilated cages (Alesco ALBR Indústria e Comércio Ltda, Monte Mor, Brazil) with 3-4 animals of the same sex per cage. *Vivarium* temperature was kept at 21 ± 2 ºC, with 60% of humidity and light/dark cycle of 12/12 hours (lights on at 7 a.m.). The animals had *ad libitum* access to standard chow (Nuvilab CR-1 Chow, Nuvital, Paraná, Brazil) and water. Cages contained a half-cardboard roll and a paper sheet, and materials were changed every week. Animals were habituated to the *vivarium* and to the experimenter's handling before the beginning of the experiments. All experiments were conducted with male and female mice along to allow proper comparison between sexes. All apparatus used (LORR vertices, Elevated Plus Maze - EPM, and Light Dark Box - LDB) were cleaned with 70% ethanol and dried between animals. Anxiety-like behavior tests and ethanol intake procedures were initiated during the ninth week of life, corresponding to early adulthood (Hefner and Holmes, 2007).

2.2. Ethanol-induced loss of righting reflex (LORR)

To model high levels of alcoholic intoxication and passing outs we used the ethanol-induced LORR protocol, which involves peripheral administrations of high ethanol doses. One day before LORR, animals were moved to the experiment room 30 minutes before receiving an intraperitoneal injection (i.p.) of saline in an equivalent volume (4.0 ml/kg) to the following administrations for habituation. The LORR protocol consisted of four intoxication episodes, with the first two intoxications spaced by 48 hours, followed by a third intoxication after five days, and a final fourth intoxication 48 hours later. The LORR intoxication happened during early adolescence (5-6 weeks). Animals received 3.2 g/kg doses of ethanol (20% v/v) (LabSynth, Sao Paulo, Brazil, prepared from 95% ethanol) (intoxicated group) or saline (0,9% w/v) (control group) through i.p injections and were placed in acrylic vertices (USA Acrylics, Sao Paulo, Brazil). Loss of reflex was defined when animals were not able to turn themselves to an upright position within three minutes after injection. Mice who failed to lose reflex were placed on warm pads and had data excluded from the LORR analysis on that day specifically (Ozburn et al., 2013). Mice were considered to have recovered the reflex when able to turn into supine position three times

in a row within one minute. After recovering the reflex, animals were taken back into their home cages and left on warm pads for at least one hour to allow complete recovery. Wet chow food was offered, and i.p. saline injections were administered to animals in case they weren't moving and/or eating normally one hour after the procedure. Animals in the control group were also placed on warm pads for one hour.

2.3. Blood Ethanol Concentration

In Experiment 1, a group of mice (n = 7-8/sex, intoxicated group) went through the LORR protocol as previously described and had their blood samples collected on days 1 and 4 to assess BECs right on the recovery time. Tail blood was collected in cooled EDTA K2 tubes and briefly vortexed and frozen at -20 ºC. Blood was stored until analysis, using gas chromatography (Shimadzu, Barueri, Brazil) at CIATox Lab of the Universidade Estadual de Campinas (Unicamp). The curve had 10 standard points, and detection ranged from 0.05 to 5.00 g/L. This experiment was done in a single iteration, and all animals from Experiment 1 were euthanized after the second blood collection.

2.4. Light-Dark Box (LDB) and Elevated Plus Maze (EPM)

Another group of animals (Experiment 2, n = 10-13/group/sex), went through the LORR protocol or received the saline administrations on their 5th and 6th weeks of age. In the 9th week, mice were submitted to the LDB test and, 48h later, to the EPM. This experiment was done in four iterations. Although some differences in the frequency with which specific behaviors were recorded, the overall behavioral trends remained consistent among iterations. Therefore, we opted to compile data from all iterations to ensure sufficient sample size per group and increase the statistical power of the analysis. Description of analyzed behaviors for both tests is listed in Supplementary Table 1.

The LDB apparatus had two equal-sized (24 cm x 24 cm) compartments with flat floors. The light side (white colored) had approximately 400 lux, and the dark side (black colored), 3 lux. Animal exploration of the light side was recorded with a Digital Camera (Sony Corporation, Japan), and behavior variables such as time spent in each side, latency to the first light side entrye, number of transitions, head out, stretch, and rearing were assessed manually with the BORIS software (Friard and Gamba, 2016, https://www.boris.unito.it/). We calculated the "total risk assessment" as the head out and stretch units sum (head outs + stretches). In addition to these measures, we also categorized risk assessment behaviors into NoGo (when animals assessed but did not enter the aversive compartment) and Go (when animals explored the aversive compartment after assessment), to better capture decision-making under approach-avoidance conflict. Half of the animals in each group (intoxicated or control) received 1.2 g/kg of ethanol (15% v/v) or saline (0,9% w/v), resulting in four groups: control-saline, control-ethanol, intoxicated-saline, and intoxicated-ethanol. Animals were placed in the dark side one minute after the i.p. administration, and after five seconds, the door between compartments was lifted. The test lasted for five minutes. One animal did not make the transition to the light side and was excluded from the analysis.

The EPM consisted of a wood apparatus elevated 50 cm from the ground with two open arms and two closed arms, with all arms measuring 50 cm. The open arms had an average light of 46 lux, and the closed arms 16 lux. Animals received the pre-test injection of either ethanol or saline as described in LDB. The test lasted five minutes and was recorded with Ethovision Noldus XT6 (Noldus Information Technology, Netherlands), which tracked animals to provide the traveled distance and velocity variables. In addition, time spent in each arm, latency to first enter the open arm, number of transitions, head outs, stretches, and rearings were manually counted using the BORIS software. The risk assessment index and Go/NoGo classifications were analyzed as described for the LDB.

2.5. Two-bottle choice Intermittent Overnight Drinking (IOD) protocol

A new group of mice (Experiment 3, n = 17-18/group/sex) went through the LORR protocol (with intoxicated and control groups, as described in 2.2) and were submitted to the IOD protocol starting on their 9th week of life. This experiment was done in three iterations and we also opted to compile data from all iterations to ensure sufficient sample size per group and increase the statistical power of the analysis.

Mice were individually placed in individual cages for 16 hours (beginning two hours before lights off, and finishing two hours after lights were on) and had access to *ad libitum* food and two bottles containing water. Then, the IOD protocol consisted of twelve intercalated nights, with no more than three sessions per week, in which mice were placed back in individual cages and had access to *ad libitum* food and one bottle containing water and one with 10% ethanol, which were switched sides every test night (Emous et al., 2025). Although isolated, animals could see and smell each other. Bottles were weighed every day before and after the experimental night, along with a separated bottle for adjusting to leakage and spilled liquid.

2.6. Statistical Analysis

We used Generalized Estimated Equations (GEE) with Auto-Regressive (AR1) correlation matrix and Gamma model type, with animal ID as subject variable and day(time) as within-subject variable for LORR and BEC data. Latency to lost reflex and duration of LORR were both analyzed in minutes. Controlling variables were experiment iteration, day, and sex. We also analyzed separated sex datasets. All comparisons were made to day one of intoxication.

For the LDB, six animals were excluded due to recording issues: three females (one from each group except for control-saline) and three males (one from each group except for intoxicated-ethanol). For the same reason, three animals were excluded from the EPM analysis: two males (one from the control-saline group and one from the intoxicated-saline group) and one female (from the intoxicated-saline group). In addition, animals whose values of time (in seconds) spent in the light side in the LDB or open arms of the EPM fell outside the range defined by the mean ± 2 standard deviations (SD) in relation to their group were considered outliers. Accordingly, five animals (all males) were excluded from the LDB test, distributed as follows: one from the control-saline group, one from the control-ethanol group, one from the intoxicated-saline group, and two from the intoxicated-

ethanol group. Regarding the EPM test, one male was excluded from the intoxicated-ethanol group. One female from the control-ethanol group was also excluded from the EPM analysis for being still during the whole test. Finally, Generalized Linear Models (GzLM) were applied to analyse LDB and EPM data, with Linear distribution for continuous numerical variables and Poisson distribution for count data (as number of stretches, for example), both with Log link function. We ran one analysis using the iteration of experiments as the predictor variable. Next, the analyses used intoxication (saline or ethanol during early adolescence), pre-test (saline or ethanol right before each behavioral test) and sex as predictor variables. We also analyzed each sex-separated dataset.

Data from IOD protocol was checked for outliers, defined as values deviating from the group mean by more than ±2 SD. We used this cutoff as it allowed us to identify procedural issues such as leakage and clogging of bottles. Animals identified as outliers for at least 6 nights were excluded from the dataset. A total of five animals were removed based on this criterion. For intake data, although the Gamma model had a better fit compared to the Linear model, water consumption dataset had 38% zeros, which would be excluded from Gamma analysis. Therefore, we used the GEELinear Model. The model was built similarly to that described for LORR.

