Hypothalamic-pituitary-adrenal axis dysregulation initiated by a binge drinking pattern, but not by acute alcohol intake, in female and male adolescents

Desregulación del eje hipotalámico-pituitario-adrenal iniciada por un patrón binge drinking*, pero no por el consumo agudo de alcohol, en mujeres y hombres adolescentes*

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Abstract

Excessive alcohol consumption is a worldwide public health problem, being adolescents and young adults the population most affected by this problem. The aim of this study was to clarify the effects of having a history of binge drinking (BD) and/or acute alcohol consumption on the stress response in female and male adolescents. Participants were 235 adolescents (143 females and 92 males). Cortisol, systolic and diastolic blood pressure (SBP and DBP), heart rate (HR) and perceived stress (PS) were evaluated in adolescents with different and similar blood alcohol concentrations (BAC). In Experiment 1, the effects of alcohol were studied separately in females and males because of differences in BAC. In Experiment 2, a direct comparison between sexes was carried out in a counterbalanced selection of participants with similar BAC. In Experiment 1, females receiving alcohol showed an increase in cortisol and HR, and binge drinkers displayed higher HR than refrainers. Male refrainers receiving alcohol showed higher HR, and binge drinkers showed higher cortisol and SBP than refrainers. In Experiment 2, similar results were observed and sex differences were evident, with males showing higher cortisol and SBP, and lower PS than females. In conclusion, the normal response of the adolescent HPA axis to alcohol consumption is an increase in cortisol levels in females, as well as HR in both sexes. In addition, a history of BD is associated with HPA axis dysregulation, which is manifested by higher values of cortisol (independently of sex), SBP in male and HR in female healthy adolescents.

Key words: Binge drinking; alcohol; stress response; adrenal-pituitary-hypothalamic axis; adolescents.

Resumen

El consumo excesivo de alcohol es un problema de salud pública mundial, siendo los adolescentes y jóvenes adultos la población más afectada. El objetivo de este estudio fue esclarecer los efectos de una historia *binge drinking* (BD) y/o del consumo agudo de alcohol sobre la respuesta de estrés en mujeres y hombres. Participaron 235 adolescentes (143 mujeres y 92 hombres). Se evaluaron cortisol, presión arterial sistólica y diastólica (PAS y PAD), frecuencia cardiaca (FC) y estrés percibido (EP). En el experimento 1, los efectos del alcohol fueron estudiados separadamente en mujeres y hombres debido a diferencias en la concentración de alcohol en sangre (CAS). En el experimento 2, una selección balanceada de mujeres y hombres con similar CAS permitió su comparación. En el experimento 1, las mujeres que recibieron alcohol mostraron un incremento de cortisol y FC, y las *binge drinkers* mostraron mayor FC que las abstemias. Los hombres abstemios que recibieron alcohol mostraron mayor FC y los *binge drinkers* tuvieron niveles más altos de cortisol y PAS que los abstemios. En el experimento 2, se observaron resultados similares y diferencias de sexo, mostrando los hombres niveles más altos de cortisol y PAS, y menos EP que las mujeres. En conclusión, la respuesta normal del eje HPA adolescente al consumo de alcohol refleja un incremento de cortisol en mujeres, así como de FC en ambos sexos. Además, una historia de consumo BD está asociada con una desregulación del eje HPA, manifestado con niveles más altos de cortisol (independientemente de sexo), PAS en varones y FC en mujeres, adolescentes sanos.

Palabras clave: Consumo intensivo de alcohol; alcohol; respuesta de estrés; eje hipotalámico-pituitario-adrenal; adolescentes.

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Excessive alcohol consumption is a worldwide public health problem, and adolescents and young adults are those most affected by this problem in Spain and other countries. Most of this population develop a pattern of alcohol consumption known as binge drinking (BD), which has been defined by the National Institute on Alcohol Abuse and Alcoholism (NIAAA, 2004) as a drinking pattern in which the blood alcohol concentration (BAC) rises to 0.8 g/L or higher. This consumption pattern consists of the ingestion of large amounts of alcohol in a short period of time (two hours), followed by a period of abstinence that can vary from one week to a month (Parada et al., 2011; Vinader-Caerols & Monleón, 2019). The prevalence of BD in Spain is high: 32.3% of Spanish students from 14 to 18 years old admitted to this type of consumption in the previous 30 days at the time of completing a recent survey (Observatorio Español de las Drogas y las Adicciones, 2020). This pattern is commonly associated with acute impairment of motor coordination and cognitive functioning, and its continuation during adolescence predicts an atypical cortisol stress response in young adulthood (Hagan et al., 2019).

Although alcohol is often consumed to alleviate stress (Keyes, Hatzenbuehler, Grant & Hasin, 2012), it activates some brain stress systems and can be a stressor itself (Becker, 2017). Study of the interactions between biological stress systems and alcohol consumption has focused on the hypothalamic-pituitary-adrenal (HPA) axis (Weera & Gilpin, 2019) and the main stress hormone, cortisol (Hellhammer & Schubert, 2012), which is used as a biochemical marker in stress research (González-Cabrera, Fernández-Prada, Iribar-Ibabe & Peinado, 2014). In light of this, there is a need for studies that evaluate HPA axis dysfunction, since this is the main mediator of the short-term effects of alcohol on the body and a potential pathway by which alcohol exerts long-term effects on biological systems (Hagan et al., 2019).

Growing evidence suggests that alcohol directly stimulates the HPA axis and has effects on glucocorticoid receptors in the extra-hypothalamic, limbic forebrain and medial prefrontal cortex circuits that contribute to the development of alcohol use disorders (AUDs) and their progression, chronicity, and relapse risk (Blaine & Sinha, 2017). The acute effect of alcohol on stress hormones has been studied through the voluntary consumption of alcohol by humans and animals, which increases cortisol and corticosterone (King, Munisamy, de Wit & Lin, 2006; Lu & Richardson, 2014). Acute alcohol has a stimulating effect on the HPA axis, resulting in an initial increase in cortisol output in adolescents (Magrys, Olmstead, Wynne-Edwards & Balodis, 2013). The literature also shows that BD during adolescence predicts an atypical cortisol stress response in young adulthood: in the short term, subjects with a history of BD exhibit significantly higher basal levels of cortisol, indicating an over-regulated HPA axis (Wemm et al., 2013); in contrast, in the long term, BD during adolescence is associated with a significantly lower cortisol stress response in young adulthood (Hagan et al., 2019). Studies evaluating the relationship between acute alcohol consumption and history of BD have associated BACs below 0.8 g/L in consumers with a history of low-to-moderate drinking (not binge drinkers) with an increased response of the HPA axis (higher blood cortisol levels); however, a reduced HPA axis response has been related to these BACs when subjects are binge drinkers (Allen, Lee, Koob & Rivier, 2011; Blaine & Sinha, 2017; Waltman, Blevins, Boyd & Wand, 1993; Zimmermann et al., 2004).

