Title

Red Bull® Energy Drink Increases Consumption of Higher Concentrations of Alcohol

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Running title: Red Bull and alcohol

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ABSTRACT

Mixing alcohol with caffeinated energy drinks is a common practice, especially among young people. In humans, the research on this issue has mainly focused on the use of the mass-marketed energy drinks themselves, whereas in animal models it has focused on the individual effects of their active ingredients (i.e., caffeine). Here we have characterized how Red Bull®, one of the most consumed caffeinated energy drink worldwide, modulates operant alcohol self-administration in Wistar rats. We found that animals readily and steadily responded for Red Bull® (mean: 90 responses, 30 min, fixed-ratio 1) which was accompanied by locomotor stimulating effects (26% increase). The higher the concentration of alcohol (3-20%), the higher the consumption of alcohol (g/kg) and associated blood alcohol levels (91,76%) in the mixed Red Bull®-alcohol group (60% increase). Blood caffeine levels in the Red Bull® group were 4,69 and 1,31 µg/mL in the Red Bull®-alcohol group after the 30 min session. Because Red Bull® also contains 11% sucrose, we examined the time course of blood glucose as well as insulin, and corticosterone. The correlation between intake of Red Bull® and blood glucose levels was higher at 90 min than 5 min after its consumption and there was no relationship with blood insulin or blood corticosterone levels. Red Bull® did not alter extinction and reacquisition of responding for alcohol, nor did it affect relapse-like drinking. Overall, our results suggest that Red Bull® might be a vulnerability factor to develop alcoholism given that it intensifies the consumption of higher concentrations of alcohol.

Keywords: Alcohol, Energy drink, Operant self-administration

INTRODUCTION

The use of energy drinks is growing. In the United States, the sales of the three most popular energy drink brands (i.e., Red Bull®, Monster® and Rockstar®) have more than doubled from 2008 to 2015 (from US$4.10 to US$9.06 million, respectively (Euromonitor International 2015). In addition, it has been reported that 27% of young alcohol users (13-18 years) consumed alcohol mixed with energy drinks (Khan et al. 2016). The consumption of combination of alcohol and energy drinks has been associated with an increased risk of binge drinking, alcohol dependence, and hazardous behavior such as driving while intoxicated and risky sexual behavior (for a review see, Marczinski & Fillmore 2014). However, interpretation of the available literature is equivocal, and studies evaluating the health impact of mixing alcohol with energy drinks are still needed.

A full understanding of the health impact of mixing caffeinated energy drinks with alcohol will require the development of good animal models. Most human studies have investigated the effect of the mixed alcohol and energy drinks on health/behaviors using marketed brands, such as Red Bull®. For this purpose, interviews, surveys, laboratory experiments, and double-blind placebo studies have been conducted (Marczinski & Fillmore 2014; Peacock et al. 2014; Striley & Khan 2014). In contrast, most animal studies have investigated the health impact of mixing alcohol with the believed key psychoactive component of energy drinks, caffeine. For instance, it has been demonstrated in mice that alcohol mixed with caffeine causes a ~4-fold increase in locomotion compared with orally administered alcohol or caffeine alone (May et al. 2015). Similar locomotor results were recently observed in adolescent mice when they were

allowed to voluntarily consume alcohol, caffeine, or its combination in a limited-access binge-like alcohol drinking paradigm (Fritz et al. 2016).

The aim of the current work was to study the effects of the most common used energy drink worldwide, Red Bull®, using an animal model of high predictive validity in humans: operant self-administration (Panlilio & Goldberg 2007). Using oral operant self-administration procedures in Wistar rats, our goal was to assess the behavioral and biochemical changes that would take place during consumption of alcohol combined with Red Bull®. We established dose-response curves for Red Bull® versus alcohol combined with Red Bull®, as well as determined the time course of glucose, and the analysis of alcohol, caffeine, insulin and corticosterone blood levels. We also studied the effect of alcohol and Red Bull® deprivation on relapse, and the extinction and reacquisition of responding for alcohol and Red Bull®. We reveal that rats can steadily self-administer Red Bull® using an operant self-administration paradigm and that mixing Red Bull® with alcohol is associated with the consumption of higher amounts and attainment of higher blood alcohol levels. Under our experimental conditions, Red Bull® did not affect relapse-like alcohol drinking and neither extinction and nor reacquisition of alcohol and Red Bull® operant self-administration.

MATERIALS AND METHODS

Animals and Housing

Seventy-two male Wistar rats (Harlan, Barcelona, Spain) were used. Rats were purchased at eight weeks old and weighted 320-360 g at the start of experimentation. All research was conducted in strict adherence to the European

Directive 2010/63/EU and Royal decree 53/2013 (BOE, 2013) on the protection of animals used for scientific purposes. The Ethics Committee of the Faculty of Psychology of the Complutense University of Madrid approved the study. Animals were housed in groups of 4 per cage in a specific pathogen free and temperature- and humidity-controlled environment (21±1°C), on a 12 h reverse light/dark cycle (lights off at 08:00 h). Experimental sessions were performed during the dark phase. Food and water were available ad libitum except as specified below. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Drugs

Red Bull® Energy Drink cans (473 mL) containing 11 g sucrose, 32 mg caffeine, 400 mg taurine, 8 mg niacin, 2 mg pantothenic acid, 2 mg vitamin B6 and 2 µg vitamin B12 per 100 mL were bought at local large supermarket chains in Madrid (Spain). Red Bull® was degassed prior to offering the solution to the animals; it was placed in a container with a magnetic bar inside on a stirring plate at room temperature. Alcohol and Red Bull® solutions were prepared every two days from 96% alcohol (Alcoholes Aroca, S.L., Madrid, Spain).

Operant Self-Administration Procedures

Apparatus and General Procedure

The operant alcohol sessions were conducted in eight modular chambers enclosed in sound-attenuating cubicles (Med Associates Inc., St. Albans, VT, USA). A fixed-ratio 1 schedule of reinforcement was used throughout all the experiments. The chambers were equipped with two retractable levers located 7 cm above a grid floor on either side of a drinking reservoir positioned in the center of the front panel of the chamber and 4 cm above the grid floor. The levers were

counterbalanced to respond as the active lever (delivering 0.1 ml) or as the inactive lever. Auditory or visual cues were not presented at any time. The rats were placed on a restricted water intake schedule for 12 hours ranging from two to four days to facilitate the training in lever pressing. For the rest of the experiments, the animals had access to food and water ad libitum. Eleven days took the whole group of rats (n=72) learning that one press in one active lever was associated to a reward delivery. Sixty rats received a 2.2% w/v sucrose solution and twelve received 0.005% w/v saccharin solution (Sigma-Aldrich, S.L., Madrid, Spain) in the dipper. Afterward, the experiment **began** (see Experimental Design subheading for details).

Deprivation, Relapse, Extinction and Reacquisition Procedures

During the period of alcohol deprivation, the animals were not introduced into the self-administration boxes for a period of seven days. After that period, the animals were reintroduced back to the self-administration boxes and the relapse was evaluated over a period of five days (relapse-like period). Once the baseline levels of consumption were reestablished, a period of extinction occurred. Animals were introduced every day into the self-administration boxes, but in this case, the active lever pressure was not followed by the release of a reward (i.e., alcohol, Red Bull®, etc.). After 11 days, the lowest number of responses (see results section, Study 5) was reached and, according to previous publications (Rodd-Henricks et al. 2002), responding behavior was considered to be extinguished. Then (day 12) the original conditions were restored; each pressure of the active lever was followed by the release of the corresponding reward. With this, behavior reacquistion was evaluated

Locomotor Activity

The locomotor activity of the rats was assessed during 30 min using six custom-made 40 x 35 x 35 cm rectangular boxes, which were equipped with eight photocells arranged in two lines (four and eight cm above the floor) that detected the locomotor activity as beam breaks.