The exported data from Boris was pre-processed using the App for Behavior Data Processing 3.0, developed in Python (3.11) with Anaconda Environment (https://github.com/mariannacecyn/App-for-Behavior-Data-Processing.git). To understand how behaviors occur and the relationships between them, we used the Markov Model (Espejo, 1997; Tejada et al., 2010). We also calculated transition probabilities from the possible transitions with each origin (start) behavior. Since the literature on how to compare groups in this model is still scarce (Wiltschko et al., 2020; Bermudez Contreras et al., 2022), we divided the problem into small parts to do a partial analysis rather than the entire stochastic system. Our analysis focused on transitions “to” and “from” aversive compartments. To construct the Markov Model graphs, we separated the transition matrices and state vectors into groups and calculated the mean (≥ 0.02) and SD.

All data was analyzed using JAMOVI 2.4.11 or IBM SPSS Statistics 21. All graphs were done in GraphPad Prism (Version 9.5.1 - 733). Alpha levels were 0.05. Where appropriated, the adjusted Bonferroni *post hoc* test was applied to identify specific differences among groups. Data are presented in Figures as mean and standard error means (SEM). Results present the Wald Chi-Square (X^2) values, degrees of freedom, and p-value when significant. When necessary, values from parameter estimates and *post hoc* are also shown.

3. Results

3.1. Heavy intoxication in adolescent animals, confirmed by high BEC

Not all mice lost the righting reflex following ethanol administration: 25% did not lose the reflex on day 1, 37% on day 2, 30% on day 3, and 31% on day 4. These rates highlight interindividual variability in sensitivity to ethanol-induced sedation. For the subset of mice that did lose the reflex, we analyzed the latency to LORR in Experiment 1 (BEC) and found no significant effect of day ($X^2_{(1)} = 1.71$), sex ($X^2_{(1)} = 0.86$) or the interaction

between day and sex ($X^2_{(3)}$ = 0.61). For the duration of LORR, the analysis did not detect a significant effect of day on recovery time overall; therefore, a separate analysis comparing days 1 and 4 was conducted. In this comparison, there was evidence of tolerance development to the motor depressant effect of ethanol, as LORR duration was significantly shorter on day 4 compared to day 1 ($X^2_{(1)}$ = 5.06; p = 0.025). No effect of sex was observed ($X^2_{(1)}$ = 3.05). BEC levels confirmed heavy intoxication, ranging around 289 mg/dL (Figure 1A), with no difference between days ($X^2_{(1)}$ = 0.03), indicating that mice reached similar BECs at the point of reflex recovery. Sex was not significant ($X^2_{(1)}$ = 1.68) (Figure 1B).

The LORR procedure for Experiments 2 and 3 yielded similar results, regarding latency and duration (Figures S1). The combined analysis of the latency to LORR showed a significant effect of day ($X^2_{(3)}$ = 10.27, p = 0.016), but no pairwise differences between days were detected in the parameter estimates (Figure 1C). There was no effect of sex ($X^2_{(1)}$ = 0.09) and no interaction between day and sex ($X^2_{(3)}$ = 2.79) (Figure 1D). Analysis of the LORR duration revealed a significant effect of day ($X^2_{(3)}$ = 14.26, p < 0.003), with shorter durations on days 3 and 4 compared to day 1, indicating the development of tolerance to the motor-depressant effects of ethanol (Figure 1E). No effect of the factor sex was observed ($X^2_{(1)}$ = 0.00) (Figure 1F). In both sexes, there was no significant effect of day on latency to LORR (Females: $X^2_{(3)}$ = 6.91; Males: $X^2_{(3)}$ = 6.89), but a significant effect on LORR duration (Females: $X^2_{(3)}$ = 16.52, p = 0.001; Males: $X^2_{(3)}$ = 12.53, p = 0.006), with day all days differing from day 1.

FIGURE 1

3.2. Effects of early adolescent intoxication on anxiety-like, exploratory and risk assessment behaviors responses to an ethanol challenge in adulthood

Initially, we analyzed variables traditionally used to infer anxiety-like behaviors. Analysis of the latency to enter the light compartment (Figure 2A) did not show an effect of the factor intoxication (adolescent saline or ethanol exposure) ($X^2_{(1)}$ = 2.52) or pre-test (saline or ethanol administration prior to testing) ($X^2_{(1)}$ = 0.86), but the interaction was significant ($X^2_{(1)}$ = 4.84, p = 0.028). Adolescent intoxicated mice that received pre-test saline took longer to enter the light side than control-saline mice (p = 0.036). Interestingly, intoxicated mice that received pre-test ethanol (intoxicated-ethanol) took a similar amount of time to enter the light side than control-saline animals. Sex was not a significant factor ($X^2_{(1)}$ = 1.46) (Figure 2B). Separate analyses of female and male mice revealed no significant differences overall, but a trend in males treated with pre-test ethanol to increased latency to the light side compared to controls, especially in previously intoxicated-ethanol males (p = 0.05). The most common variable to indicate anxiety-like behavior is the percentage of time spent in the light side, which was not affected by ethanol exposure (Figure 2C), revealed by no significant effects of intoxication ($X^2_{(1)}$ = 0.82), pre-test administration ($X^2_{(1)}$ = 1.16) or interaction ($X^2_{(1)}$ = 0.22). Sex was also not significant ($X^2_{(1)}$ = 0.47) and the analyses for each sex did not indicate any specific differences (Figure 2D).

We also analyzed exploratory behaviors by counting the transitions between compartments and rearing behaviors in the light compartment of the LDB. The analysis of transitions did not indicate intoxication as a significant factor ($X^2_{(1)} = 0.12$), but it revealed a significant pre-test effect ($X^2_{(1)} = 45.10$, $p < 0.001$) and interaction ($X^2_{(1)} = 10.62$, $p = 0.001$) (Figure 2E). The *post hoc* test showed that, among intoxicated groups, pre-test ethanol induced more transitions than pre-test saline ($p < 0.001$). The analysis detected a significant effect of sex ($X^2_{(1)} = 34.46$, $p < 0.001$), with females exhibiting a higher number of transitions compared to males (Figure 2F). Separate analysis of female data showed significant pre-test ($X^2_{(1)} = 20.27$, $p < 0.001$) and interaction effects ($X^2_{(1)} = 5.77$, $p = 0.016$), consistent with the effect described above ($p < 0.001$). For males, a significant pre-test factor ($X^2_{(1)} = 30.60$, $p < 0.001$) was observed, but not the interaction ($X^2_{(1)} = 3.49$). Considering the rearings counts, the analysis did not reveal a significant pre-test effect ($X^2_{(1)} = 0.91$), but it showed significant effects of adolescent intoxication ($X^2_{(1)} = 10.03$, $p = 0.002$) and interaction ($X^2_{(1)} = 5.75$, $p = 0.017$) (Figure 2G). Intoxicated mice did more rearing than controls when receiving pre-test ethanol ($p < 0.001$). No sex effect was observed ($X^2_{(1)} = 0.10$) (Figure 2H). When analyzing females separately, there were significant effects of intoxication ($X^2_{(1)} = 16.86$, $p < 0.001$) and interaction ($X^2_{(1)} = 5.74$, $p = 0.017$). In control mice, pre-test ethanol decreased rearings ($p = 0.034$), but this effect was not observed in previously intoxicated mice. The ethanol pre-test effect in rearing was higher in intoxicated mice ($p < 0.001$). In contrast, when analyzing males, only the pre-test factor was significant ($X^2_{(1)} = 10.79$, $p = 0.001$), as pre-test ethanol increased rearing compared to pre-test saline.

An important goal of this study was to better understand the effects of ethanol exposure during early adolescence on risky decision-making in adulthood, using risk assessment measures. Pre-test ($X^2_{(1)} = 1.64$) and interaction ($X^2_{(1)} = 0.52$) were not detected as significant factors; however, the analysis detected a significant effect of intoxication ($X^2_{(1)} = 5.13$, $p = 0.024$) (Figure 2I). Intoxicated animals exhibited significantly fewer risk assessment behaviors compared to controls ($p = 0.024$), regardless of pre-test drug administration. The factor sex was not significant ($X^2_{(1)} = 1.59$) (Figure 2J). Analysis of the female dataset revealed only a pre-test ethanol effect ($X^2_{(1)} = 5.73$, $p = 0.017$), as females who received ethanol before the test performed fewer risk assessments than those treated with saline ($p = 0.017$). The intoxication effect, as described above, was significant only for males ($X^2_{(1)} = 5.54$, $p = 0.019$), as males made less risk assessment. We evaluated the relative percentage of NoGo behaviors (NoGo/Risk, %) within the total risk assessment (Go + NoGo). The analysis revealed significant effects of pre-test ($X^2_{(1)} = 107.23$, $p < 0.001$) and intoxication ($X^2_{(1)} = 25.85$, $p < 0.001$) (Figure 2K). Thus, ethanol heavy intoxication during adolescence reduced the proportion of safe decision-making behaviors (NoGo) later in life ($p < 0.001$). However, when animals received a low dose of ethanol prior to testing, they showed a higher proportion of NoGo responses relative to total risk assessments, suggesting a shift toward safer decision-making ($p < 0.001$). No significant effect of sex was observed ($X^2_{(1)} = 3.30$) (Figure 2L). Interestingly, female separate analysis detected significance for the pre-test factor ($X^2_{(1)} = 43.49$, $p < 0.001$), while the male dataset analysis revealed significant effects of both pre-test ($X^2_{(1)} = 67.84$, $p < 0.001$) and adolescent intoxication ($X^2_{(1)} = 45.41$, $p < 0.001$).