Cortisol also facilitates an increase in cardiovascular activity, raising the heart rate and blood pressure (Hagan et al., 2019; Ulrich-Lai & Herman, 2009). Several studies have demonstrated that an acute intake of alcohol (Bau et al., 2011; Sher, Bartholow, Peuser, Erickson & Wood, 2007; Vinader-Caerols, Monleón, Carrasco & Parra, 2012) or a sustained BD pattern (King, Houle, de Wit, Holdstock & Schuster, 2002) increases heart rate (HR) in young adults. It has also been noted that frequent BD during adolescence can increase the risk of developing high blood pressure (BP) in young adults, regardless of other risk factors such as a smoking habit or obesity (Hayibor, Zhang & Duncan, 2019).

Perceived stress has been associated with the risk of alcohol misuse in adolescents and young adults (Tavolacci et al., 2013). In line with this, students with a marked BD pattern of alcohol consumption have been found to score almost twice as high as the general adult population on the stress perception scale (Bidwal, Ip, Shah & Serino, 2015).

Experimental results of research performed with only one sex are sometimes extrapolated to both sexes. Sex should be considered an important biological variable in basic and preclinical research before results are applied to both men and women (Lee, 2018). Thus, we decided to compare the sexes with respect to the effects of alcohol on the HPA axis according to the history of BD. With this goal in mind, two experiments were designed: the first one analysed separately females and males with different BAC; and the second one evaluated a selection of females and males with similar BAC, which allowed the effect of gender to be studied.

Taking into consideration the aforementioned published evidence, there is a need to systematically examine the effects of acute alcohol consumption on the HPA axis and autonomic arousal. Such experimental studies in the adolescent population are scarce, which is why the main objective of this work was to clarify the effects of a history of BD and/or acute alcohol consumption on the functionality of the HPA axis (cortisol) and other variables of the stress response (HR, BP and PS) in female and male adolescents. Another goal was to provide further evidence about the changes triggered in the HPA axis and

the stress response in healthy adolescents by BD when initiated from an early age, specifically cortisol levels, HR, BP and PS. These changes are of great importance, as they can contribute to the transition from BD to the compulsive loss of control over alcohol intake seen in severe AUDs. For the mentioned goals, a sample of binge drinkers (with a history of BD) and non-binge drinkers (refrainers and occasional consumers without history of BD) was selected and they received a control drink or an alcoholic drink as experimental conditions.

We have recreated the conditions in which BD usually occurs –i.e., a risk alcohol consumption (38.4 g) in a short time (20 min)– and have measured the functionality of the HPA axis (cortisol levels). We expected to see an increase in cortisol levels, HR, BP and PS in the female and male adolescents without a history of BD, due the natural reaction of the stress response after acute alcohol intake. On the other hand, the participants with a history of BD only (no acute alcohol intake) would have higher basal levels of the variables evaluated, due to over-regulation of the HPA axis. Finally, in accordance with the literature, we assumed that our participants with a one-year history of BD would display lower values (cortisol levels, HR, BP and PS) after acute alcohol consumption.

Method

Participants

Two hundred and thirty-five healthy 18-19-year-old adolescent students (143 females and 92 males) at the University of Valencia (Spain) participated in this study. The volunteers were recruited by means of a self-report of their alcohol consumption habits and general health. Participants were classified as refrainers if they had never previously consumed alcoholic drinks (pure refrainers) or had consumed very sporadically (occasional consumers) (see Table 1); or as consumers of alcohol with a BD pattern according to the NIAAA criteria for Spain (López-Caneda et al., 2014) if they had drunk six or more SDUs (standard drink unit) of distilled spirits (alcohol content ≥ 40% vol.), according to the BD habits referred by the subjects, in a row in the case of males and five or more SDUs in a row in the case of females on a minimum of three occasions per month during the previous 12 months (Vinader-Caerols & Monleón, 2019).

Strict inclusion/exclusion criteria were applied to the sample selection. The following inclusion criteria were applied: age 18–19 years old; a healthy body mass index (mean of 22.88 ± 0.30 in males and 21.44 ± 0.23 in females) and good health (reporting a state of emotional and physical well-being, without major medical problems or diagnosed pathology). The exclusion criteria were as follows: taking medication; a history of mental disorders (diagnosed by a health professional according to DSM criteria); an irregular sleep pattern (non-restorative sleep and/or an irregular schedule); having consumed, even sporadically, any drug (apart from alcohol or tobacco) or having a history of substance abuse, including caffeine (our criterion: ≤ 2 stimulant drinks/day), tobacco (our criterion: ≤ 10 cigarettes/day) or alcohol; having suffered from an intense stressful event within a year of the experiment and having first degree relatives with history of alcoholism. A telephone interview of approximately 15 min was conducted with each subject to confirm the information provided in the self-report and to arrange the date and time of the test. Participants were told to follow their normal sleep pattern and their usual meal routine, and have lunch at one hour before the experimental session.

The data of the female participants' menstrual cycle were registered in the self-report and telephone interview, and the subject's cycle phase was considered in the test to counterbalance this variable in each group, checking that the number of females in each cycle phase was similar in every group. No females taking contraceptives were included in the study.