Experimental Design

The first study aimed to investigate whether the animals could self-administer Red Bull® in the operant self-administration paradigm. Once all the animals were trained, the solution of Red Bull® or sucrose was progressively increased every three days up to 100% of Red Bull® or 11% of sucrose. Red Bull® contains 11% sucrose, therefore, we used it as the control solution here and throughout all the studies. A group of rats responding for saccharin was used as an additional control. Rat body weight was measured every day before to introduce the animals into the operant chambers. Changes in body weight were calculated for each individual as % deviation from its previous body weight before introducing Red Bull® or sucrose. The last day (i.e., the 15th day – see **Figure 1A**) locomotor activity was evaluated immediately after the 30-min session of Red Bull® or sucrose.

The second study would permit to determine the effects of Red Bull® on different concentrations of alcohol. It begun immediately after the first study and the concentration of alcohol was progressively increased every four days, from 3% up top 20%. Animal's locomotor activity was evaluated the last day (i.e., the 20th day – see **Figure 2B**) immediately after the 30-min operant self-administration session.

The third and fourth studies would allow to describe the interaction between alcohol and Red Bull® in the blood glucose curve and alcohol, caffeine, insulin and corticosterone levels (shown in **Figure 3 and 4**). For the blood glucose levels measures food was withdrawn the night before the sample collection. The blood was taken by tail vein puncture and analyzed by a glucometer Accu-Chek® Aviva (Roche Diagnostics S.L. Spain) before (basal) and after (5, 30, and 90 minutes) the self-administration session. To determine blood alcohol and insulin levels, 250 µL of blood was collected from the rat tail vein into a capillary tube (Microvette CB 300 K2E) immediately after the alcohol self-administration session after the final operant session. The complete blood sample and plasma collection procedures were described in our previous study (Calleja-Conde et al. 2016). The alcohol and insulin concentration were measured using the EnzyChrom ethanol assay kit (Bioassay Systems, Hayward, CA, USA) and the Rat Insulin Wide Range ELISA (BioVendor research and diagnostic products LM), respectively, following the protocol recommended by the manufacturers. All measurements were performed in duplicate. Simple HPLC method for determination of caffeine was used for sample preparation and caffeine Standards and quality controls. Chromatographic conditions were adapted to Hitachi Elite LaChrom HPLC system with Diode Array Detector, and deproteinized samples was injected over reverse-phase analytical column (phenomenex Luna 100x4.6 mm, 5 um size particule) with guard column. Elution was done in gradient condition (time=0 min, 15/75/10 [acetonilrile/water/buffer 10 mM potassium phosphate] and time= 7.0 min 50/40/10 and flow 0,7 ml/mim. After each run, 6 min of initial condition was run. Measurement of Caffeine was done at 270 nm, with 99% of similarity spectrum over 3,88 min. Blood from the rat trunk

(400-450 µL) after decapitation was collected in VACUTEST tubes (Vacutest Kima S.r.l., Arzergrande, Italy) that contain K3 EDTA. Following, plasma was obtained as described earlier. Plasma corticosterone was measured by radioimmunoassay (RIA) using a commercial kit from MP Biomedicals, LLC (Orangeburg, NY, USA), following the manufacturer's protocol.

In the last study, to investigate whether Red Bull® would be able to modulate the relapse on alcohol consumption, we carried out a relapse-like drinking situation known as the Alcohol Deprivation Effect (ADE). This model consists in introducing a period of deprivation of alcohol, i.e., a forced abstinence. Here, the animals were not introduced into the operant boxes. The baseline corresponded to the average of the last five operant alcohol self-administration sessions (Figure 5A). According to previous results from our laboratory we used a seven-day period of alcohol deprivation. It results in a significant transient peak of alcohol self-administration (López-Moreno et al. 2004; López-Moreno et al. 2007). After five days monitoring alcohol relapse and ten more additional days for returning to their baseline, we investigated whether Red Bull® would alter the processes of the extinction and reacquisition for responding for alcohol. The animals were introduced into the operant chambers in the extinction process, but there was not a delivery of the reinforcing solution (e.g., Red Bull®+alcohol) after pressing the former active lever. That implies that the animals should made a new learning. Once completed the extinction period (Figure 5B), the following day (12^{th}), the initial conditions of operant self-administration were reinstituted. Every press lever was followed by the delivery of the respective reinforcing solution (e.g., Red Bull®+alcohol).

Statistical Analysis

Data from **Figure 1B** were analyzed using a one-way ANOVA (between-groups: drinking solutions) and from **Figure 1C** using a two-way mixed ANOVA (between-groups: drinking solutions; within-subjects: days). Data from **Figure 2A,C** and **2B** were analyzed using a two and three-way mixed ANOVAs respectively (between-groups: drinking solution; within-subjects: days and alcohol concentration). Data from **Figure 3A** were analyzed using a two-way mixed ANOVA (between-groups: drinking solutions; within-subjects: time-point) and correlations from **Figures 3B-D** were determined by Pearson's correlations analysis. Data from **Figures 4A and 4D** were analyzed by *t*-Student test, those from **Figures 4G and 4J** were analyzed using a one-way ANOVA (between-groups: drinking solutions); and all the correlations from **Figure 3** were determined by using Pearson's analysis. Data from **Figures 5A-C** were analyzed by a two-way mixed ANOVA (between-groups: drinking solutions; within-subjects: days). A significance level of $p<0.05$ was applied to all ANOVA statistical analyses and, when significant, the results were followed by Tukey's post hoc tests. All the analyses were performed after controlling for assumptions (e.g., Levene´s test to assess variance homogeneity among groups), and the anomalous values detected through the SPSS box plot analysis were discarded. The SPSS statistical software package (version 20.0) for Windows (Chicago, IL, USA) was used for all statistical analyses.

RESULTS

Study 1. Dose response curve for Red Bull®

A dose-response curve for self-administration of Red Bull® and sucrose -as the control group- was established. We obtained a reliable and robust operant self-

administration of both fluids (**Figure 1A**). The mean number of lever presses to obtain the 100% Red Bull® solution was between 90 (4.09 ± SEM) and 105 (3.96 ± SEM). This is the first characterization of the intake of an energy drink on animals under an operant self-administration procedure. Regarding the mean number of the inactive lever responses, there were no significant differences between both groups. *For that reason, data from the inactive lever will be omitted from the rest of the figures.* Red Bull® caused a significant increase in the spontaneous locomotor activity of around 26% compared with the saccharin and sucrose control groups (**Figure 1B**) (locomotor activity: one-way ANOVA: drinking solution $F_{(2,75)}$=15.92, p<0.001). Red Bull® did not alter weight gain. The increase in body weight was similar to the saccharin control group whereas the sucrose group was the group that gained more weight, a 2.5% more (**Figure 1C**) (two-way mixed ANOVA: drinking solution $F_{(2,72)}$=8.84, p<0.001; days $F_{(5,360)}$=1,904.97, p<0.001; interaction $F_{(10,360)}$=4.47 p<0.001).