For NoGo risk assessment, the analysis showed a significant effect of adolescent intoxication ($X^2_{(1)}$ = 9.23, p = 0.002), but no effect of pre-test ($X^2_{(1)}$ = 3.45) or interaction ($X^2_{(1)}$ = 0.73) (Figure 2M). Animals exposed to ethanol during adolescence displayed significantly fewer NoGo risk assessments than control animals (p = 0.003). There was no significant effect of sex ($X^2_{(1)}$ = 0.02) (Figure 2N). Although no significant effect was observed in females, a significant effect of intoxication ($X^2_{(1)}$ = 9.16, p = 0.002) and pre-test ($X^2_{(1)}$ = 6.88, p = 0.009), but no interaction, was found among males. Male mice that received pre-test ethanol performed more NoGo risk assessments than saline-treated controls (p = 0.009). Additionally, intoxicated males exhibited fewer NoGo risk assessment compared to control (p = 0.003). We also analyzed stretch-attend postures and head-out behaviors separately (Figure S2). The analysis for NoGo stretches revealed significant effects of pre-test ($X^2_{(1)}$ = 8.32, p = 0.004) and intoxication ($X^2_{(1)}$ = 7.40, p = 0.007) (Figure S2A). Pre-test ethanol induced more NoGo stretches *versus* pre-test saline (p = 0.004), whereas intoxicated mice exhibited fewer NoGo stretches compared to controls (p = 0.007). The analysis revealed a significant effect of sex ($X^2_{(1)}$ = 6.03, p = 0.014), with males performing significantly fewer NoGo stretches than females (p = 0.015) (Figure S2B). Among females, no significant differences were observed. Among males, the analysis detected a similar pattern described for all mice (intoxication: $X^2_{(1)}$ = 5.21, p = 0.022; pre-test: $X^2_{(1)}$ = 8.80, p = 0.003). Considering NoGo head-outs, the analysis revealed no significant effects of intoxication ($X^2_{(1)}$ = 1.57), pre-test ($X^2_{(1)}$ = 0.34) or interaction ($X^2_{(1)}$ = 0.18) (Figure S2C). The sex factor was significant ($X^2_{(1)}$ = 6.29, p = 0.012), as females did more NoGo head outs than males. The analysis for each sex did not indicate any specific differences (Figure S2D).

We also analyzed Go-type risk assessment behaviors, which occurred less frequently than NoGo risk assessment behaviors. The analysis showed no effect of intoxication ($X^2_{(1)}$ = 0.67) or interaction ($X^2_{(1)}$ = 1.41), but there was a significant effect of pre-test ($X^2_{(1)}$ = 20.66, p < 0.001) (Figure 2O). Pre-test ethanol decreased Go risk assessments compared to saline-treated animals (p < 0.001). The sex was significant ($X^2_{(1)}$ = 5.80, p = 0.016), with males performing more Go risk assessments than females (p = 0.017) (Figure 2P). Among females, only the pre-test factor was significant ($X^2_{(1)}$ = 17.70, p < 0.001), revealing that pre-test ethanol administration induced fewer Go risk assessments compared to pre-test saline(p < 0.001). A similar effect was observed in males ($X^2_{(1)}$ = 5.76, p = 0.016). Considering the Go stretches, the analysis showed a significant effect of pre-test ($X^2_{(1)}$ = 15.27, p < 0.001), but not of intoxication ($X^2_{(1)}$ = 0.00) or interaction ($X^2_{(1)}$ = 0.02) (Figure S2E). Pre-test ethanol induced fewer Go stretches compared to saline (p < 0.001). A significant effect of sex was observed ($X^2_{(1)}$ = 10.06, p = 0.002), with males performing more Go stretches than females (p = 0.002) (Figure S2F). A significant effect of pre-test administration was also observed for females ($X^2_{(1)}$ = 13.55, p < 0.001) and for males ($X^2_{(1)}$ = 4.43, p = 0.035). Regarding the number of Go head-outs, neither the intoxication ($X^2_{(1)}$ = 0.72) nor interaction ($X^2_{(1)}$ = 1.61) were significant, but there was a pre-test effect ($X^2_{(1)}$ = 8.90, p = 0.003) (Figure S2G). Pre-test ethanol induced fewer head-outs when compared to pre-test saline (p = 0.004). No significant sex effect was observed ($X^2_{(1)}$ = 0.03) (Figure S2H). Among females, a significant pre-test effect was found ($X^2_{(1)}$ = 6.67, p = 0.010), similar to the effect described above. No differences were found among males.

FIGURE 2

We analyzed the transition probabilities to and from risk assessment behaviors (Figure S3), which allowed us to build Markov Models representing the complexity of behavioral organization within each experimental group (Figure 3). We observed subtle differences in the transition probabilities of behaviors across groups. For example, animals that received pre-test ethanol exhibited Go risk assessment behaviors in less than 2% of the total test duration, which explains their absence from the Markov Models. In contrast, those animals which received pre-test saline showed more stretch Go behaviors in their chains, although NoGo risk assessment behaviors were present in all groups. The probabilities of transitions among events remained statistically similar across groups.

FIGURE 3

Again, we first analyzed variables traditionally used to infer anxiety-like behaviors. The analysis of the percentage of time spent in the open arms did not show a significant effect of the intoxication ($X^2_{(1)} = 1.10$) or the interaction ($X^2_{(1)} = 0.08$), but the pre-test factor was significant ($X^2_{(1)} = 36.82$, $p < 0.001$) (Figure 4A). Pre-test ethanol produced the expected anxiolytic effect, as mice spent significantly more time in the open arms compared to those that received pre-test saline ($p < 0.001$). There was no significant effect of sex ($X^2_{(1)} = 0.27$) (Figure 4B). Similar results of pre-test were observed for females ($X^2_{(1)} = 12.52$, $p < 0.001$) and males ($X^2_{(1)} = 25.99$, $p < 0.001$). We also evaluated the open arm entries and observed the pre-test factor as significant ($X^2_{(1)} = 38.94$, $p < 0.001$) but not the intoxication ($X^2_{(1)} = 0.88$) or interaction ($X^2_{(1)} = 0.43$) (Figure 4C). Pre-test ethanol increases open arm entries when compared to saline ($p < 0.001$). There was no significant effect of sex ($X^2_{(1)} = 2.55$) (Figure 4D). Analyzing the separated dataset revealed a stimulant effect of pre-test ethanol in both females ($X^2_{(1)} = 10.48$, $p = 0.001$) and males ($X^2_{(1)} = 46.78$, $p < 0.001$) mice.

Exploratory behavior can be inferred by the number of center crossings and rearing. The analysis of center crossings did not detect intoxication ($X^2_{(1)} = 3.45$) or interaction ($X^2_{(1)} = 2.62$) as significant factors, but it showed a significant pre-test factor ($X^2_{(1)} = 70.70$, $p < 0.001$) (Figure 4E). Pre-test ethanol induced higher center crossings compared to saline-treated mice ($p < 0.001$). Analyses showed a sex effect ($X^2_{(1)} = 9.33$, $p = 0.002$), as female mice made more center crossings than males ($p = 0.002$) (Figure 4F). Separate analyses of the datasets revealed a similar stimulant effect of pre-test ethanol in both females ($X^2_{(1)} = 35.60$, $p < 0.001$) and males ($X^2_{(1)} = 36.23$, $p < 0.001$). Considering rearing behavior, the analysis revealed no significance of intoxication ($X^2_{(1)} = 2.56$) or interaction ($X^2_{(1)} = 1.60$) but pre-test factor was significant ($X^2_{(1)} = 32.96$, $p < 0.001$) (Figure 4G). Pre-test ethanol induced fewer rearings than the saline group ($p < 0.001$). The sex factor was not significant ($X^2_{(1)} = 1.38$) (Figure 4H). When analyzing females separately, the pre-test factor was significant ($X^2_{(1)} = 9.34$, $p = 0.002$), indicating similar results as described above. Among males the pre-test factor was significant ($X^2_{(1)} = 27.60$, $p < 0.001$), as well as the interaction ($X^2_{(1)} = 5.51$, $p = 0.019$). Pre-test ethanol induced fewer rearing behavior

in male mice that had not been exposed to ethanol during adolescence (control) ($p < 0.001$).