Tests and Apparatus

The activity of the HPA-axis was measured by analysing salivary cortisol levels using a competitive solid phase radioimmunoassay test. Salivette® was employed as a hygienic method of collecting saliva by means of a synthetic swab specially designed for cortisol determination. Three saliva samples were collected from all our subjects: one prior to consumption (COR0'), a second 20 min (COR20') after drink intake, and a third 50 min (COR50') after intake, considering that high levels of BAC are observed at the 20-50 min interval (Vinader-Caerols, Talk, Montañés, Duque & Monleón, 2017a; Vinader-Caerols, Duque, Montañés & Monleón 2017b). The samples were frozen at −18°C until they were sent to the laboratory for analysis by a competitive solid phase radioimmunoassay (tube coated) with the commercial kit Coat-A-Count C (DPC, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). Assay sensitivity was 0.5 ng/mL (1.38 nmol/L). Data were expressed in nanomolar units (nmol/L). All the samples from each participant were analysed in the same trial; the within and inter assay variation coefficients were all below 5.5%. Salivary cortisol levels were determined at Echevarne Analysis Laboratory, Valencia (Spain).

The Alcohol Use Disorders Identification Test (AUDIT) (Saunders, Aasland, Babor, de La Fuente & Grant, 1993) was employed to measure a problematic use of alcohol among the subjects. The AUDIT consists of 10 questions that evaluate the quantity and frequency of alcohol intake and alcohol-related behaviours and consequences. It uses a range of 0-40 points, in which a score of 8 or more indicates a problematic use of alcohol. A higher score is related to a greater severity of problematic drinking.

A digital automatic blood pressure monitor (M10-IT, OMRON, Spain) was employed to measure systolic BP (SBP), diastolic BP (DBP) and HR in all the subjects.

Participants were assessed by means of the Perceived Stress Scale (PSS-14) (Cohen, Kamarck & Mermelstein, 1983), a standardized self-report questionnaire composed of 14 items designed to measure stress and which evaluate how unpredictable, uncontrollable and overloaded respondents consider their lives to have been in the previous month. A higher direct score on the PSS-14 indicates a higher level of PS. All subjects completed the standardized Spanish version of PSS-14. The internal consistency of this scale was calculated for our data, obtaining a Cronbach's alfa coefficient of .836 for females and .860 for males in Experiment 1, and .861 in Experiment 2 (females and males together).

An alcoholmeter (Alcoquant® 6020, Envitec, Germany) was employed to measure BAC of each participant before and after (20 min and 50 min) intake of a beverage (control drink or alcoholic drink).

Procedure

The experimental procedure was approved by the Research Ethics Committee of the University of Valencia (Certification number: H1485172642673; approved on July 7th, 2017), and was in accordance with the Helsinki Agreement. All participants provided written informed consent to take part in the study. According to their drinking pattern (refrainers and binge drinkers) and the beverage received (control drink or alcoholic drink), subjects were assigned to one of four experimental conditions: Refrainers-Control (R-Co) (pure refrainers-refreshment intake); Refrainers-Alcohol (R-A) (occasional consumers-alcoholic drink intake); Binge Drinkers-Control (BD-Co) (subjects with a history of BD-refreshment intake) or Binge Drinkers-Alcohol (BD-A) (subjects with a history of BD-alcoholic drink intake).

Participants were instructed to abstain from any intake of alcohol, caffeinated beverages, drugs or medication and strenuous exercise for 24 h before the experimental session, and to refrain from eating and smoking at least 1 h prior to the session. At the beginning of the session BAC was measured in all subjects using the alcoholmeter to ensure that they had not consumed alcohol. In addition, a problematic use of alcohol among the BD subjects was assessed using the AUDIT test (mean score: 6.72 ± 0.33 in females and 7.69 ± 0.47 in males). None of the subjects was found to be alcohol-dependent. All the participants provided a first saliva sample for cortisol determination (COR0') just before the intake. Each subject received a flavoured refreshment (lime, orange or cola, without caffeine) contained in cans of 330 ml, alone or mixed (according to the experimental group) with distilled drinks with an alcohol content of 40% vol. (vodka or gin) in risk doses of 120 ml (equivalent to 38.4 g of alcohol). The subjects were instructed to consume their drink within a period of 20 min, during which they ate a light snack (the same for all participants) and the beverages were always consumed in the presence of a research assistant. After finishing the drink, all subjects rinsed their mouths with water and underwent a 20-min waiting period.

After this 20-min waiting interval, the second saliva sample was collected for cortisol determination (COR20') and BAC was measured in all the subjects. Subsequently, we measured SBP, DBP and HR (all were recorded 3 times and their average calculated) and perception of stress by means of the PSS-14. Finally, the participants provided a third saliva sample (COR50') after which BAC was measured once again. The duration of the experimental protocol was around 2 hours and all measurements were performed between 16:00 and 18:00h, during descendent BAC. Members of the groups that received alcohol remained on the premises until their alcohol concentration dropped to legal limits for driving.

The BAC was 0.00 g/L for females and males before the alcoholic drink, and the mean was 0.53 ± 0.12 g/L for females and 0.34 ± 0.01 g/L for males after drinking. It is important to point out that, although all subjects consumed the same amount of alcohol, the statistical differences in BAC between females and males did not allow a direct comparison between sexes; however, it was possible to study the effects of alcohol within each sex (Experiment 1). A counterbalanced selection of females and males with similar BAC 0.38 ± 0.01 g/L was carried out to study the gender factor (Experiment 2). A summary of the characteristics of the study population (represented in its entirety in Experiment 1) is presented in Table 1.