Study 2. Effects of Red Bull® on different concentrations of alcohol

Red Bull® increased the alcohol intake at higher concentrations. **Figure 2A** shows that while at low concentrations of alcohol (3 and 6% v/v) consumption is greater when combined with sucrose, at higher concentrations of alcohol (15 and 20% v/v) alcohol consumption is increased when combined with Red Bull® (mean: 60% more alcohol is consumed when Red Bull is combined with the highest alcohol concentration). The ANOVA reveals a significant interaction between the type of drinking solution and the level of alcohol concentration (two-way mixed ANOVA: drinking solution $F_{(1,30)}$=0.54, NS; alcohol concentration $F_{(4,120)}$=51.57, p<0.001; interaction $F_{(4,120)}$=35.71 p<0.001), suggesting that the

effects of Red Bull® on alcohol intake depend on the alcohol concentration. This interactive effect can also be observed in **Figure 2B** which shows the number of the active lever responses (three-way mixed ANOVA: drinking solution $F_{(3,44)}$=25.78, p<0.001; alcohol concentration $F_{(4,176)}$=59.80, p<0.001; and days $F_{(3,132)}$=1.04, NS; the only significant interaction was between the alcohol concentration and the type of drinking solution, $F_{(12,176)}$=38.19, p<0.001). The inset in the graphic depicts the structure of a standard 30 min session of operant self-administration at 5-min intervals. **Figure 2C** shows the total amount of caffeine consumed by the two Red Bull® groups. At the highest concentration of alcohol (20% v/v), there was a reduction of 61.8% in the amount of caffeine consumed in the Red Bull®+alcohol group (two-way mixed ANOVA: drinking solution $F_{(1,27)}$=4.80, p<0.05; alcohol concentration $F_{(4,108)}$=29.16, p<0.001; interaction $F_{(4,108)}$=24.87 p<0.001). Immediately after the final operant session on day 15, there were no significant differences detected between groups in the animal's spontaneous locomotor activity (**Figure 2D**).

Study 3. Effects of Red Bull® self-administration on blood glucose levels

Figure 3A shows the time course of blood glucose levels. Five min before the initiation of the operant self-administration session, there were no differences between the groups (basal levels). Blood glucose levels were around 100 mg/dL. Blood glucose levels were elevated 5 min after conclusion of the operant session for all groups except for the saccharin control group, and decreased as a function of time thereafter (two-way mixed ANOVA: drinking solution $F_{(4,43)}$=26.57, p<0.001; time-point $F_{(2,86)}$=14.62, p<0.001; interaction $F_{(8,86)}$=6.19 p<0.001). Blood glucose levels from the saccharin control group remained close to 100

mg/dL throughout all the time-points. **Figures 3B-D** depict the significant positive correlations between rat's blood glucose levels and number of reinforces obtained during the operant self-administration sessions. Correlation coefficients and their respective levels of significance were higher 90 min after operant self-administration (r=0.84 / r=0.74; p<0.001) than five min after operant self-administration (r=0.60 / r=0.55; p<0.01).

Study 4. Effects of Red Bull® self-administration on blood alcohol, caffeine, insulin and corticosterone levels

To determine the levels of alcohol, caffeine and insulin reached by the animals in their peripheral blood following the 30 min operant self-administration sessions, blood samples from the rat tail vein were collected *in vivo*. **Figure 4A** shows that the mean of blood alcohol levels from the animals responding to the Red Bull®+alcohol mixture was 91,8 mg/dL, whereas that for the animals responding for the sucrose+alcohol mixture was 58,1 mg/dL (p<0.05, Student's t-test). Both groups of animals exhibited a significant correlation between the number of reinforces obtained and their blood alcohol levels (**Figures 4B-C**, $r_{(30)}$=0.76, p<0.005; and $r_{(30)}$=0.72, p<0.005). According to their number of responses and reinforcers obtained, blood caffeine levels in the animals drinking Red Bull® alone were higher than those drinking the Red Bull®+alcohol mixture (**Figure 4D**, p<0.001, Student's t-test). Also, there were significant correlations between the number of reinforcers and blood caffeine levels (**Figures 4E-F**, $r_{(28)}$=0.79, p<0.005; and $r_{(28)}$=0.88, p<0.001). Sucrose self-administration, with or without alcohol, resulted in increased blood insulin levels (**Figure 4G**). Red Bull® also increased blood insulin levels, but not significantly when it was combined with

alcohol (one-way ANOVA $F_{(4,69)}$=3.68, p<0.01). Finally, there was no correlation between the number of reinforces and blood insulin levels (**Figures 4H-I**).

Because we wondered whether chronic Red Bull® intake might be linked to biological stress, corticosterone levels were assessed at the end of the experiment from blood collected from the trunk body, *post mortem*. No significant differences were found between groups in blood corticosterone levels (**Figure 4J**), nor did the number of reinforces correlate with blood corticosterone levels (**Figures 4K-L**).

Study 5. Effects of Red Bull® on alcohol relapse, extinction and reacquisition

To investigate the effects of Red Bull on relapse-like drinking we used the Alcohol Deprivation Effect paradigm. We observed that the seven-day period of deprivation of sucrose or Red Bull® was not associated with an increase in their consumption after reintroducing the animals to the operant chambers (**Figure 5A**). There were no significant differences in the number of responses in the alcohol groups at the baseline. Furthermore, both alcohol groups, either combined with sucrose or Red Bull®, exhibited an Alcohol Deprivation Effect. After the alcohol deprivation period the self-administration levels were higher, particularly in the sucrose+alcohol group (**Figure 5B**), (two-way mixed ANOVA: drinking solution $F_{(1,30)}$=10.57 p<0.001; days $F_{(5,150)}$=3.88, p<0.01; interaction $F_{(5,150)}$=2.51, p<0.05).

Next, we studied whether Red Bull® was able to modify the extinction process of alcohol responses and its corresponding reacquisition (**Figure 5C**). For the extinction period, the animals were introduced into the operant chambers

but their responses on the active lever were not followed by any reinforcer. In this situation, it was not until the fourth day that the significant differences in the number of responses between groups disappeared (p=0.09). The lowest levels of responses were in the 11th day. At that point, it was considered that the behavior was extinguished, according also with previous reports (Rodd-Henricks et al. 2002). Then, the following day (12th), the initial conditions of operant self-administration were reinstituted; every press lever was followed by the delivery of its respective reinforcer. All the groups of animals reacquired the previous self-administration patterns, suggesting that Red Bull® did not alter the reacquisition of this extinguished behavior (two-way mixed ANOVA: drinking solution $F_{(3,52)}$=54.93 p<0.001; days $F_{(4,208)}$=11.49, p<0.001; interaction $F_{(12,208)}$=2.72, p<0.005)

DISCUSSION

Here we report the first characterization of operant energy drink self-administration, as well as its effects on alcohol self-administration. Given that this method has a very high predictive validity in humans (Tkacs & Thompson 2006), the feasibility of operant energy drink and mixed alcohol/energy drink self-administration constitutes a further advance that likely will help us understandthe impact of these beverages on human health. Furthermore, in order to mimic more precisely human energy drink consumption, we used one of the most consumed energy drink worldwide, i.e., Red Bull®. One key finding was that the effects of Red Bull® on alcohol self-administration depend on the concentration of alcohol: at low alcohol concentrations (3, 6%) the sucrose+alcohol exhibited greater active lever presses and consumed more alcohol per body weight. But at high

alcohol concentrations (15, 20%), it was the Red Bull®+alcohol group which displayed more active lever presses and consumed more alcohol (**Figure 2A and 2B**). We believe there are two possible explanations for these findings. The first and simplest one is that Red Bull® masks the strong taste of alcohol to a greater extent than does sucrose alone. That is, palatability could explain the current results. It would make sense given that young people from several countries (e.g., UK, USA, Australia) report the main reason for mixing alcohol with energy drink is “I like the taste” (Verster et al. 2014; Bonar et al. 2015; Johnson et al. 2016). Another possibility is that one or more of the ingredients in energy drinks increase the rewarding properties of alcohol, or relieve alcohol-induced-negative effects. Among those ingredients that could modulate the animal’s alcohol consumption are taurine, caffeine and group B vitamins (B3, B5, B6 and B12). Caffeine is the ingredient that has received the most interest from researchers (for review see, McKetin et al. 2015). Researchers argue that caffeine may counteract the depressant effects of alcohol (Arria & O’Brien 2011; Peacock & Bruno 2013; Fritz et al. 2014; Fritz et al. 2016). The modulatory and interactive effects of the other energy drink ingredients on alcohol intake and related behavior remain greatly unexplored. Therefore, further research is warranted to explore the ingredients and mechanisms by which Red Bull® increases alcohol consumption at high alcohol concentrations.

