Chronic Ethanol Vapor Exposure Potentiates the Cardiovascular Responses to Acute Stress in Male but Not in Female Rats

Paula Cristina Bianchi (paula.cbianchi@gmail.com)  
Universidade Federal de Sao Paulo Escola Paulista de Medicina  
https://orcid.org/0000-0003-3412-4514

Lucas Gomes de Souza  
Universidade Estadual Paulista Julio de Mesquita Filho

Willian Costa-Ferreira  
Universidade Estadual Paulista Julio de Mesquita Filho

Paola Palombo  
Universidade Estadual Paulista Julio de Mesquita Filho

Paulo E. Cameiro de Oliveira  
Universidade Federal de Sao Carlos

Sheila A. Engi  
Universidade Federal de Sao Paulo Escola Paulista de Medicina

Rodrigo M. Leão  
Universidade Federal de Uberlandia

Cleopatra S. Planeta  
Universidade Estadual Paulista Julio de Mesquita Filho

Carlos C. Crestani  
Universidade Estadual Paulista Julio de Mesquita Filho

Fabio C. Cruz  
Universidade Federal de Sao Paulo Escola Paulista de Medicina

Research

Keywords: alcohol, blood pressure, heart rate, restraint stress, sex

DOI: https://doi.org/10.21203/rs.3.rs-90023/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. 
Read Full License
Abstract

**Background:** Ethanol use is related to a wide variety of negative health outcomes, including cardiovascular diseases. Stress is also involved in numerous pathologies such as cardiovascular diseases and psychiatric disorders. Sexual dimorphism is an important factor affecting the cardiovascular response and have been proposed as a potentially risk factor for sex-specific health problems in human. Here, we evaluated the effect of prolonged ethanol vapor inhalation in arterial pressure, heart rate and tail skin temperature responses to acute restraint stress, investigating differences between male and female rats.

**Methods:** We exposed male and female Long-Evans rats to ethanol vapor for 14 hours, followed by ethanol withdrawal for 10 hours, for 30 consecutive days or room air (control groups). The animals underwent surgical implantation of a cannula into the femoral artery for assessment of arterial pressure and heart rate values. The tail skin temperature was measured as an indirect measurement of sympathetic vasomotor response.

**Results:** Chronic ethanol vapor inhalation reduced basal heart rate in both female and male rats. Sex-related difference was observed in the decrease of tail cutaneous temperature evoked by stress, but not in the pressor and tachycardiac responses. Furthermore, prolonged ethanol inhalation enhanced the blood pressure and heart rate increase caused by acute restraint stress in male, but not in female rats. However, no effect of chronic ethanol vapor was observed in the tail cutaneous temperature response to restraint in both sexes.

**Conclusion:** Chronic ethanol vapor exposure increased the cardiovascular reactivity to stress in male, but not in female rats.

Background

Excessive ethyl alcohol (ethanol) consumption is related to a wide variety of negative health outcomes and premature deaths [1]. According to the World Health Organization, among worldwide ethanol-related deaths in 2016, 19% were due to cardiovascular diseases [1]. Clinical and preclinical studies have demonstrated that alterations in contractile/relaxant properties of the vascular smooth muscle, changes in neuroendocrine function, impairment of baroreflex activity and autonomic imbalance constitute important mechanisms underlying the negative cardiovascular effects of heavy ethanol consumption [1–7].

Stress is a complex and multidimensional phenomenon of great biological importance that requires an appropriate and coordinated set of physiological responses for the maintenance of homeostasis [8–11]. Restraint is one of the most commonly employed stressors to investigate stress-evoked behavioral and physiological changes in laboratory animals [12–14]. This model is characterized by unconditioned and unavoidable stress-elicited neuroendocrine and cardiovascular responses, the latter being characterized by sustained blood pressure and heart rate (HR) increases that last throughout the restraint period [10, 15,
In addition, cutaneous vasoconstriction during restraint leads to a fall in the tail skin temperature [17–19].

Sexual dimorphism is an important factor affecting the cardiovascular response induced by both stress and chronic ethanol access [20–27]. For example, susceptibility to hypertension in men is generally associated with increased vascular response to stress when compared to women [28]. In addition, preclinical results showed that females are more resistant than males to stress-induced cardiovascular disorders [20]. Regarding the association between ethanol and cardiovascular impairments, results have demonstrated that hypertensive effect of ethanol in men is manifested in a linear dose-dependent manner [29–31], whereas a slight protective effect of ethanol is observed in women at moderate doses [32–34]. Accordingly, studies in rodents demonstrated that high blood alcohol levels (BALs) induced by chronic ethanol consumption evoked hypertension, increased sympathetic neural activity and enhanced baroreflex tachycardic response in males [3–5, 7], while effects considered protective to cardiovascular function were reported in female, including hypotension, increased cardiac parasympathetic dominance and bradycardic reflex response [21, 35–38]. Regarding stress responses, decreased stress-evoked cardiovascular changes was reported following acute ethanol administration [39]. Nevertheless, the impact of chronic ethanol exposure in cardiovascular reactivity during aversive threats has never been reported.