Statistical Analyses

Data for females and males were analysed separately, as BAC was statistically different in the two groups (Experiment 1), and together when BAC was similar (Experiment 2). The data were subjected to parametric analysis after confirming they met the criteria for normality and homogeneity of variances. Statistically significant differences were established at $p < .05$ and the statistical power was calculated using alpha = 0.05. A repeated measures ANOVA was performed for cortisol COR20' and COR50' (COR0' measure was not included in this ANOVA because the first register of cortisol was taken before treatment –alcohol or control drink– was administered). A one-way ANOVA was performed for each measure of stress response (SBP, DBP, HR and PS) for females and males, separately (Experiment 1) or together (Experiment 2). Each analysis contained the between-subject factors 'Drinking Pattern' (refrainers and binge drinkers) and 'Treatment' (control drink and alcohol) as independent variables. Factor 'Sex' was also included as a third between-

Table 1. *Characteristics of the study population (Experiment 1).*

	Refrainers (occasional consumers) (n = 38)			Binge Drinkers (n = 130)		
	Females (n = 21)	**Males** (n = 17)	**Females + Males** (n = 38)	**Females** (n = 81)	**Males** (n = 49)	**Females + Males** (n = 130)
Age at first alcohol consumption	15.809 ± 0.255	16.058 ± 0.326	15.921 ± 0.185	14.654 ± 0.125&&	14.714 ± 0.157&&	14.676 ± 0.097++
Mean number of occasions per month	0.565 ± 0.146	0.482 ± 0.155	0.528 ± 0.097	2.666 ± 0.084&&	2.612 ± 0.119&&	2.646 ± 0.068++
Mean number of drinks per occasion	2.952 ± 0.381	2.0 ± 0.332	2.562 ± 0.246	6.259 ± 0.232&&	7.265 ± 0.325&&	6.638 ± 0.194++
Mean number of BD episodes per month	NA	NA	NA	2.666 ± 0.084	2.612 ± 0.119	2.646 ± 0.068
Mean duration of BD pattern (in months) until the beginning of experiment	NA	NA	NA	11.333 ± 0.262	11.326 ± 0.267	11.330 ± 0.084

	Refrainers (pure refrainers + occasional consumers) (n = 105)			Binge Drinkers (n = 130)		
	Females (n = 62)	**Males** (n = 43)	**Females + Males** (n = 105)	**Females** (n = 81)	**Males** (n = 49)	**Females + Males** (n = 130)
Mean number of stimulant drinks: Coke, tea, or coffee / day	1.048 ± 0.160	0.953 ± 0.188	1.009 ± 0.121	0.950 ± 0.100	0.714 ± 0.148	0.861 ± 0.084
Smoker: no / yes	62/0	43/0	105/0	61/20	42/7	83/27
Stressful event in the last year: no / yes	47/15	38/5	85/20	63/18	44/5	87/23
Nervous: no / yes	45/17	34/8	79/25	73/8	42/5	97/13
Good Sleep: no / yes	6/56	6/37	12/93	9/72	4/45	13/97
Sports activity: no / yes	53/9	39/4	92/13	66/15	36/13	82/28
Breakfast: no / yes	9/53	7/36	16/89	13/68	5/44	18/92

The results are expressed as number or mean ± SEM for Refrainers and Binge Drinkers. NA: not applicable.
&& $p < .001$ *vs* occasional consumers in the same sex. ++ $p < .001$ *vs* occasional consumers.

subject factor in Experiment 2. When any interaction between these factors was statistically significant, pairwise comparisons were carried out by Student's t-test. All correlations of measures of stress response registered at 20 minutes of treatment were explored. All analyses were performed using the 'SPSS' Statistics software package, version 26 for Windows (IBM, 2019).

Results

Experiment 1: Effects of a risk dose of alcohol on cortisol, BP, HR, and PS in female (BAC: 0.53 ± 0.12 g/L) and male (BAC: 0.34 ± 0.01 g/L) adolescents with or without a history of BD.

A summary of descriptive statistics and results for the main factors in stress responses in Experiment 1 is presented in Table 2.

Cortisol (COR0', COR20' and COR50')

The factor Drinking Pattern in COR0' was not statistically significant, neither in females ($F_{(1,79)} = 0.122$, $p = .727$) nor in males ($F_{(1,53)} = 2.308$, $p = .135$).

COR20' and COR50': The repeated measures ANOVA showed that Cortisol was statistically significant in females ($F_{(1,77)} = 16.842$, $p = .001$) and males ($F_{(1,51)} = 32.155$, $p = .001$), showing a significant decrease of COR50' with respect to COR20' in both females and males (Figure 1A). The interactions Cortisol X Drinking Pattern, Cortisol X Treatment, and Cortisol X Drinking Pattern X Treatment were not statistically significant, neither in females ($F_{(1,77)} = 0.790$, $p = .377$), ($F_{(1,77)} = 0.472$, $p = .494$), ($F_{(1,77)} = 0.014$, $p = .907$), respectively; nor in males ($F_{(1,51)} = 1.313$, $p = .257$), ($F_{(1,51)} = 0.823$, $p = .369$), ($F_{(1,51)} = 0.551$, $p = .461$), respectively.

The main factor Drinking Pattern in females was not found to be statistically significant ($F_{(1,77)} = 0.629$, $p = .430$), while the factor Treatment was statistically significant ($F_{(1,77)} = 6.222$, $p = .015$), with higher cortisol levels detected in the alcohol groups (see Table 2). The interaction Drinking Pattern X Treatment was not statistically significant ($F_{(1,77)} = 0.739$, $p = .393$).

The main factor Drinking Pattern in males was statistically significant ($F_{(1,51)} = 4.071$, $p = .049$), with binge drinkers showing higher cortisol levels than refrainers (see Table 2); while Treatment was not statistically significant ($F_{(1,51)} = 2.151$, $p = .149$). The interaction Drinking Pattern X Treatment was not significant ($F_{(1,51)} = 0.932$, $p = .339$).

Blood Pressure (SBP and DBP)

SBP: In females, neither the main factors –Drinking Pattern ($F_{(1,139)} = 1.379$, $p = .242$) and Treatment ($F_{(1,139)} = 0.993$, $p = .321$)– nor their interaction ($F_{(1,139)} = 1.491$, $p =$

Table 2. *Summary of descriptive statistics and results for COR, SBP, DBP, HR and PS (Experiment 1).*