Although the consumption of sucrose solution was double that of Red Bull® (**Figure 2B**), the blood glucose peak was similar at five min after the operant self-administration session (**Figure 3A**). However, by 90 min later blood glucose adjusted better to the levels of animal’s consumption. That is, the higher the sucrose consumption, the higher the blood glucose levels. That would

suggest, as the correlations support, that a better predictor of total sucrose consumed is 90 min after operant self-administration. The presence of alcohol did not change blood glucose levels as both alcohol groups did not differ significantly from each other (**Figure 3A**).

Sucrose consumption did not seem to alter the metabolism of alcohol as blood alcohol levels were only linked to the amount of alcohol consumed (**Figure 4A**). There was a positive correlation between blood caffeine levels and number of reinforces (**Figure 4E-F**). It has been proposed that a moderate caffeine user consumes between 200-400 mg/day of caffeine, which is 2.9-5.7 g/kg per body weight (Cappelletti et al. 2015). Here, we observed that the mean caffeine intake in the Red Bull® group was 5.0 g/kg per body weight (mean: 4.7 µg caffeine/mL blood). That means that the animals' caffeine intake was relatively high considering that this amount of caffeine was consumed in just a period of 30 min. However, the mean intake of caffeine in the Red Bull®+alcohol group was 1.9 g/kg per body weight, suggesting that Red Bull®-induced intake of higher concentrations of alcohol is not linked directly to the intake of high doses of caffeine. It was surprising that the sucrose and sucrose+alcohol groups did not differ in blood insulin levels (**Figure 4G**) given the sucrose group consumed five times more sucrose than the sucrose+alcohol group (see **Figure 2B**). The correlation between blood insulin levels and number of sucrose reinforcers obtained was also not significant. This might be explained by the fact that animals had an extended history of sucrose self-administration. Insulin secretion has been shown to decrease after repeated exposures to glucose (Santos Junior et al. 1989). Current studies are invetigating whether insulin levels are linked to alcohol craving in alcohol dependent individuals (Leggio et al. 2008; Haass-

Koffler et al. 2016). Here, Red Bull®-induced intake of higher concentrations of alcohol was not associated to blood insulin levels, therefore, our results wouldn't support the hypothesis that insulin levels are correlated with alcohol craving (Haass-Koffler et al. 2016). As a final blood result analysis, the fact that blood corticosterone levels were unaffected by any drinking solution (**Figure 4J**) suggests the absence of any significant stress response.

The Alcohol Deprivation Effect (ADE) is characterized by a transient peak increase in alcohol self-administration after a period of abstinence. That is, it is a within-subject effect; the animals' consumption after the deprivation period is compared to their previous alcohol consumption before the deprivation period. The ADE is typically used as a model of loss of control over alcohol drinking resulting in higher amounts of alcohol consumed (Agabio et al. 2000; Bell et al. 2008). Our results showed that sucrose deprivation did not significantly increase subsequent sucrose self-administration, regardless of whether Red Bull was present (**Figure 5A**). However, as expected, both alcohol groups showed the ADE. Intriguingly, the sucrose alcohol group showed a more long-lasting ADE compared with the Red Bull®+alcohol group (**Figure 5B**). Thus, Red Bull® does not be appear to significantly affect the intake of alcohol after a single period of deprivation. In the same way, the extinction period and the reacquisition was not altered by the presence of Red Bull®. It seems only that the resistance to the extinction of the behavior was more linked to the higher number of responses. That is, the higher the number of responses to obtain a reward, the higher the number of sessions are needed to extinguish such responses. Further studies should address whether Red Bull® would affect alcohol-paired cues during cue-induced reinstatement and the relapse after repeated periods of abstinence.

Taken together, our results indicate, for the first time, that animals will readily self-administer Red Bull® in an operant self-administration paradigm. Importantly, Red Bull® self-administration was associated with the consumption of higher concentrations of alcohol which led to higher blood alcohol levels. This supports the meta-analysis by Verster and colleagues in humans, who concluded that heavy alcohol consumption is one of the phenotypic differences between consuming alcohol mixed with energy drinks and alcohol only (Verster et al. 2016). Therefore, and in conclusion, it seems that mixing Red Bull® with alcohol makes one more likely to reach higher blood alcohol levels, and consequently, either lead to more risky and dangerous behaviors during a single alcohol drinking episode or increasing the risk to develop alcohol addiction.

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AUTHORS CONTRIBUTION

JAL-M, MR, KMB and VE-A were responsible for the study concept and design. MR, VE-A, JC-C, PO, contributed to the acquisition of animal data. IJS-D, CS, FG-G and EG performed the biochemcial analysis. RM, FRdeF and SLB assisted with data analysis and interpretation of findings. MR and JAL-M drafted the manuscript. RM, FRdF, and SLB provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version for publication.

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FIGURE LEGENDS

Figure 1. Dose-response curve for Red Bull®, and locomotor activity and body weight changes assessment.

Data represent the mean ± SEM. **(A)** Dose-response curve for Red Bull® using an operant self-administration paradigm. Red Bull® was steadily self-administered (Active Lever Responses). The solution concentration of Red Bull® or sucrose, was increased by 20% every three days up to 100% for Red Bull® (the percentage of sucrose in a can of Red Bull® is 11%). There were no differences between Red Bull® and sucrose in the number of inactive lever responses. **(B)** Red Bull® increased locomotor activity. ***p < 0.005 compared with the saccharin and sucrose groups. The saccharin group was added as an additional control group. The activity was evaluated immediately after the operant self-administration session during 30 min. No significant differences were found between Red Bull® or sucrose self-administration at baseline. At the end of the dose-response curve for Red Bull® a significant psychomotor stimulant effect was observed in those animals responding for Red Bull®. **(C)** Percentage of body weight change through the dose response curve for Red Bull®. **p < 0.01 compared with the Red Bull® and saccharin groups. The baseline (0% weight change) corresponds to the animal's weight before responding for Red Bull® or sucrose. Each point represents the mean of the weight for every three-day drink solution period.