We also analyzed the risk assessment behaviors in the EPM. For the total amount of risk assessment, the analysis indicated no effect of the intoxication ($X^2_{(1)} = 0.0$) or interaction ($X^2_{(1)} = 0.13$), but a significant pre-test effect ($X^2_{(1)} = 24.38$, $p < 0.001$) (Figure 4I). Mice that received pre-test ethanol did less risk-assessment behaviors than controls ($p < 0.001$). No significant effect of sex was observed ($X^2_{(1)} = 0.04$) (Figure 4J). Similar results were observed when analyzing females ($X^2_{(1)} = 12.29$, $p < 0.001$) or males ($X^2_{(1)} = 11.56$, $p < 0.001$) only. When analyzing the percentage of NoGo risk assessments relative to the total risk assessments (% NoGo/Risk), the analyses revealed significant effects of pre-test ($X^2_{(1)} = 9.37$, $p = 0.002$), but not of the intoxication ($X^2_{(1)} = 0.09$) or the interaction ($X^2_{(1)} = 0.84$) (Figure 4K). Pre-test ethanol induced a lower percentage of NoGo risk assessments than saline ($p = 0.002$). Sex was not significant ($X^2_{(1)} = 0.35$) (Figure 4L). Among females, a significant pre-test effect was observed ($X^2_{(1)} = 6.68$, $p = 0.010$), with ethanol-treated females performing a lower percentage of NoGo risk assessments than saline controls ($p = 0.010$). Among males, the analyses revealed no significant effects.

Considering only the NoGo risk assessment behaviors, the analysis showed no significant effects of intoxication ($X^2_{(1)} = 0.09$) or interaction ($X^2_{(1)} = 0.00$), but a significant effect of pre-test ($X^2_{(1)} = 30.25$, $p < 0.001$) (Figure 4M). Pre-test ethanol induced fewer NoGo behaviors compared to pre-test saline ($p < 0.001$). The factor sex was not significant ($X^2_{(1)} = 0.14$) (Figure 4N). A similar effect of the pre-test was observed for females ($X^2_{(1)} = 19.54$, $p < 0.001$) and males ($X^2_{(1)} = 11.49$, $p < 0.001$). The analysis of NoGo stretches revealed significant effects of pre-test ($X^2_{(1)} = 9.83$, $p = 0.002$), but not of intoxication ($X^2_{(1)} = 1.06$) or the interaction ($X^2_{(1)} = 0.03$) (Figure S4A). Mice receiving pre-test ethanol did less NoGo stretches when compared to saline-treated animals ($p = 0.002$). Sex was not significant ($X^2_{(1)} = 2.89$) (Figure S4B). Among females, the analysis revealed significant effects of pre-test ($X^2_{(1)} = 9.82$, $p = 0.002$), but among males, no significant differences were observed. Considering the NoGo head outs, the analysis showed a significant effect of pre-test ($X^2_{(1)} = 23.72$, $p < 0.001$), but not intoxication ($X^2_{(1)} = 0.61$) or interaction ($X^2_{(1)} = 0.03$) (Figure S4C). Pre-test ethanol induced fewer NoGo head outs compared to saline ($p < 0.001$). The sex factor was significant ($X^2_{(1)} = 8.35$, $p = 0.004$), as males performed more NoGo head outs than females ($p = 0.004$) (Figure S4D). Analyzing each sex separately, similar effects were observed for the pre-test factor in females ($X^2_{(1)} = 9.00$, $p = 0.003$) and males ($X^2_{(1)} = 12.78$, $p < 0.001$).

We also analyzed Go-type risk assessment behaviors. In this case, the analysis revealed no significant effects of intoxication ($X^2_{(1)} = 0.30$), pre-test ($X^2_{(1)} = 0.80$), or interaction ($X^2_{(1)} = 0.35$) (Figure 4O). Sex was also not significant ($X^2_{(1)} = 0.00$), and the analysis for each sex did not indicate any specific differences (Figure 4P). When analyzing Go stretches, no significant effects of intoxication ($X^2_{(1)} = 0.03$), pre-test ($X^2_{(1)} = 0.27$), or interaction ($X^2_{(1)} = 0.08$) (Figure S4E) were found. Sex was also not significant ($X^2_{(1)} = 0.01$) and the analysis for each sex did not indicate any specific differences (Figure S4F). The same lack of significance was observed when analyzing Go head outs (intoxication: $X^2_{(1)} = 0.62$; pre-test: $X^2_{(1)} = 0.79$; interaction: $X^2_{(1)} = 0.49$; sex: $X^2_{(1)} = 0.32$) (Figure S4G). Separate analyses of females and males also showed no effects (Figure S4H).

FIGURE 4

We also analyzed the transition probabilities to and from risk assessment behaviors (Figure S5) and built the Markov Models (Figure 5) for EPM behaviors. Frequency of events varied across groups, particularly for exploratory behaviors such as sniffing and head dipping. The anxiolytic effect of acute ethanol was evident, as animals that received the drug pre-test engaged in more walks in the open arms and did more head dips compared to controls. Notably, the Markov chains of acute ethanol animals did not display risk assessment behaviors, because they accounted for less than 2% of the total test duration. In fact, Go risk assessment behaviors were also observed in less than 2% of the time across all groups, which explains their absence from the chains. Despite these behavioral differences, the probabilities of transitions between events did not differ significantly across groups.

FIGURE 5

3.3. Early adolescence intoxication increased adulthood voluntary ethanol consumption in females

For water consumption, although the analysis did not find significance for the factor group ($X^2_{(1)} = 2.35$) or for the interaction between group and night ($X^2_{(11)} = 9.65$), there was a significant effect of the factor night ($X^2_{(11)} = 143.45$, $p < 0.001$). Animals drank less water over time, especially from night 3 onward compared to night 1 ($p < 0.001$) (Figure 6A). There was a significant effect of sex ($X^2_{(1)} = 10.09$, $p = 0.002$), as females drank more water than males ($p = 0.002$) (Figure 6B). When analyzing each sex separately, similar patterns were observed. For females, no effect of group ($X^2_{(1)} = 1.21$) or interaction ($X^2_{(11)} = 18.59$) was observed, but the factor night was significant ($X^2_{(11)} = 86.39$, $p < 0.001$), with females drinking less water on night 3 onward compared to night 1 ($p < 0.001$). For males, there was no effect of group ($X^2_{(1)} = 1.31$) or interaction ($X^2_{(11)} = 15.19$), but the factor night was significant ($X^2_{(11)} = 87.45$, $p < 0.001$); males drank less water on nights 4, 6-10 and 12 compared to night 1 ($p < 0.001$).

Regarding ethanol consumption, there was a significant effect of night ($X^2_{(11)} = 134.21$, $p < 0.001$), with animals escalating their intake from night 3 onward ($p < 0.001$). The analysis also revealed a significant main effect of the factor group ($X^2_{(1)} = 13.55$, $p < 0.001$); however, *post hoc* analysis did not detect differences between groups. No interaction between night and group was observed ($X^2_{(11)} = 8.36$) (Figure 6C). The factor sex was significant ($X^2_{(1)} = 42.88$, $p < 0.001$), as females consumed more ethanol than males ($p < 0.001$) (Figure 6D). Analysis of the female dataset revealed a significant effect of night ($X^2_{(11)} = 92.86$, $p < 0.001$), indicating an escalation in ethanol intake, and a significant group effect ($X^2_{(1)} = 21.31$, $p < 0.001$), with previously intoxicated females consuming more ethanol than controls ($p < 0.001$). No significant interaction was found ($X^2_{(11)} = 7.46$). In contrast, among males, the effect of night was significant ($X^2_{(11)} = 83.90$, $p < 0.001$), indicating increased ethanol consumption over time; however, there were no significant effects of group ($X^2_{(1)} = 1.77$) nor interaction ($X^2_{(11)} = 16.28$).

Regarding ethanol preference, there was a significant effect of night ($X^2_{(11)}$ = 152.61, $p < 0.001$), with animals showing increased preference for ethanol from night 3 onward ($p < 0.001$). The group was also significant ($X^2_{(1)}$ = 5.90, $p = 0.015$), although *post hoc* analysis did not detect differences between groups. No significant interaction between group and night was observed ($X^2_{(11)}$ = 16.74) (Figure 6E). There was no effect of sex ($X^2_{(1)}$ = 1.78). Similar patterns were observed in the sex-specific analyses. For females, there was no group effect ($X^2_{(1)}$ = 2.88), but a significant effect of night ($X^2_{(11)}$ = 111.42, $p < 0.001$), with ethanol preference increasing from night 3 onward compared to night 1 ($p < 0.001$); and no interaction was found ($X^2_{(11)}$ = 16.41). Likewise, for males, there was no group effect ($X^2_{(1)}$ = 3.22), but a significant effect of night ($X^2_{(11)}$ = 70.76, $p < 0.001$), with ethanol preference emerging from night 3 onward ($p < 0.001$) and the interaction was not significant ($X^2_{(11)}$ = 11.40).