	FEMALES (BAC: 0.53 ± 0.12 g/L)					
	Drinking Pattern			Treatment		
	Refrainers	Binge Drinkers	Statistical Power[1]	Control	Alcohol	Statistical Power[1]
COR (n = 81)	4.59 ± 0.34	4.77 ± 0.37	.982	4.18 ± 0.29	5.45 ± 0.42+	.982
SBP (n = 143)	106.45 ± 1.32	104.49 ± 1.09	.219	104.58 ± 1.14	106.24 ± 1.25	.137
DBP (n = 143)	68.96 ± 0.97	68.14 ± 0.85	.068	68.78 ± 0.81	68.16 ± 0.94	.088
HR (n = 138)	72.36 ± 1.20	77.05 ± 1.11#	.656	72.93 ± 1.01	77.55 ± 1.33*	.659
PS (n = 137)	24.67 ± 0.93	24.08 ± 0.86	.109	23.26 ± 0.83	25.50 ± 0.94	.409
	MALES (BAC: 0.34 ± 0.01 g/L)					
	Drinking Pattern			Treatment		
	Refrainers	Binge Drinkers	Statistical Power[1]	Control	Alcohol	Statistical Power[1]
COR (n = 55)	5.89 ± 0.54	7.88 ± 0.84$	1.000	7.37 ± 0.57	6.11 ± 0.85	1.000
SBP (n = 86)	116.90 ± 1.71	122.39 ± 1.60&	.314	118.87 ± 1.81	121.00 ± 1.51	.069
DBP (n = 86)	67.32 ± 1.37	71.06 ± 1.37	.424	68.00 ± 1.45	70.92 ± 1.59	.218
HR (n = 89)	74.04 ± 1.82	71.25 ± 1.42	.332	72.04 ± 1.49	73.15 ± 1.77	.080
PS (n = 92)	18.39 ± 1.15	20.00 ± 1.15	.122	18.20 ± 1.05	20.50 ± 1.27	.148

Notes. Salivary cortisol concentrations (COR). Systolic (SBP) and Diastolic (DBP) Blood Pressure. Heart Rate (HR). Stress Perception (PS). Not applicable (NA). The results are expressed as mean ± SEM. [1]The Statistical Power is calculated using alpha = 0.05. Females: + $p < .05$ increase of cortisol in Alcohol group *vs* Control group. # $p < .05$ increase of HR in Binge Drinkers *vs* Refrainers. * $p < .05$ increase of HR in Alcohol group *vs* Control group. Males: $ $p < .05$ increase of cortisol in Binge Drinkers *vs* Refrainers. & $p < .05$ increase of SBP in Binge Drinkers *vs* Refrainers.

.224) were statistically significant. In males, Drinking Pattern was statistically significant ($F_{(1,82)} = 4.892$, $p = .03$), with binge drinkers displaying higher SBP than refrainers (see Table 2). Neither the factor Treatment nor the interaction Drinking Pattern X Treatment were statistically significant ($F_{(1,82)} = 0.660$, $p = .419$; $F_{(1,82)} = 0.256$, $p = .615$; respectively).

DBP: In females, neither the main factors –Drinking Pattern ($F_{(1,139)} = 0.165$, $p = .685$) and Treatment ($F_{(1,139)} = 0.253$, $p = .616$)– nor their interaction ($F_{(1,139)} = 0.729$, $p = .395$) were statistically significant. In males, Drinking Pattern was not statistically significant ($F_{(1,82)} = 3.143$, $p = .08$), and neither were the factor Treatment or the interaction Drinking Pattern X Treatment ($F_{(1,82)} = 2.062$, $p = .155$; $F_{(1,82)} = 0.323$, $p = .572$; respectively).

Heart Rate (HR)

In females, the factor Drinking Pattern was statistically significant ($F_{(1,134)} = 5.625$, $p = .019$), with binge drinkers showing higher HR than refrainers (see Table 2). The factor Treatment was also statistically significant ($F_{(1,134)} = 5.588$, $p = .02$) among females, with those who drank alcohol presenting higher HR (see Table 2). The interaction Drinking Pattern X Treatment was not statistically significant ($F_{(1,134)} = 0.016$, $p = .898$). In males, neither Drinking Pattern ($F_{(1,85)} = 2.319$, $p = .132$) nor Treatment ($F_{(1,85)} = 0.605$, $p = .439$) were statistically significant, while the interaction Drinking Pattern X Treatment was ($F_{(1,85)} = 4.877$, $p = .03$), with refrainers receiving alcohol presenting a higher HR than all the other groups (R-Co, BD-Co and BD-A) (Figure 2).

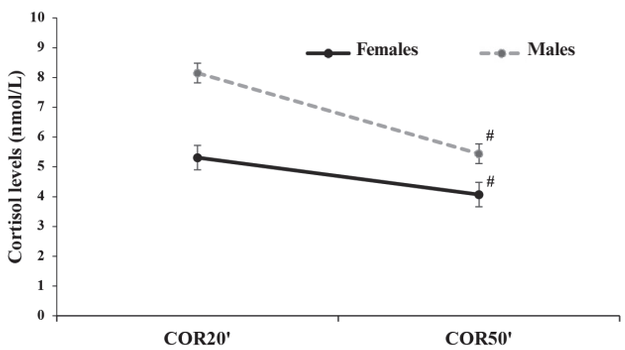

Figure 1A. Salivary cortisol concentrations mean at 20 minutes (COR20') and 50 minutes (COR50') after treatment in females and males separately (Experiment 1). # $p < .05$ *vs* COR20'.

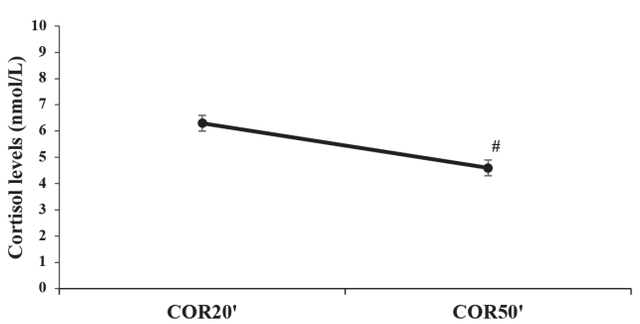

Figure 1B. Salivary cortisol concentrations mean at 20 minutes (COR20') and 50 minutes (COR50') after treatment in females and males together (Experiment 2). # $p < .05$ *vs* COR20'.