Besides, despite the evidence of differences on cardiovascular changes to chronic ethanol between females versus males [40–46], a possible influence of sexual dimorphisms in the effect of ethanol on stress-evoked cardiovascular changes is unknown. Thus, the present study aimed to evaluate the effect of chronic ethanol vapor inhalation in blood pressure, HR and tail skin temperature responses to acute stress, investigating differences between male and female rats.

**Methods**

**Animals**

We used 25 male and 27 female Long Evans rats at post-natal day (PND 60), obtained from the animal breeding facility at School of Pharmaceutical Sciences, São Paulo State University (UNESP) (Araraquara, SP, Brazil) and were housed in standard rat cages (plastic cages) in a temperature-controlled room at 24 °C in the Animal Facility of the Physic Institute of São Carlos, University of São Paulo (USP) (São Carlos, SP, Brazil). They were kept under a 12:12 h light-dark cycle (lights on between 7:00 h and 19:00 h) with food and filtered water *ad libitum*. Housing conditions and experimental procedures were carried out following protocols approved by the Ethical Committee for Use of Animal and Subjects of the Physic Institute of São Carlos-USP (approval# 2014/01), which complies with Brazilian and international guidelines for animal use and welfare.

**Drugs and solutions**
Ethanol 95% (Labsynth, Diadema, SP, Brazil). Isoflurane, USP (99.9% v.v). Tribromoethanol (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in saline (NaCl 0.9%). Flunixine meglumine (Banamine®; Schering-Plough, Cotia, SP, Brazil) and the poly-antibiotic preparation (Pentabiotico®; Fort Dodge, Campinas, SP, Brazil) were used as provided.

**Ethanol Vapor Inhalation**

Animals were exposed to chronic intermittent ethanol vapor in an attempt to induce a state of ethanol dependence in rodents, which is characterized by the presence of withdrawal signs, tolerance and negative emotional symptoms upon cessation of ethanol vapor exposure [47–52]. Furthermore, comparing to other methods, ethanol vapor inhalation offers advantages to the ethanol researcher, including the circumvention of rodents' natural aversion to ethanol and the easily maintenance of consistent BALs [47–52].

We adapted the protocol from Leão et al. [53]. Briefly, animals were housed in standard rat cages that were placed into separate sealed clear acrylic chamber (n = 4 per chamber), where the animals were exposed to controlled ethanol vapor. Evaporated ethanol values were adjusted as necessary to maintain animal BALs in the 150–350 (mg/dL) range. Animals were daily exposed to ethanol vapor inhalation for 14 hours (7 pm – 9am) followed by 10 hours withdraw (no ethanol vapor inhalation) for 30 days. Blood samples were collected every week to confirm BALs. Control animals were not exposed to ethanol vapor.

**Surgical Preparation**

Animals were anesthetized with tribromoethanol (250 mg/kg, i.p.) and a polyethylene cannula (a 4 cm segment of PE-10 heat-bound to a 13 cm segment of PE-50) (Clay Adams, Parsippany, NJ, USA) filled with a solution of heparin (50UI/ml, Hepamax-S®, Blausiegel, Cotia, SP, Brazil) diluted in saline (0.9% NaCl) was inserted into the abdominal aorta through femoral artery for cardiovascular recording. The catheter was tunneled under the skin and exteriorized on the animal’s dorsum. After the surgery, rats were treated with a poly-antibiotic formulation containing streptomycins and penicillins (560 mg/ml/kg, i.m.) to prevent infection; and flunixin meglumine (0.5 mg/ml/kg, s.c.) - a non-steroidal anti-inflammatory drug - for postoperative analgesia.

**Blood pressure and heart rate recording**

The catheter implanted into the femoral artery was connected to a pressure transducer (DPT100, Utah Medical Products Inc., Midvale, UT, USA). Pulsatile blood pressure was recorded using an amplifier (Bridge Amp, ML224, ADInstruments, Australia) and a digital acquisition board (PowerLab 4/30, ML866/P, ADInstruments, NSW, Australia). Mean arterial pressure (MAP) and HR values were derived from the pulsatile blood pressure recording.

**Tail skin temperature measurement**

The cutaneous temperature of the tail was recorded using a thermal camera (IRI4010, InfraRed Integrated Systems Ltd., Northampton, UK). The temperature was measured on five points of the animal’s tail and
the mean value was calculated for each recording [19, 54].