FIGURE 6

4. Discussion

Ethanol exposure can influence behavior in complex ways, depending on factors such as dose, age and sex. Therefore, it is important to investigate if and how/which behavioral changes take place when subjects have been previously exposed to ethanol, particularly during adolescence, and/or are under its acute effects later in life. In the present study, heavy ethanol intoxications in early adolescence induced decreased NoGo risk assessment behaviors later in life in the LDB, specifically in males, while no effect was observed in the EPM. The well-established immediate anxiolytic effect of a low ethanol dose was accompanied by increased exploratory behavior and reduced male No-Go risk assessment behaviors in the EPM. Interestingly, the acute anxiolytic effect of ethanol was not observed in the LDB. However, ethanol administered prior to the LDB increased exploratory behavior in both sexes, which was accompanied by an increase in male No-Go risk assessment and a decrease in Go risk assessment behaviors in both sexes. Finally, we observed that early adolescent heavy intoxication increased voluntary ethanol intake in adult females, an effect not observed in males.

To induce heavy intoxication, we used a model of repeated high-dose ethanol administrations during early adolescence, which led to LORR (Swartzwelder et al., 1998; Crabbe et al., 2006) and may represent repeated episodes of alcohol-induced passing outs in humans. Importantly, losing the righting reflex is different from sleep, as 4.0 g/kg of ethanol causes a brain activity state different from sleep in mice (Abrahao et al., 2020). While the majority of work using LORR has been performed with adult mice, only a few studies have used LORR protocols in adolescents (Hefner and Holmes, 2007). We observed a shorter LORR duration in adolescents (postnatal days 36 to 45) compared to what is typically reported in the literature, even in studies using slightly higher doses of ethanol (Spear and Varlinskaya, 2005; Hefner and Holmes, 2007; Linsenbardt et al., 2009). It is important to note that animals can develop LORR tolerance with repeated ethanol administrations, as indicated by faster reflex recovery. Our protocol may have induced a metabolic tolerance, as the BEC levels on days 1 and 4 were similar, even though animals showed a shorter recovery time on day 4 compared to the first day of

LORR. In general, functional tolerance is observed in adult animals while metabolic tolerance is observed in adolescents (Silvers et al., 2003; Quoilin et al., 2013). Few studies have used LORR to investigate ethanol tolerance in female and male mice (Linsenbardt et al., 2009). In our study, we did not observe sex differences in the time to lose or recover the righting reflex.

Adolescent ethanol exposure has been linked to increased anxiety-like behavior, impulsivity, and risk-taking, though these effects vary depending on the exposure protocol and the method of ethanol administration (for review, see Towner and Varlinskaya, 2020). The specific long-term behavioral consequences of repeated alcohol-induced passing-outs during adolescence, induced by heavy ethanol intoxications, remain incompletely understood, particularly regarding sex differences and the use of both frequentist and structural ethological analyses to assess adult behavior. Thus, the structural analysis of different types of behaviors in models that evoke conflict helps to better assess the complexity of ethanol's effects. In traditional anxiety tests, measures such as latency to enter an aversive area, percentage of time spent in that environment, and number of entries are commonly used as indicators of anxiety-like behavior. However, these variables are affected by the animal's exploratory drive, as seen in changes in transitions between compartments and rearing behavior. Risk assessment, in particular, occupies a distinct but related domain. As defined by Blanchard and Meyza (2019), it involves an approach–avoidance conflict in response to an ambiguous or threatening stimulus. The behavioral repertoire associated with risk assessment includes cautious engagement with the environment - such as stretch-attend postures and head outs towards the aversive environment - reflecting a balance between defensive and exploratory trends. In the present study, anxiety-like, exploratory, and risk assessment behaviors were evaluated using two behavioral tests, each involving a conflict scenario: the choice between exploring a brightly lit, novel area or remaining in a dark, safe zone (LDB), and the exposure to elevated open arms versus enclosed areas (EPM). These paradigms enabled us to assess how previous ethanol exposure affected decision-making under potential threat. We also propose the definition of two categories of risk assessment behaviors: those followed by a return to the safe environment (NoGo) and those followed by taking the risk to enter the aversive environment (Go), allowing us to study risk-based decision-making.

The LDB test is traditionally used to evaluate escaping behavior, by measuring the latency of rodents to pass from the light side to the dark side of the box (Kulesskaya and Voikar, 2014). Using this protocol, Younis and colleagues reported that male and female mice exposed to voluntary ethanol drinking during adolescence did not differ in the time to escape the light side, although mice ran faster to the dark side after an ethanol i.p. administration immediately before the test (Younis et al., 2019). In rats, only female adolescents exposed to ethanol showed decreased latency to enter the dark side when they were adults (Amodeo et al., 2018). We used an adapted version of the LDB: an "avoid" paradigm rather than an "escape" one, which consisted of allocating the animal first to the dark side of the apparatus, allowing them to evaluate the risk to go to the light side (Takao and Miyakawa, 2006). Intoxicated mice - particularly males - exhibited increased latency to enter the light compartment compared to other groups, suggesting that heavy ethanol exposure during adolescence may increase anxiety-like responses towards novel,

aversive environments in adulthood. However, this effect was not supported by differences in the number of entries or total time spent in the light side of the LDB. Previous studies have reported that adolescent ethanol exposure increases avoidance of the aversive (light) compartment, especially in male rats (Pandey et al., 2015; Vetreno et al., 2016; Sakharkar et al., 2019). Interestingly, the present study shows that male mice which were intoxicated during adolescence exhibited fewer NoGo risk assessment behaviors in adulthood, which may suggest that, despite a potential increase in anxiety, these animals display a greater propensity to take risks. Regarding acute ethanol administration prior to testing, no anxiolytic effect was observed; however, it increased the relative percentage of No-Go risk assessment behaviors, primarily due to a reduction in Go risk assessment behaviors in both sexes. We also did not observe substantial effects on the overall behavioral structure measured in the LDB; however, this conclusion should be interpreted with caution, as behaviors in the dark compartment were not evaluated due to methodological constraints.

The EPM is a widely used test for assessing anxiety-like behavior in rodents (Kraeuter et al., 2019). In this test, increases in anxiety-like behavior in adulthood following adolescent ethanol exposure have been reported in both rats (Varlinskaya et al., 2020; review in Towner and Varlinskaya, 2020) and mice (Rice et al., 2019; Van Hees *et al.*, 2022). However, our results suggest that in C57BL/6 mice, four ethanol intoxications during early adolescence did not alter anxiety-like, exploratory, or risk assessment behaviors in adulthood, regardless of sex. Similarly, Younis et al. (2019) reported that voluntary ethanol drinking during adolescence did not affect the latency to enter or time spent in the open arms of the EPM in adulthood. It is noteworthy that, unlike the previously mentioned studies, both Younis et al (2019) and the present study involved i.p. injection administered prior to EPM testing. Even saline, used as a control, is a procedural manipulation that can influence behavioral outcomes compared to experiments in which there is no pre-test injection (Moraes et al., 2025). Regarding the effects of a pre-test low dose of ethanol, as broadly shown by the literature (Younis *et al.*, 2019; Gilpin, et al., 2012), we found that an increased time spent in the open arms, indicative of the anxiolytic acute effect of ethanol. Surprisingly, this effect was observed only in males. In females, ethanol induces an increased time in the open arms if intoxicated during adolescence. Interestingly, the acute anxiolytic effect of ethanol is followed by increases in exploration and decreases in NoGo risk assessment behaviors. These effects profoundly change the behavior structure, as displayed on the Markov Model in the present study.

Repeated ethanol-induced intoxications during adolescence, along with the development of tolerance to ethanol's sedative effects, have been shown to influence drinking behavior later in life (Spear and Varlinskaya, 2005; Crews et al., 2016; Wetherill and Fromme, 2016). While many adolescence ethanol exposure protocols have been shown to increase ethanol intake in adulthood (Strong et al., 2010; Amodeo et al., 2018), this outcome is not consistently replicated across studies (Jury et al., 2017b; Cozzoli et al., 2014). In our study, we did not observe a general effect of early adolescent intoxication on adult ethanol consumption. However, intoxicated female mice consumed significantly more ethanol than non-intoxicated females, suggesting a sex-specific vulnerability to risky drinking patterns. It is broadly replicated that female mice drink more ethanol than males, which already points to possibly a more prone development of increased drinking. Our

results further highlight the importance of considering both sex and developmental timing when investigating the long-term effects of adolescent ethanol exposure. Literature from both rodent and human studies supports robust sex differences in behaviors related to addiction (Schulte et al., 2009; Becker and Koob, 2016). Moreover, the specific timing of ethanol exposure - whether during early, mid, or late adolescence - can result in distinct behavioral outcomes later in life (Varlinskaya and Spear, 2004; Spear, 2015).