Figure 2. Effects of Red Bull® on different concentrations of alcohol

Data represent the mean ± SEM. **(A)** Effects of Red Bull® on accumulated grams of alcohol consumed per kilo of body weight through four-day periods of increasing doses of alcohol (3-20%). *p < 0.05, **p < 0.01, ***p < 0.001 compared

between groups. **(B)** Effects of Red Bull® on the active lever responses day-by-day. Within the two alcohol groups, the number of responses depended on the concentration of alcohol: at higher concentrations of alcohol, compared to the sucrose+alcohol, RedBull® caused a greater number of responses. **p < 0.01, compared with the Red Bull®+alcohol mixture; ###p < 0.001 compared with the Red Bull®-group; &&&p < 0.001 compared with the sucrose-group. The inset depicts the accumulated percentage of responses within one standard 30 min session at 5 min intervals (20% alcohol concentration in the alcohol-groups). The alcohol groups gave around 50% of their responses within the first five min and 90% within the first 15 min. **(C)** Accumulated milligrams of caffeine per kilo of body weight through four-day periods of increasing doses of alcohol (3-20%). #p < 0.05, ###p < 0.001 compared between groups **(D)** At that point, locomotor activity, which was evaluated immediately after the operant self-administration session during 30 min, did not show any significant effect by any treatment.

Figure 3. Effects of Red Bull® self-administration on blood glucose levels

Data represent the mean ± SEM. **(A)** Time course of blood glucose levels after operant self-administration of sucrose (11% w/v), sucrose+alcohol, Red Bull®, or Red Bull®+alcohol. Higher sucrose consumption was associated with long-lasting elevated levels of glucose in blood rather than a higher peak level. The concentration of alcohol was 20%. A control group under saccharin self-administration (0,005%) was added. **p < 0.01, compared with the four solutions. &p < 0.05, &&p < 0.01, &&&p < 0.001 compared with the sucrose-group. (**B-E**) Correlations between blood glucose levels and the number of sucrose or Red Bull® reinforces obtained at 5 and 90 min after operant self-administration. The

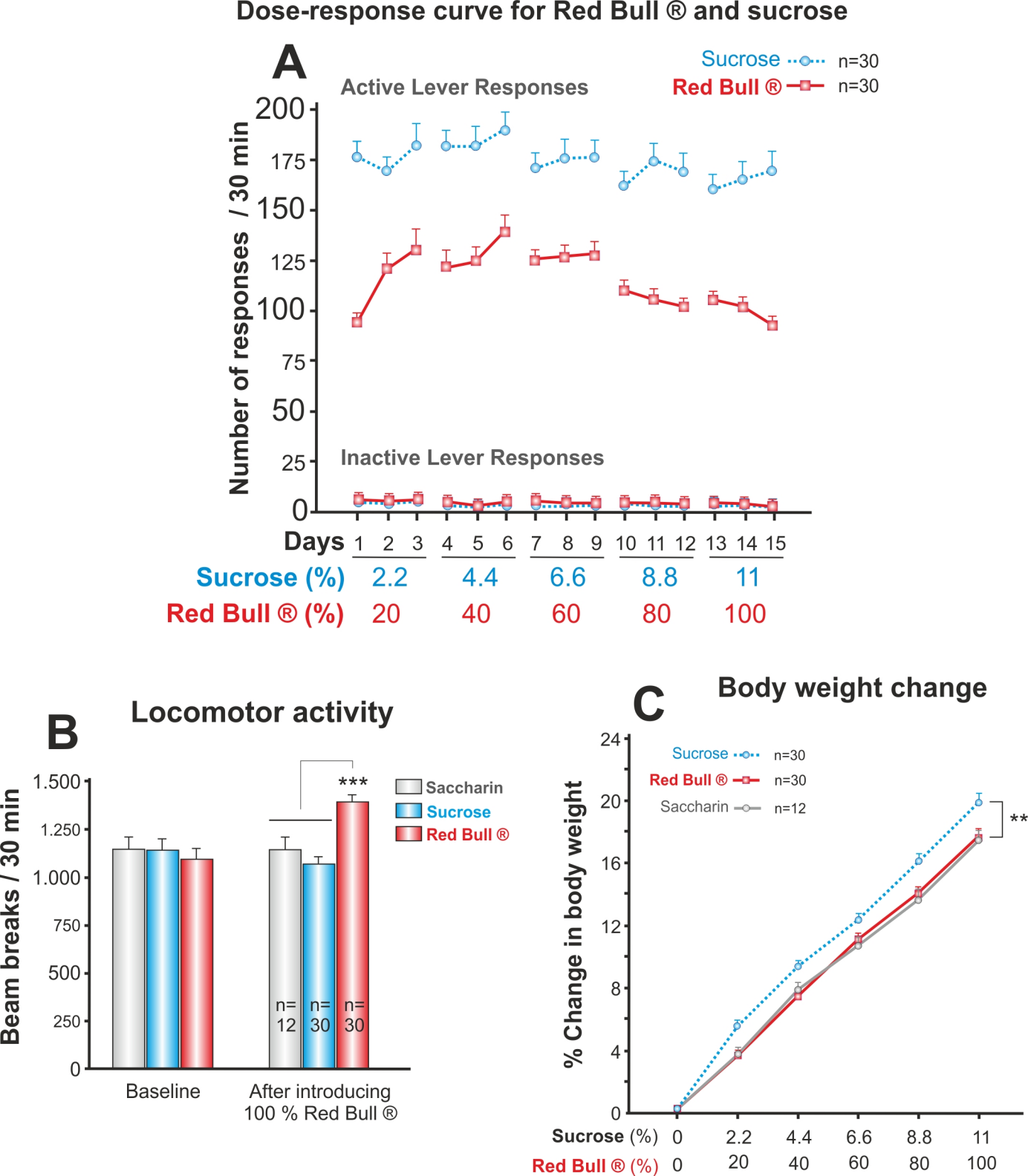
correlations were higher at 90 min after than 5 min after operant self-administration.

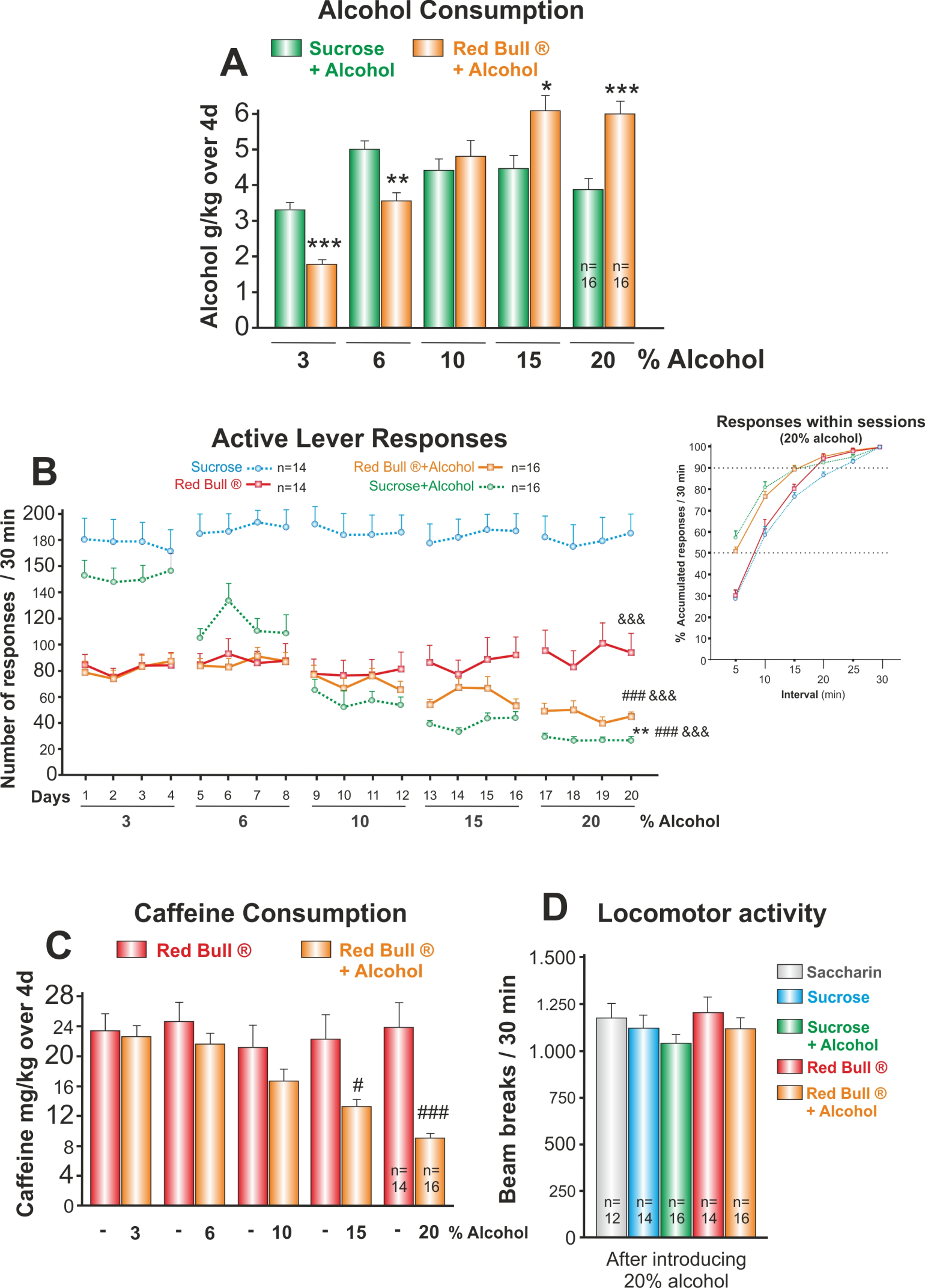
Figure 4. Effects of Red Bull® self-administration on blood alcohol, caffeine, insulin and corticosterone levels