**Restraint stress**

For acute restraint stress, each rat was placed in a plastic cylindrical restraint tube (diameter 6.5 cm, length 15 cm), ventilated by holes (1 cm diameter) that made up approximately 20% of the tube surface. Restraint lasted 30 min [54, 55], and immediately after the end of the stress exposure rats were returned to their home cages. Each rat was submitted to only one session of restraint in order to avoid habituation [56, 57].

**Experimental protocols**

Different set of female and male animals were randomly allocated in four experimental groups: (i) **female control**, which animals were kept in their home cage without ethanol vapor exposure (n = 8); (ii) **female ethanol vapor**, which animals were submitted to ethanol vapor chamber daily (n = 9); (iii) **male control**, which animals were kept in their home cage without ethanol vapor exposure (n = 5); and (iv) **male ethanol vapor**, which animals were submitted to ethanol vapor chamber daily (n = 9). A schematic representation of the experimental design is presented in Fig. 1. First, animals were exposed to intermittent ethanol vapor for four weeks. Blood samples were collected every week to confirm BALs (Fig. 2). Twenty-four hours after the last ethanol vapor exposure day, animals in all experimental groups were subjected to surgical preparation. The next day, rats were brought to the experimental room in their own home cages. Rats were allowed one hour to adapt to the conditions of the experimental room, such as sound and illumination, before starting arterial pressure and HR recording. The experimental room was temperature controlled (25 °C) and was acoustically isolated from the other rooms. Cardiovascular recording of MAP and HR of freely moving rats began at least 30 min before the onset of the restraint, and was performed throughout the session of stress. The tail skin temperature was measured 10, 5 and 0 min before the restraint for baseline values, and at 5, 10, 15, 20, 25 and 30 min during restraint [16, 19]. At the end of the experiment, the rats were anesthetized with isoflurane inhalation and decapitated.

**Data analysis**

Basal values of MAP, HR, and cutaneous temperature were compared using two-way ANOVA, with sex (female x male) and vapor (control x ethanol vapor) as independent factors. The time–course curves of cardiovascular and cutaneous temperature changes during pre-stress period (basal) and restraint stress session were analyzed using three-factor mixed ANOVA, with sex and vapor as main factor and time (basal x stress) as repeated measurement. ANOVA analysis were conducted on GraphPad Prism 8.0.2 software. Results of statistical tests with p < 0.05 were considered significant.

Generalized Estimation Equations (GEE) were used to assess the effects of time, sex and vapor during restraint stress session on cardiovascular parameters and cutaneous tail temperature. For that, the robust estimator (Huber-White estimator) was used under the first-order autoregressive correlation matrix structure. The GEE analysis were conducted on the software SPSS v. 20. The adopted statistical significance level (α) was 0.05. Data were submitted to contrast analysis with Bonferroni adjustment for
multiple comparisons when effect of time was detected. When two or three-way interactions were detected, data from different experimental groups (vapor or sex) were analyzed separately.

Results

**Effects of ethanol vapor exposure on basal values of arterial pressure, heart rate and tail skin temperature**

Figure 3 depict the mean ± standard error of mean (SEM) of MAP, HR and tail temperature during basal period in female and male rats. Analysis of MAP indicated no significant effect for sex ($F_{(1, 27)} = 1.12, p > 0.05$), vapor ($F_{(1, 27)} = 0.21, p > 0.05$), or interaction between factors ($F_{(1, 27)} = 0.06, p > 0.05$) (Fig. 3A).

Analysis of HR revealed a main effect of vapor ($F_{(1, 27)} = 5.24, p < 0.05$), with no effect for sex ($F_{(1, 27)} = 0.09, p > 0.05$), or interaction between factors ($F_{(1, 27)} = 0.13, p > 0.05$) (Fig. 3B). No significant effect of sex ($F_{(1, 40)} = 0.005, p > 0.05$), vapor ($F_{(1, 40)} = 0.10, p > 0.05$), or interaction between factors ($F_{(1, 40)} = 0.34, p > 0.05$) were observed for tail skin temperature (Fig. 3C).

**Effects of ethanol vapor exposure on cardiovascular responses to acute restraint stress**

Independently of sex or treatment, acute restraint stress increased MAP ($F_{(9, 150)} = 58.15, p < 0.05$) and HR ($F_{(9, 220)} = 19.01, p < 0.05$) and decreased tail cutaneous temperature ($F_{(1, 44)} = 25.35, p < 0.05$), as observed by a significant effect of time in all this parameters (Fig. 4).