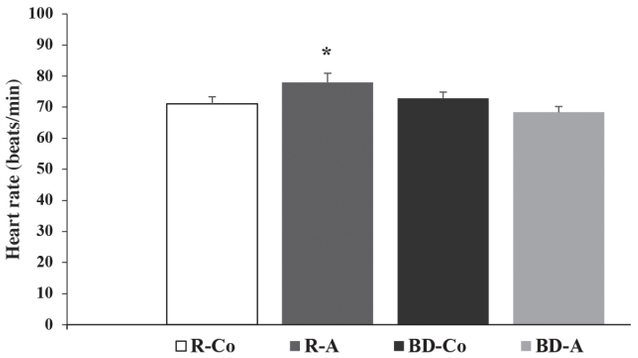

Figure 2. Heart Rate (HR) in males in Experiment 1. Values are expressed as means (+ SEM). R-Co: Refrainers-Control; R-A: Refrainers-Alcohol; BD-Co: Binge Drinkers-Control; BD-A: Binge Drinkers-Alcohol. * $p < .05$ *vs* R-Co, BD-Co, and BD-A.

Perceived Stress (PS)

Neither the main factors –Drinking Pattern and Treatment– nor their interaction was statistically significant in females (Drinking Pattern: $F_{(1,133)} = 0.508$, $p = .477$; Treatment: $F_{(1,133)} = 3.057$, $p = .083$; Interaction: $F_{(1,133)} = 0.140$, $p = .709$) or in males (Drinking Pattern: $F_{(1,88)} = 0.633$, $p = .428$; Treatment: $F_{(1,88)} = 1.687$, $p = .197$; Interaction: $F_{(1,88)} = 0.030$, $p = .862$).

Correlations between measures

Positive correlations 20 min after treatment were detected in females, demonstrating a mutual increase of the following variables: SBP and DBP (r = 0.617, $p = .001$), and DBP and HR (r = 0.222, $p = .046$). Positive correlations were also found in males between COR20' and SBP (r = 0.344, $p = .013$), SBP and DBP (r = 0.417, $p = .002$), and DBP and HR (r = 0.434, $p = .001$).

Experiment 2: Effects of a risk dose of alcohol on cortisol, BP, HR and PS in female and male adolescents (BAC: 0.38 ± 0.01 g/L) with or without a history of BD.

A summary of descriptive statistics and results for the main factors in stress responses in Experiment 2 is presented in Table 3.

Cortisol (COR0', COR20' and COR50')

In COR0', neither the factor Drinking Pattern ($F_{(1,92)} = 1.6470$, $p = .203$) nor the factor Sex ($F_{(1,92)} = 0.057$, $p = .811$) were statistically significant. Similarly, the interaction Drinking Pattern X Sex was not statistically significant ($F_{(1,92)} = 0.004$, $p = .948$).

COR20' and COR50': The repeated measures ANOVA showed that Cortisol was statistically significant ($F_{(1,88)} = 20.764$, $p = .001$), showing a significant decrease of COR50' with respect to COR20' (Figure 1B). None of the possible interactions between Cortisol, Drinking Pattern, Treatment and Sex was statistically significant ($ps > .05$ in all cases).

The main factors Drinking Pattern and Treatment were not found to be statistically significant ($F_{(1,88)} = 2.584$, $p = .112$; and $F_{(1,88)} = 0.460$, $p = .500$; respectively); while Sex was significant ($F_{(1,88)} = 4.538$, $p = .036$), with higher cortisol levels detected in male groups (see Table 3). The interaction Drinking Pattern X Treatment was also statistically significant ($F_{(1,88)} = 4.492$, $p = .037$), with binge drinkers who received control drink showing higher cortisol levels than the rest of the subjects (Figure 3). The rest of interactions were not statistically significant (Drinking Pattern X Sex: $F_{(1,88)} = 0.116$, $p = .735$; Treatment X Sex: $F_{(1,88)} = 3.578$, $p = .062$; and Drinking Pattern X Treatment X Sex: $F_{(1,88)} = 1.370$, $p = .245$).

Blood Pressure (SBP and DBP)

SBP: No significant results were observed for the main factors Drinking Pattern ($F_{(1,88)} = 0.006$, $p = .939$) or Treatment ($F_{(1,88)} = 0.418$, $p = .520$), but the factor Sex was statistically significant ($F_{(1,88)} = 56.639$, $p = .001$), with males showing higher SBP than females (see Table 3). The interaction Drinking Pattern X Sex was statistically

Table 3. *Summary of descriptive statistics and results for COR, SBP, DBP, HR and PS (Experiment 2).*

	FEMALES AND MALES (BAC: 0.38 ± 0.01 g/L)								
	Drinking Pattern			**Treatment**			**Sex**		
	Refrainers (n = 48)	**Binge Drinkers** (n = 48)	**Statistical Power**[1]	**Control** (n = 48)	**Alcohol** (n = 48)	**Statistical Power**[1]	**Females** (n = 48)	**Males** (n = 48)	**Statistical Power**[1]
COR	4.94 ± 0.37	5.37 ± 0.55	.995	5.67 ± 0.45	5.23 ± 0.50	.995	4.77 ± 0.32	6.14 ± 0.58#	.995
SBP	113.75 ± 1.54	113.60 ± 1.86	.051	114.29 ± 1.80	113.06 ± 1.61	.098	106.52 ± 1.27	120.83 ± 1.44*	1.000
DBP	68.70 ± 0.92	68.83 ± 1.25	.051	69.02 ± 0.90	68.52 ± 1.22	.062	68.08 ± 0.93	69.45 ± 1.23	.144
HR	72.12 ± 1.55	74.93 ± 1.46	.278	70.33 ± 1.41	76.72 ± 1.56$	.875	75.16 ± 1.38	71.89 ± 1.69	.357
PS	21.93 ± 1.14	23.12 ± 1.28	.083	21.77 ± 1.22	23.29 ± 1.19	.100	24.39 ± 1.07&	20.66 ± 1.29	.636

Notes. Salivary cortisol concentrations (COR). Systolic (SBP) and Diastolic (DBP) Blood Pressure. Heart Rate (HR). Stress Perception (PS). Not applicable (NA). The results are expressed as mean ± SEM. [1]The Statistical Power is calculated using alpha = 0.05. # $p < .05$ increase of COR *vs* females. * $p < .05$ increase of SBP *vs* females. $ $p < .05$ increase of HR in Alcohol group *vs* Control group. & $p < .05$ increase of PS *vs* males.