The mechanisms by which early alcohol use increases risky decision-making, including alcohol abuse, are yet to be fully clarified. Among youth, drinking to the point of blackout or even passing out is often viewed as socially acceptable or even desirable - used as a form of celebration or a coping mechanism for stress (Riordan et al., 2019; Miller et al., 2020; Carpenter and Merrill, 2021; Patrick and Terry-McElrath, 2021). It is therefore essential to characterize how high-intensity ethanol exposure during adolescence interferes with the drug's response in adulthood. Our findings suggest that heavy intoxication during early adolescence can interact with adulthood drinking to change anxiety-like, exploratory and risk assessment behaviors in males, and enhance ethanol consumption in females.

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6. Declaration of competing interests

The authors declare no conflict of interest.

7. Acknowledgments

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8. Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Figure Legends

Figure 1: Repeated ethanol administration during adolescence induced high BEC levels and tolerance to the depressant effects of ethanol. All graphs show means ± standard error means (SEM). Black symbols and lines represent compiled data from both sexes, while orange represents data from females and blue from male mice. (*) indicates difference from day 1 ($p < 0.05$). **(A-B)** BEC level (mg/dL) at the time mice recovered the reflex in Experiment 1 (n = 7-8/sex). **(C-D)** Latency and Duration of LORR in minutes for experiments 2 and 3 (n = 27-31/sex). LORR: loss of righting reflex. *: $p < 0.05$ compared to day 1.

Figure 2: Anxiety-like, locomotion and risk assessment behaviors in the light–dark box (LDB). All graphs show means ± standard error means (SEM). The grayscale graphs represent data from all animals, the orange-toned graphs represent data from females, and the blue-toned graphs represent data from males. Hatched bars represent the intoxicated groups, which received ethanol during adolescence. Light colors (light gray, light orange, and light blue) represent animals that received saline in adulthood, while dark colors (gray, orange, and blue) represent animals that received ethanol in adulthood. **(A-B)** Latency to pass to the light compartment. **(C-D)** Percentage of time spent in the light compartment. **(E-F)** Number of transitions between compartments. **(G-H)** Number of rearings. **(I-J)** Number of total risk assessments. **(K-L)** Percentage of NoGo risk assessments relative to the total risk assessments. **(M-N)** NoGo risk assessments. **(O-P)** Go risk assessments. n = 10-13/group/sex. intox: intoxicated during adolescence. #: $p < 0.05$ for main factor significance. *: $p < 0.05$ *post hoc* analysis.

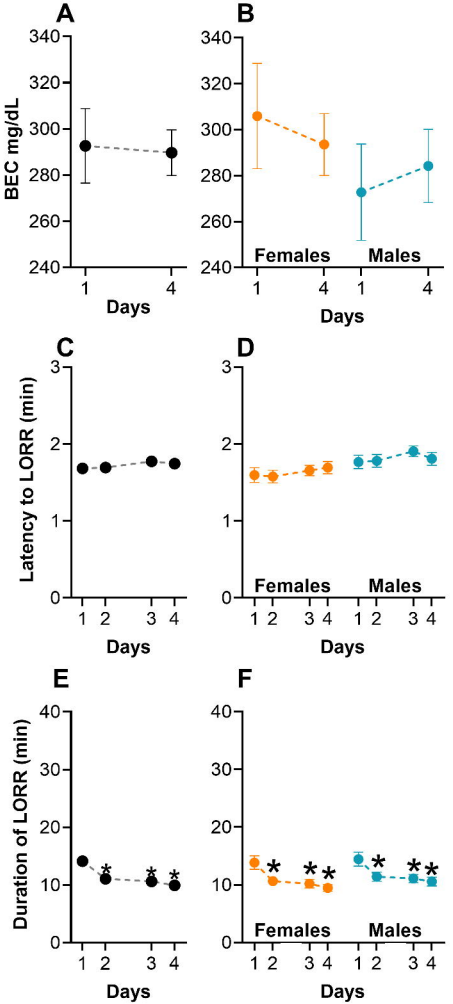
Figure 3: Markov chains representing the sequencing and chaining of behavioral events during the LDB test. Black circles represent behaviors in the dark side and gray circles represent behaviors in the light compartment. Circles' sizes are proportional to the number of occurrences of each behavior, while the arrows' color gradient represents the probability of each transition. HO: head out, St: stretch, Rr: rear, Sn: sniff, W: walk. n = 10-13/group/sex.

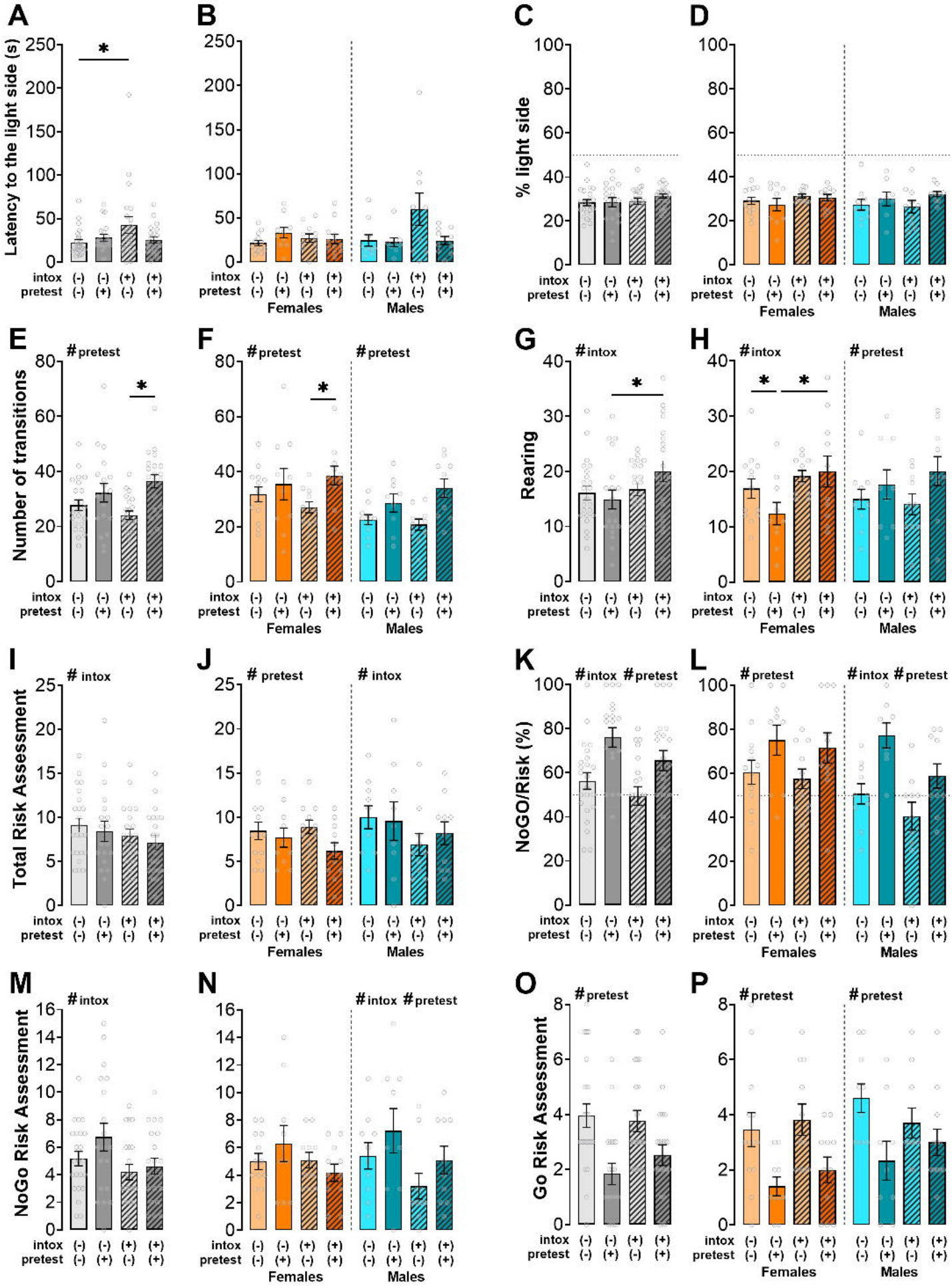
Figure 4: Anxiety-like, locomotion and risk assessment behaviors in the elevated plus maze (EPM). All graphs show means ± standard error means (SEM). The grayscale graphs represent data from all animals, the orange-toned graphs represent data from females, and the blue-toned graphs represent data from males. Hatched bars represent the intoxicated groups, which received ethanol during adolescence. Light colors (light gray, light orange, and light blue) represent animals that received saline in adulthood, while dark colors (gray, orange, and blue) represent animals that received ethanol in adulthood. **(A-B)** Percentage of time spent in the open arms. **(C-D)** Number of entrances in the open arms. **(E-F)** Number of center crosses. **(G-H)** Number of rearings. **(I-J)** Number of total risk assessments. **(K-L)** Percentage of NoGo risk assessments relative to the total risk assessments. **(M-N)** NoGo risk assessments. **(O-P)** Go risk assessments. n = 10-13/group/sex. #: $p < 0.05$ for main factor significance. *: $p < 0.05$ *post hoc* analysis.