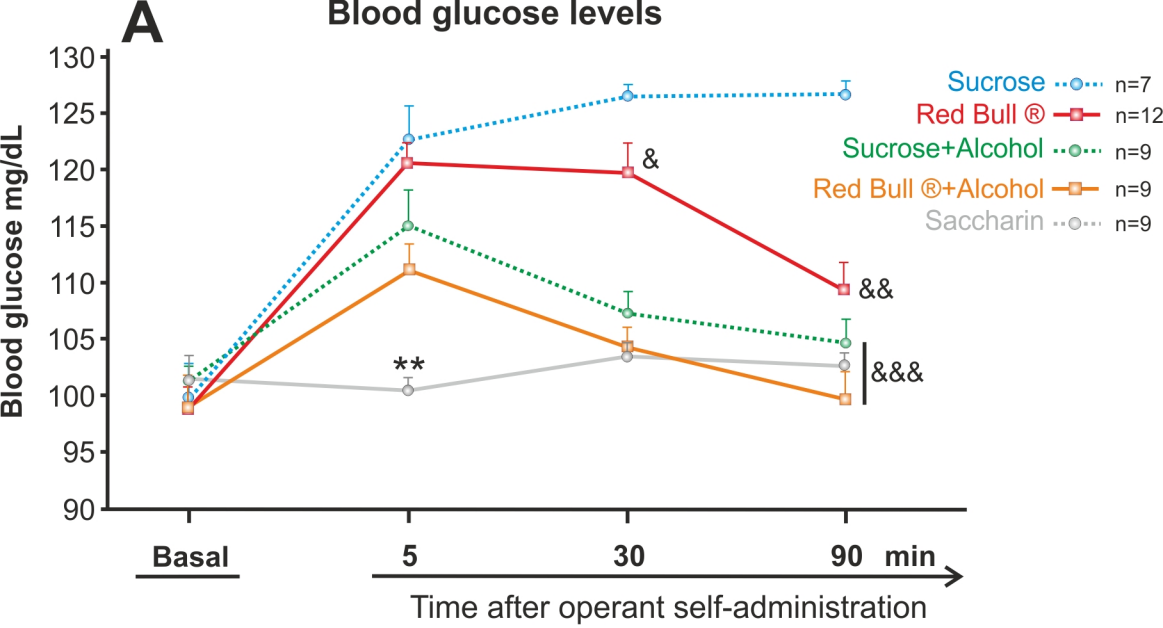
Data represent the mean ± SEM. Blood samples were collected *in vivo* from the rat tail vein immediately after the 30 min operant self-administration session for the analysis of alcohol, caffeine and insulin. Correlations were established using the reinforces obtained for the animals throughout their corresponding session. **(A-C)** Red Bull®+alcohol self-administration resulted in higher blood alcohol levels compared with the sucrose+alcohol, *p < 0.05. There was a significant correlation between the number of reinforces obtained in the 30 min session and blood alcohol levels in both groups. **(D-F)** Blood caffeine levels compared within the two Red Bull® groups, ***p < 0.001. There was a significant correlation between the number of reinforces and blood caffeine levels in both groups. **(G-I)** Self-administration of sucrose and Red Bull® resulted in an increase of blood insulin levels when compared with the saccharin-control group,*p < 0.05, **p < 0.01. However, there were no correlations between the number of reinforces and blood insulin levels. **(J-L)** Blood samples for corticosterone assessment were collected from the trunk body immediately after the sacrifice of the animal. Correlations were established using the mean of the last four sessions of operant self-administration. There were no significant differences between groups in blood corticosterone levels and neither a correlation between the number of reinforces obtained by the animals in the last four sessions and blood cortiscosterone levels.

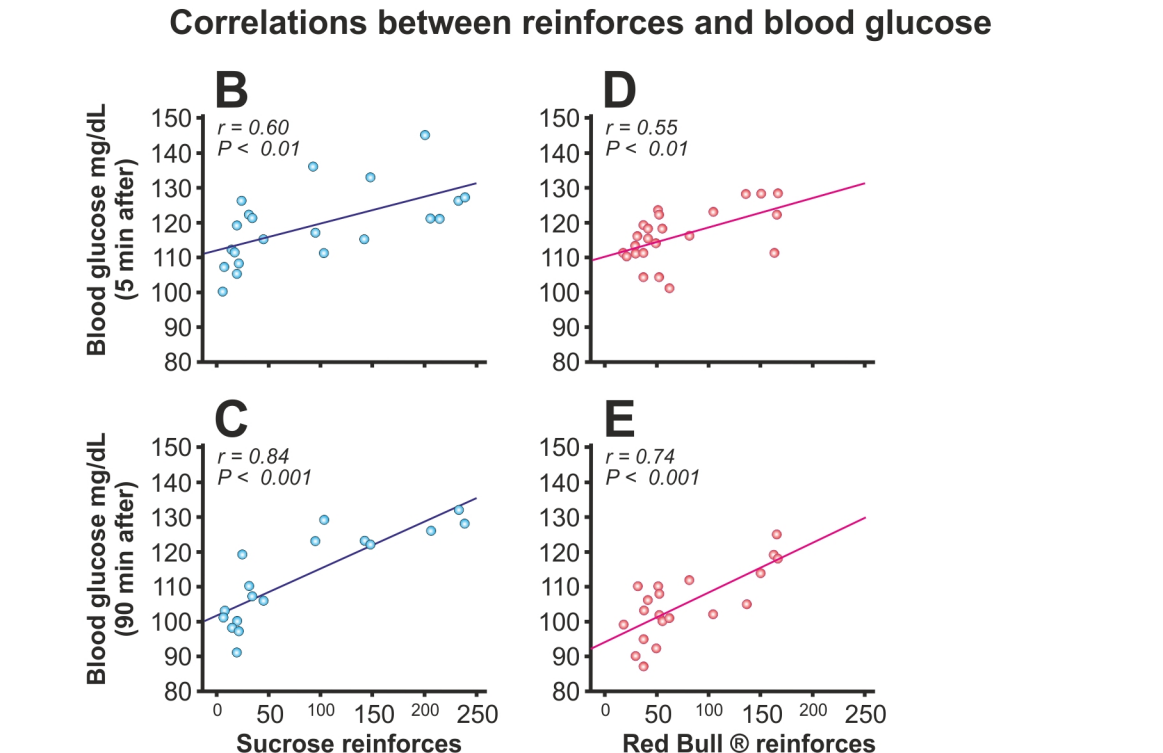
Figure 5. Effects of Red Bull® on alcohol relapse, extinction and reacquisition