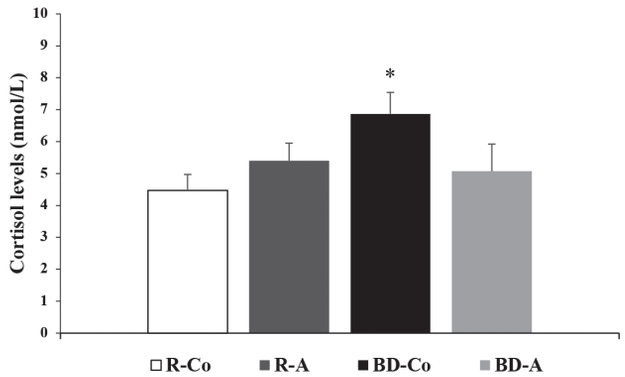

Figure 3. Salivary cortisol concentrations (females and males together) in Experiment 2. Values are expressed as means (+ SEM). R-Co: Refrainers-Control; R-A: Refrainers-Alcohol; BD-Co: Binge Drinkers-Control; BD-A: Binge Drinkers-Alcohol. * $p < .05$ *vs* R-Co.

significant ($F_{(1,88)} = 3.675$, $p = .05$), with male binge drinkers displaying higher SBP than their female counterparts (Figure 4). The interactions Drinking Pattern X Treatment ($F_{(1,88)} = 1.273$, $p = .262$), Treatment X Sex ($F_{(1,88)} = 0.640$, $p = .426$) and Drinking Pattern X Treatment X Sex ($F_{(1,88)} = 2.123$, $p = .149$) were not statistically significant.

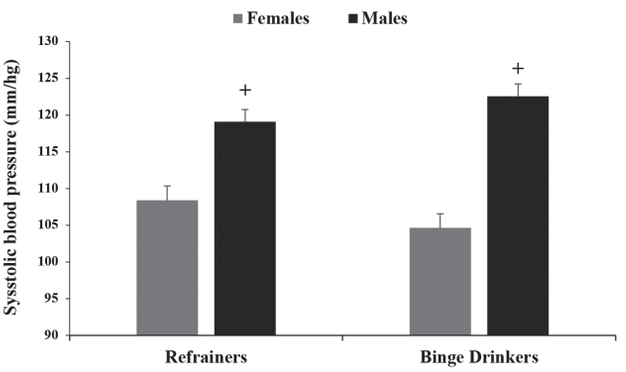

Figure 4. Systolic blood pressure (SBP) in females and males in Experiment 2. Values are expressed as means (+ SEM). + $p < .05$ *vs* females.

DBP: Significant results were not obtained for the main factors Drinking Pattern ($F_{(1,88)} = 0.007$, $p = .935$), Treatment ($F_{(1,88)} = 0.106$, $p = .746$), and Sex ($F_{(1,88)} = 0.802$, $p = .373$). The interactions Drinking Pattern X Treatment ($F_{(1,88)} = 3.816$, $p = .054$), Drinking Pattern X Sex ($F_{(1,88)} = 3.305$, $p = .072$), Treatment X Sex ($F_{(1,88)} = 0.851$, $p = .359$), and Drinking Pattern X Treatment X Sex ($F_{(1,88)} = 0.027$, $p = .871$) were not statistically significant.

Heart Rate (HR)

HR was not statistically significant for the main factors Drinking Pattern ($F_{(1,88)} = 1.915$, $p = .170$) and Sex ($F_{(1,88)} = 2.590$, $p = .111$), but it was for the factor Treatment ($F_{(1,88)} = 9.902$, $p = .002$), with higher HR observed among the subjects that received alcohol during the experiment (see Table 3). The interaction Drinking Pattern X Treatment was not statistically significant ($F_{(1,88)} = 3.442$, $p = .067$), but Drinking Pattern X Sex was ($F_{(1,88)} = 4.245$, $p = .042$), with higher HR in female binge drinkers *vs* the rest of the groups (Figure 5). The rest of interactions were not statistically significant (Treatment X Sex: $F_{(1,88)} = 0.318$, $p = .574$; Drinking Pattern X Treatment X Sex: $F_{(1,88)} = 1.342$, $p = .250$).

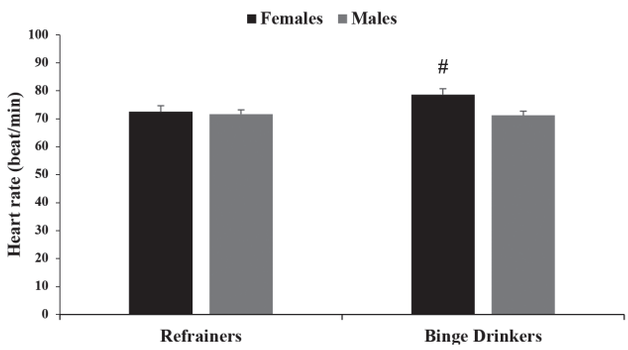

Figure 5. Heart Rate (HR) in females and males in Experiment 2. Values are expressed as means (+ SEM). # $p < .05$ *vs* all groups.

Perceived Stress (PS)

The results obtained for PS were not statistically significant for Drinking Pattern ($F_{(1,88)} = 0.488$, $p = .487$) or Treatment ($F_{(1,88)} = 0.800$, $p = .373$), but was for factor Sex ($F_{(1,88)} = 4.811$, $p = .031$), with females obtaining higher PS scores than males (see Table 3). None of the interactions was statistically significant (Drinking Pattern X Treatment: $F_{(1,88)} = 0.523$, $p = .472$; Drinking Pattern X Sex: $F_{(1,88)} = 0.025$, $p = .874$; Treatment X Sex: $F_{(1,88)} = 0.109$, $p = .742$; Drinking Pattern X Treatment X Sex: $F_{(1,88)} = 1.243$, $p = .268$).

Correlations between measures

Positive correlations 20 min after treatment were detected between the following variables: COR20' and SBP ($r = 0.314$, $p = .002$), SBP and DBP ($r = 0.493$, $p = .001$), and DBP and HR ($r = 0.235$, $p = .021$).