Figure 5: Markov chains representing the sequencing and chaining of behavioral events during the EPM test. Black circles represent behaviors in the closed arms and gray circles represent behaviors in the open arms. Circles' sizes are proportional to the number of occurrences of each behavior, while the arrows' color gradient represents the probability of each transition. Gr: grooming, HO: head out, St: stretch, Rr: rear, Sn: sniff, W: walk, HD: head dips. n = 10-13 /per group/per sex.

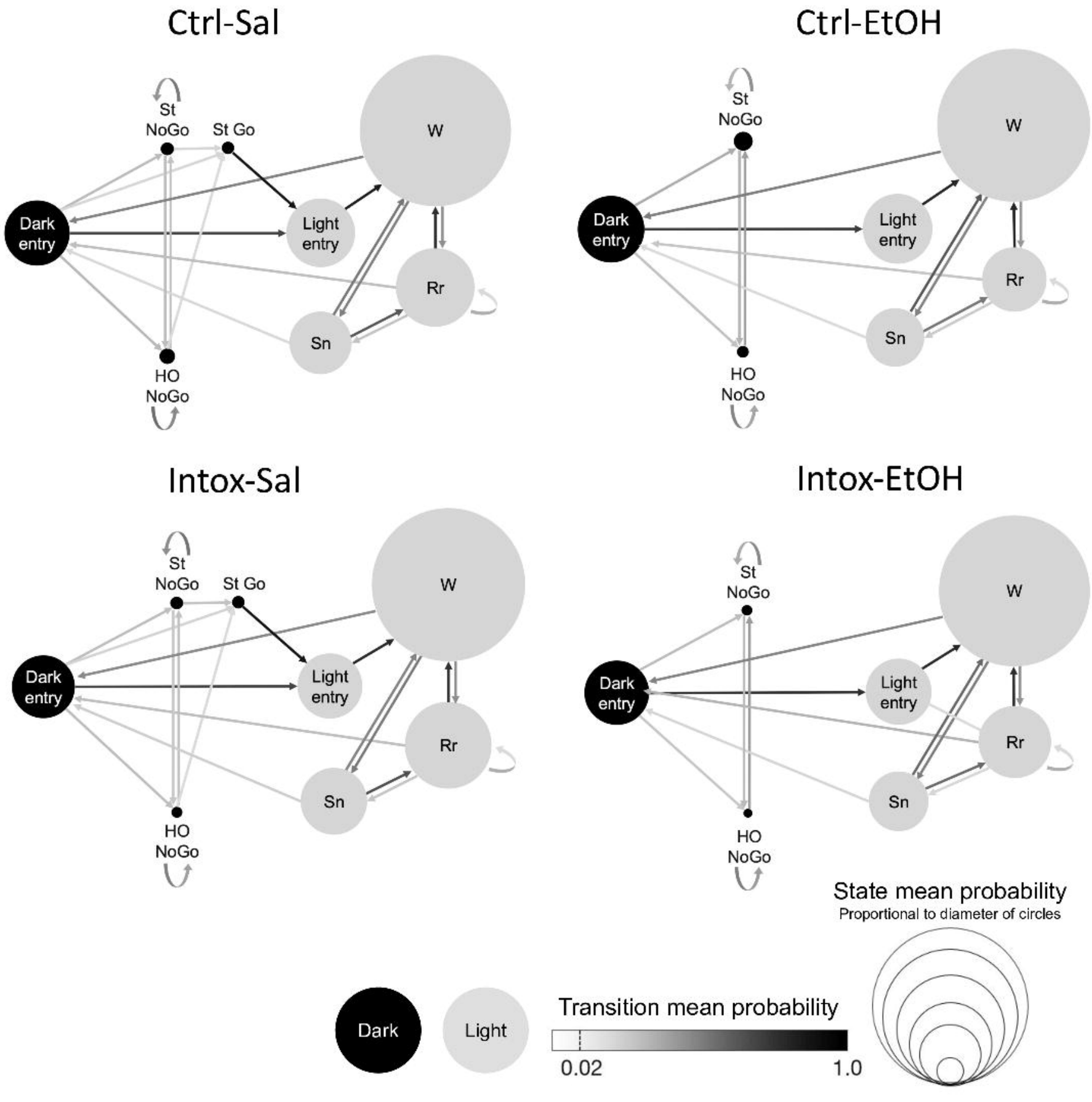
Figure 6: Effects of heavy intoxications during adolescence increases adulthood ethanol drinking in females. All graphs show means ± standard error means (SEM). Black and white symbols and lines represent compiled data from both sexes, while orange represents data from females and blue from males. White circles represent control groups, while circles filled with black,

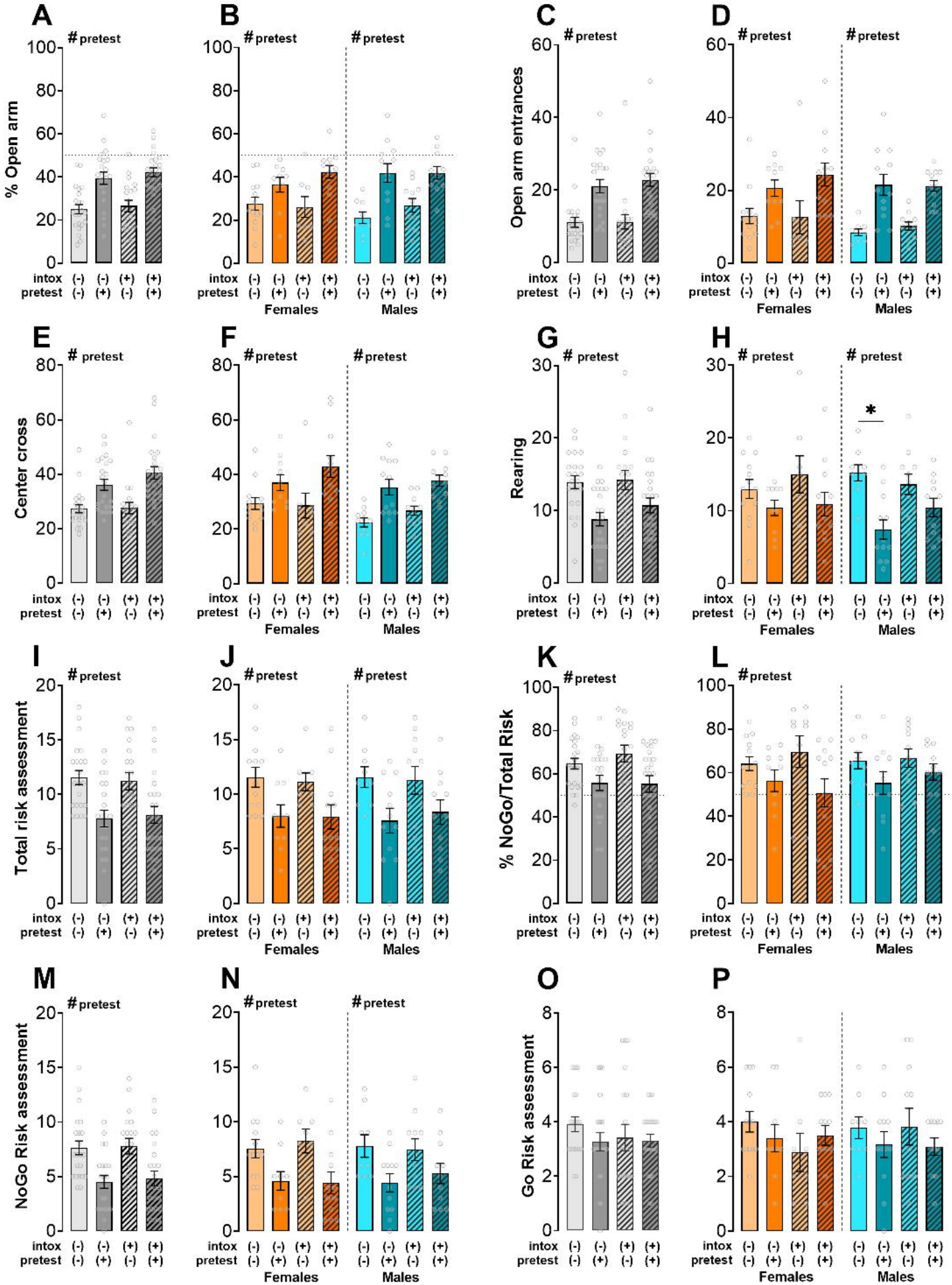
orange or blue represent adolescence-intoxicated groups. **(A-B)** Water intake (g/kg). **(C-D)** Ethanol intake (g/kg) from 10% (v/v) solution. **(E-F)** n = 17-18/group/sex. *: $p < 0.05$ for group factor significance.