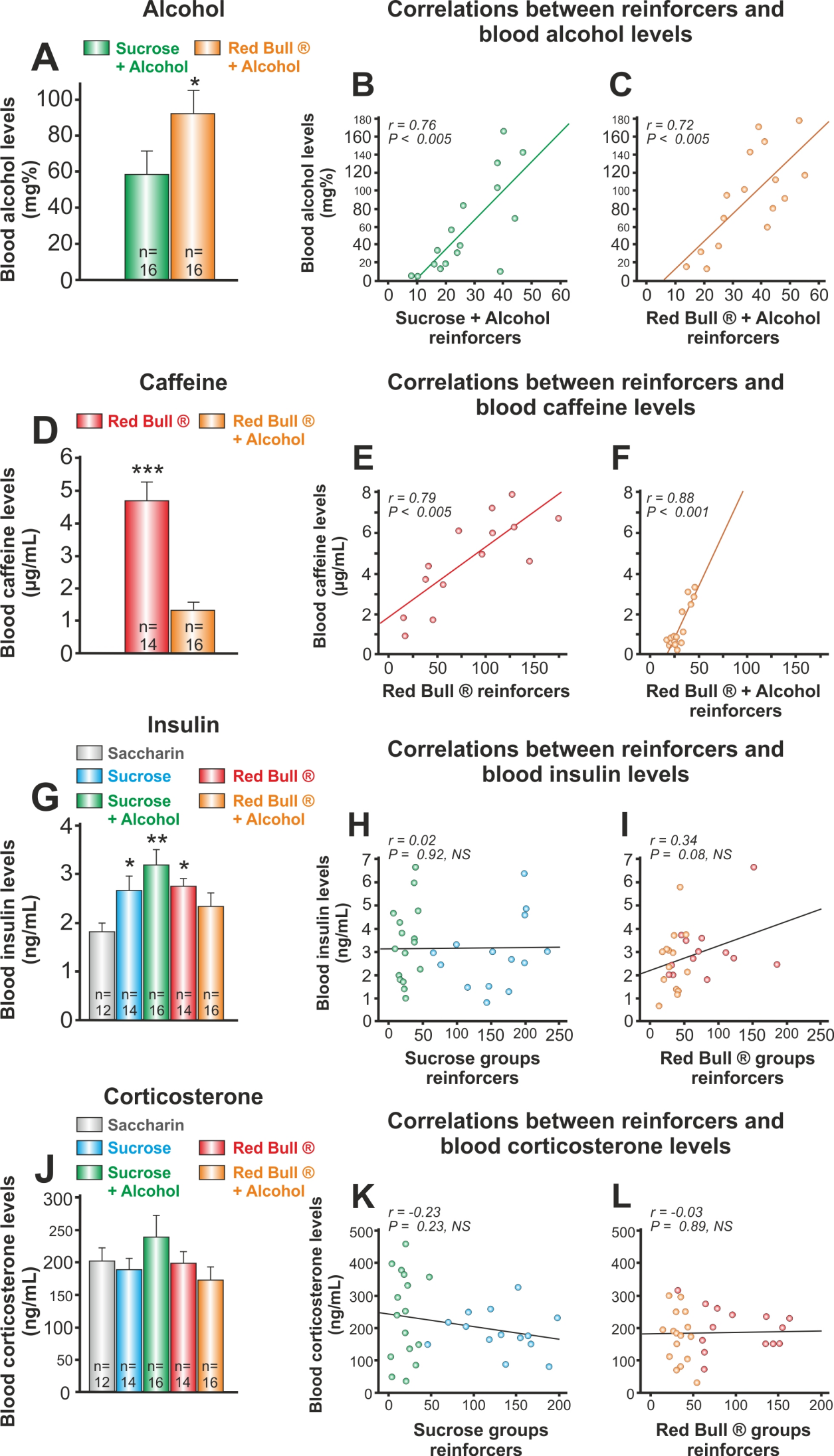
Data represent the mean ± SEM. **(A)** There was not an increase in the operant self-administration of the sucrose solution (11%) or Red Bull® after a seven-day period of deprivation. **(B)** The increase of alcohol self-administration after a seven-day period of deprivation was higher within the group responding for the sucrose+alcohol than within the group responding for the Red Bull®+alcohol mixture. The baseline corresponds to the average of the last five days before to the deprivation period. *p < 0.05, compared with their respective baseline (extended arrow); #p < 0.05, ###p < 0.001 compared between drinking solutions. **(C)** Pattern of responses during the period of extinction and reacquisition responding for sucrose, Red Bull®, sucrose+alcohol or Red Bull®+alcohol. Red Bull® did not cause any alteration in the extinction of alcohol responding and neither in its reacquisition, **p < 0.01, compared with Red Bull®+alcohol; ###p < 0.001 compared with Red Bull®; &&&p < 0.001 compared with sucrose.