Discussion

This study was designed to clarify the effects of a history of BD and/or the effects of acute alcohol on the functionality of the HPA axis (cortisol) and other variables of the stress response (HR, BP, and PS) in female and male adolescents. Our hypothesis was that there would be an increase of cortisol levels, HR, BP, and PS in the participants without a history of BD, due to the normal stress response to acute alcohol consumption (Hagan et al., 2019; King et al., 2006; Lu & Richardson, 2014; Ulrich-Lai & Herman, 2009). Our

results partly support this hypothesis: cortisol and HR were higher in females who drank alcohol during Experiment 1 and HR was higher among participants of both sexes who drank alcohol during Experiment 2. The outcome of our investigation is in line with previous research showing alcohol to have a direct influence on the HPA axis by elevating HPA hormone levels (King et al., 2006; Lu & Richardson, 2014; Richardson, Lee, O'Dell, Koob & Rivier, 2008). Although a significant correlation between cortisol and heart rate was not observed in our study, the increase in cardiovascular activity in our female subjects could be due to the rise in HPA hormone levels (cortisol), since such a finding has been reported by other groups (Bau et al., 2011; Sher et al., 2007).

Our results after acute alcohol intake are related to a normal stress response system, in the line of other studies (King et al., 2006; Lu & Richardson, 2014; Richardson et al., 2008). Furthermore, our findings show that a history of BD (not the effects of acute alcohol intake) can also have a significant effect, as some variables of the stress response system appeared to already be dysregulated due to past BD episodes. In this way, our results in Experiment 1 revealed higher HR levels in BD females, and higher basal cortisol and SBP in BD males. In Experiment 2, basal cortisol levels were higher in BD participants who received control drink in comparison to the other groups. This suggests that adolescents with a history of BD (without considering any acute alcohol intake) have higher cortisol levels due to over-regulation of the HPA axis (Wemm et al., 2013). Therefore, we have achieved the other major goal of our study, as we have demonstrated that BD triggers changes in the HPA axis and in the stress response in healthy adolescents who began a consumption pattern BD in adolescence. In contrast to our findings, other studies have reported lower values of cortisol levels, HR, BP, and PS in subjects with a history of BD engaging in acute alcohol consumption, arguing that the response system is regulated by a sustained history of BD (e.g., Hagan et al., 2019; Orio et al., 2018). We did not observe such a finding in our study, perhaps because our participants had not reached that point of regulation: i.e., a one-year history of BD was not long enough for the aforementioned regulation (lower levels in stress response measures) to be achieved.

The interactions that we observed are in line with the rest of the results in our study, pointing to an increased HR in male refrainers who drank alcohol, as reported in previous research (Bau et al., 2011; Sher et al., 2007; Vinader-Caerols, et al., 2012). This result, observed in Experiment 1, can be interpreted as a phenomenon of tolerance among male binge drinkers, as their HR did not increase when they drank alcohol. In Experiment 2, the interaction Drinking Pattern X Treatment led to higher cortisol le-vels in the BD group receiving control drink, compared to the other groups. This confirms the over-regulation of the HPA axis basal state reported in a previous study (Wemm et al., 2013), and provides evidence that the BD pattern triggers alterations in the HPA axis, independently of sex.

The main goal of Experiment 2 was to study the sex factor directly. Considering that the variations in cortisol over time observed in our study are in the same direction in both sexes (lower levels of COR50' than COR20' in females and males separately, as well as in both sexes together), the main sex difference was the higher levels of cortisol in males, perhaps because salivary cortisol increases are up to twice as high in males as in females. This sex difference is supported by the literature: the typical mean response in males has been shown to range from 200 to 400% with respect to baseline, while changes from 50 to 150% are usually seen in females (Kudielka, Hellhammer & Wüst, 2009). Another sex difference was observed in the case of BP, with SBP proving to be higher in males than in females. According to previous research, the BP of young females is typically lower than that of young males, even among healthy normotensive people (Joyner, Wallin & Charkoudian, 2016). In addition, SBP was higher among our male BD participants in comparison to the female BD and refrainers groups. Finally, our female participants showed higher levels of stress (PS) than males and the female BD group showed higher HR than their male counterparts, as reported by other studies (Anbumalar, Dorathy, Jaswanti, Priya & Reniangelin, 2017; Hogan, Carlson & Dua, 2002).

Several studies have reported that cortisol facilitates an increase in cardiovascular activity, raising heart rate and blood pressure (Hagan et al., 2019; Ulrich-Lai & Herman, 2009). The findings of our study partly support this issue through positive correlations between cortisol levels and cardiovascular activity (COR20' and SBP).

Among the limitations of this study, it must be mentioned that we measured cortisol levels after treatment, but we did not measure them early in the morning, when levels are highest because of the circadian rhythm of cortisol variations (Weitzman et al., 1971; Yamanaka, Motoshima & Uchida, 2019). Nevertheless, the saliva cortisol samples were within the normal range of basal values (2.7586-8.2758 nmol/L) at the time they were taken (16:00h-18:00h) (Aardal & Holm, 1995). In addition, a bigger sample would have been desirable in the second experiment to back up the results obtained in females and males with similar BAC. Finally, longitudinal studies are necessary in the future in order to study the long-term effects of HPA axis dysregulation.

Considering all the results, and despite the aforesaid limitations, our study infers that:

a) A normal HPA axis in adolescents reacts to alcohol consumption by increasing cortisol levels in females, as well as HR in both sexes, as a normal reaction of the stress response system.

b) Higher values of cortisol (independently of sex), HR in females and SBP in males are observed in subjects with a history of BD maintained at least one year, even if they are healthy teenagers, due to an emerging dysregulation in the HPA axis. However, these changes are not sufficient for diagnosing a disease.

c) The main sex differences were found in cortisol, SBP and PS, with males showing higher levels of cortisol and SBP, and lower PS than females.

d) Our results contribute to a better knowledge of the changes related to alcohol consumption that occur at this stage of life (late adolescence) and may help to devise strategies to prevent the changes in the HPA axis triggered by BD, considering the sex factor and thus implementing more effective prevention programs aimed at this risk group.

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Conflict of interests

The authors declare they have no conflict of interest.

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