A
B
BEC mg/dL
340
320
300
280
260
240
1
4
Days
Females
Males
C
D
Latency to LORR (min)
3
2
1
0
1 2
3 4
E
F
Duration of LORR (min)
40
30
20
10
*

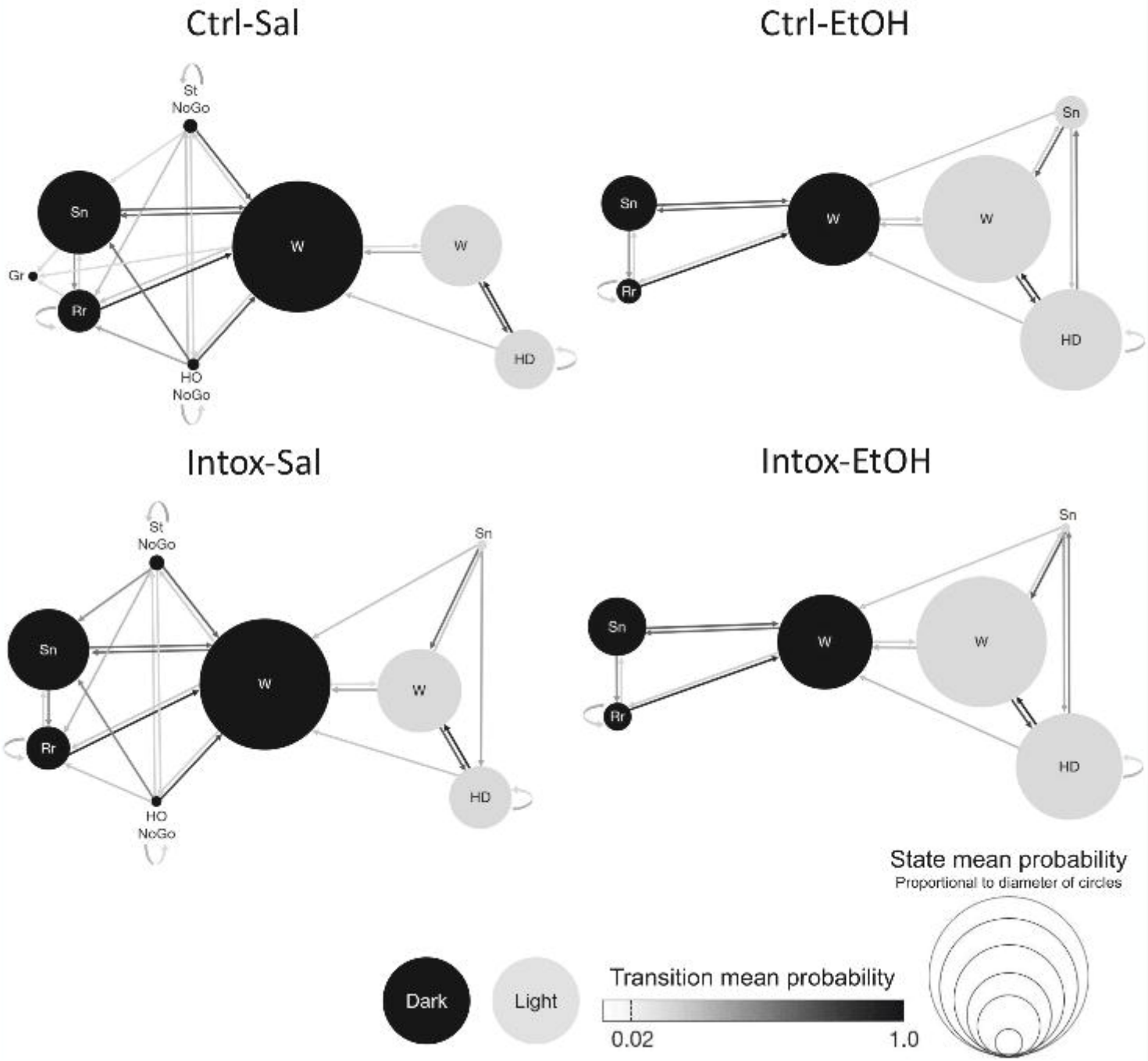

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Latency to the light side (s)
% light side
Number of transitions
Rearing
Total Risk Assessment
NoGO/Risk (%)
NoGo Risk Assessment
Go Risk Assessment
pretest
intox
*
intox (-) (-) (+) (+)
pretest (-) (+) (-) (+)
Females
Males

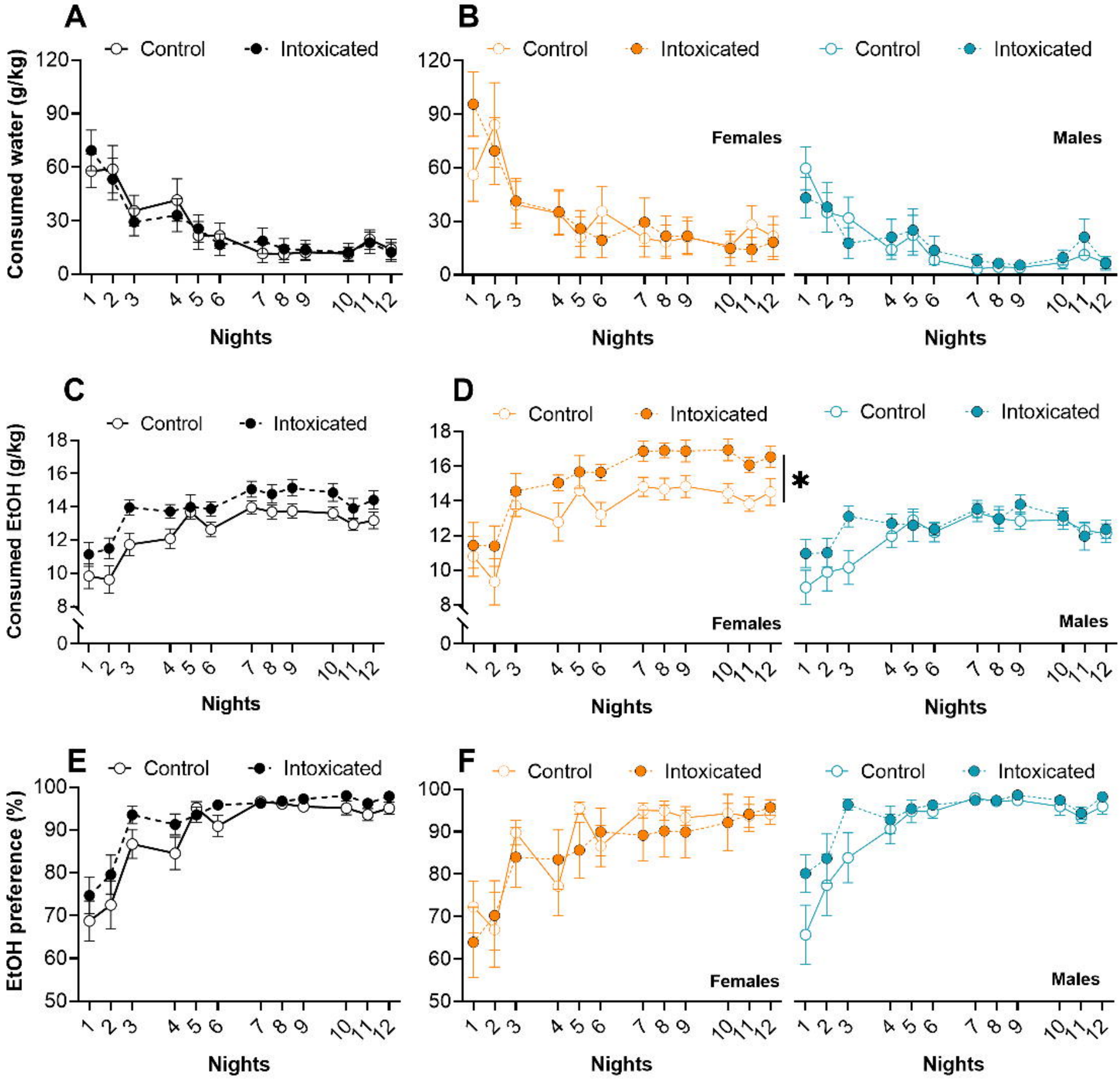
Light-Dark Box




A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
pretest
% Open arm
Open arm entrances
Center cross
Rearing
Total risk assessment
% NoGo/Total Risk
NoGo Risk assessment
Go Risk assessment
intox (-) (-) (+) (+)
pretest (-) (+) (-) (+)
Females
Males
*

Elevated Plus-Maze




A
B
C
D
E
F
Control
Intoxicated
Consumed water (g/kg)
Consumed EtOH (g/kg)
EtOH preference (%)
Nights
Females
Males
*