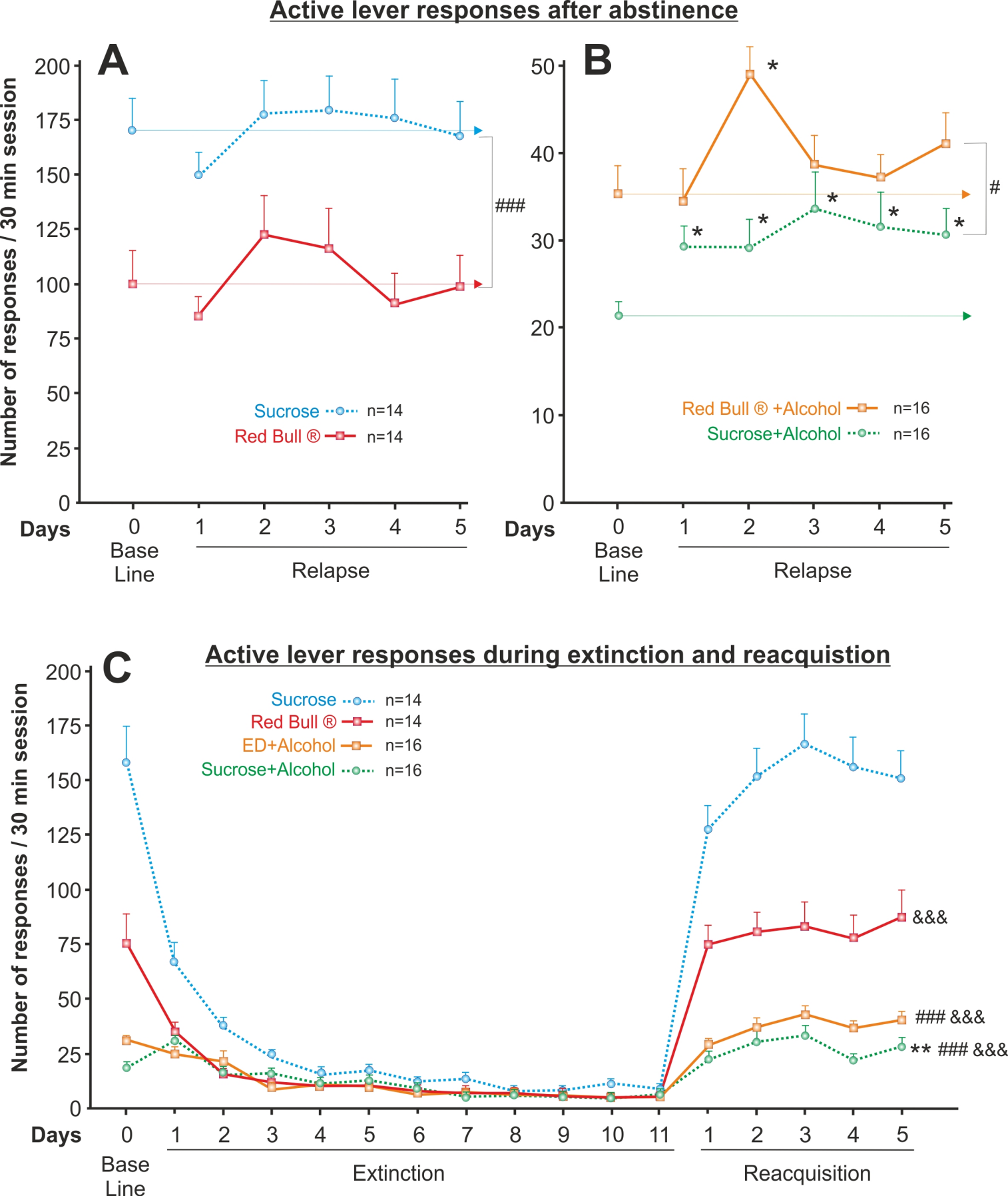

Dose-response curve for Red Bull ® and sucrose
A
Active Lever Responses
Sucrose n=30
Red Bull ® n=30
Number of responses / 30 min
200
175
150
125
100
75
50
25
0
Inactive Lever Responses
Days 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Sucrose (%) 2.2 4.4 6.6 8.8 11
Red Bull ® (%) 20 40 60 80 100
B
Locomotor activity
Beam breaks / 30 min
1.500
1.250
1.000
750
500
250
0
***
Saccharin
Sucrose
Red Bull ®
n= 12
n= 30
n= 30
Baseline
After introducing 100 % Red Bull ®
C
Body weight change
% Change in body weight
24
20
16
12
8
4
0
Sucrose n=30
Red Bull ® n=30
Saccharin n=12
**
Sucrose (%) 0 2.2 4.4 6.6 8.8 11
Red Bull ® (%) 0 20 40 60 80 100


Alcohol Consumption
A
Sucrose + Alcohol
Red Bull ® + Alcohol
Alcohol g/kg over 4d
0
1
2
3
4
5
6
***
**
*
***
n= 16
n= 16
3
6
10
15
20
% Alcohol
Active Lever Responses
B
Sucrose n=14
Red Bull ® n=14
Red Bull ®+Alcohol n=16
Sucrose+Alcohol n=16
Number of responses / 30 min
0
20
40
60
80
100
120
140
150
180
200
&&&
&&&
** ### &&&
Days
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
3
6
10
15
20
% Alcohol
Responses within sessions
(20% alcohol)
% Accumulated responses / 30 min
0
10
20
30
40
50
60
70
80
90
100
5
10
15
20
25
30
Interval (min)
Caffeine Consumption
C
Red Bull ®
Red Bull ® + Alcohol
Caffeine mg/kg over 4d
0
4
8
12
16
20
24
28
#
###
n= 14
n= 16
- 3
- 6
- 10
- 15
- 20
% Alcohol
D
Locomotor activity
Beam breaks / 30 min
0
250
500
750
1.000
1.250
1.500
n= 12
n= 14
n= 16
n= 14
n= 16
After introducing 20% alcohol
Saccharin
Sucrose
Sucrose + Alcohol
Red Bull ®
Red Bull ® + Alcohol


A
Blood glucose levels
Blood glucose mg/dL
130
125
120
115
110
105
100
95
90
Sucrose n=7
Red Bull ® n=12
Sucrose+Alcohol n=9
Red Bull ®+Alcohol n=9
Saccharin n=9
&
&&
&&&
**
Basal
5
30
90 min
Time after operant self-administration


Correlations between reinforces and blood glucose
B
r = 0.60
P < 0.01
D
r = 0.55
P < 0.01
C
r = 0.84
P < 0.001
E
r = 0.74
P < 0.001
Blood glucose mg/dL (5 min after)
Blood glucose mg/dL (90 min after)
150
140
130
120
110
100
90
80
0
50
100
150
200
250
Sucrose reinforces
Red Bull ® reinforces


Alcohol
A
Sucrose + Alcohol
Red Bull ® + Alcohol
Blood alcohol levels (mg%)
n= 16
n= 16
Correlations between reinforcers and blood alcohol levels
B
r = 0.76
P < 0.005
Blood alcohol levels (mg%)
Sucrose + Alcohol reinforcers
C
r = 0.72
P < 0.005
Red Bull ® + Alcohol reinforcers
Caffeine
D
Red Bull ®
Red Bull ® + Alcohol
Blood caffeine levels (µg/mL)
n= 14
n= 16
Correlations between reinforcers and blood caffeine levels
E
r = 0.79
P < 0.005
Blood caffeine levels (µg/mL)
Red Bull ® reinforcers
F
r = 0.88
P < 0.001
Red Bull ® + Alcohol reinforcers
Insulin
G
Saccharin
Sucrose
Red Bull ®
Sucrose + Alcohol
Red Bull ® + Alcohol
Blood insulin levels (ng/mL)
n= 12
n= 14
n= 16
n= 14
n= 16
Correlations between reinforcers and blood insulin levels
H
r = 0.02
P = 0.92, NS
Blood insulin levels (ng/mL)
Sucrose groups reinforcers
I
r = 0.34
P = 0.08, NS
Red Bull ® groups reinforcers
Corticosterone
J
Saccharin
Sucrose
Red Bull ®
Sucrose + Alcohol
Red Bull ® + Alcohol
Blood corticosterone levels (ng/mL)
n= 12
n= 14
n= 16
n= 14
n= 16
Correlations between reinforcers and blood corticosterone levels
K
r = -0.23
P = 0.23, NS
Blood corticosterone levels (ng/mL)
Sucrose groups reinforcers
L
r = -0.03
P = 0.89, NS
Red Bull ® groups reinforcers


Active lever responses after abstinence
A
Number of responses / 30 min session
200
175
150
125
100
75
50
25
0
Sucrose n=14
Red Bull ® n=14
###
Days
0
Base Line
1 2 3 4 5
Relapse
B
50
40
30
20
10
0
*
#
Red Bull ® +Alcohol n=16
Sucrose+Alcohol n=16
Days
0
Base Line
1 2 3 4 5
Relapse
C
Active lever responses during extinction and reacquistion
Sucrose n=14
Red Bull ® n=14
ED+Alcohol n=16
Sucrose+Alcohol n=16
&&&
&&&
** ### &&&
Days
0
Base Line
1 2 3 4 5 6 7 8 9 10 11
Extinction
1 2 3 4 5
Reacquisition