During restraint stress, GEE analysis of MAP revealed significant effect of sex ($W_{(1)} = 4.99, p = 0.025$), treatment ($W_{(1)} = 5.79, p = 0.016$) and time ($W_{(14)} = 806.67, p < 0.001$). Significant interactions between sex and time ($W_{(14)} = 155.38, p < 0.001$), treatment and time ($W_{(14)} = 42.64, p < 0.001$) and sex, treatment and time ($W_{(14)} = 96.42, p < 0.001$) were also detected. Subsequent analyzes revealed a significant effect of treatment in males ($b = 7.38, p < 0.002$), but not in females ($b = 0.85, p > 0.05$). Furthermore, there was a significant effect of sex in the group treated with vapor ($b = -6.48, p < 0.05$), but not in the control group ($b = -0.29, p > 0.05$). This data indicates that chronic ethanol vapor inhalation potentiates the effect of acute stress on MAP in male, but not in female rats (Fig. 4).

Analysis of HR during the stress session indicated a main effect of time ($W_{(14)} = 447.65, p < 0.001$) and significant interactions between sex and time ($W_{(14)} = 116.907, p < 0.001$), treatment and time ($W_{(14)} = 88.87, p < 0.001$) and sex, treatment and time ($W_{(14)} = 62.33, p < 0.001$). In female rats the effect of time was detected in both, control and ethanol vapor groups ($W_{(5)} = 70.51, p < 0.001$ and $W_{(6)} = 902.13, p < 0.001$, respectively). However, in male groups the effect of time was observed only in controls ($W_{(7)} = 369.53, p < 0.001$), but not in ethanol vapor ($W_{(2)} = 1.94, p > 0.05$). Thus, male rats exposed to ethanol enhanced and sustained the tachycardic response to acute restraint, when compare to control males (Fig. 4).

The tail temperature analyses during acute restraint stress period revealed a main effect of time ($W_{(6)} = 42.7, p < 0.001$) and a significant interaction between sex and time ($W_{(6)} = 14.26, p = 0.027$). GEE
analyzes also revealed significant effects of time for both male ($W_{(6)} = 47.45, p < 0.001$) and female ($W_{(6)} = 20.66, p < 0.05$) groups. Subsequent analyses indicated that in female groups the decrease in cutaneous temperature occurred only in the first five minutes of restraint, while in male groups, the decrease was observed throughout the stress session (Fig. 4).

**Discussion**

Present findings provide evidence of the effects of chronic ethanol vapor exposure on cardiovascular function during acute restraint stress in female and male rats. Our main findings were as follows: (i) females and males exposed to ethanol vapor presented a decrease in basal HR when compared to control groups; (ii) sex-related difference was observed in the time-course of decrease in the cutaneous temperature under stress condition; (iii) chronic ethanol vapor enhanced the MAP and tachycardiac response caused by acute restraint stress in males, but not in females; and (iv) chronic ethanol vapor did not change the tail cutaneous temperature response to acute restraint stress in both sexes.

An association between chronic ethanol consumption and hypertension in males is well documented [2–7,58]. However, we did not observe alterations in MAP values neither in males nor females. As demonstrated previously by our group [21], four weeks of ethanol vapor exposure was not enough to promote increases in basal blood pressure in male rats. Previous studies have provided evidence that long-term ethanol exposure (e.g., 6, 8, or 12 weeks) is required to induce hypertension in males with the BALs levels (100 to 300 mg/dl) reached during ethanol inhalation [2,5,58,59]. In addition, different from other models of chronic forced ethanol exposure, such as liquid diet or ethanol in drinking water, the ethanol vapor is an intermittent model of ethanol access. In agreement with our results, Engi et al. [60] showed that 6 weeks of intermittent voluntary ethanol consumption did not induce an increase in blood pressure in male rats.

We observed decreased basal HR values in females and males exposed to ethanol vapor. The bradycardia effect of ethanol in females was described by other authors [21,61–63], and might be followed by reductions in cardiac output and contractile force. For example, El-Mas and colleagues [61,62] showed that females exposed to ethanol presented up-regulation of cardiac nitric oxide synthase, which resulted in reductions in cardiac output. Further, Duan et al. [63] observed that ethanol metabolic product acetaldehyde-induced cardiac contractile depression in females. Other studies showed that decreases in basal HR are associated with inadequate tissue perfusion, arrhythmias, higher mortality and sudden death [64,65]. Although previous studies did not report resting bradycardia in male rodents [2,4,5,7,21], similar mechanisms and consequences as identified in females might be related to the HR decrease identified in the present study in males.

Our findings are in line with previous studies that reported blood pressure and HR increases and decrease in the skin temperature as physiological changes during restraint stress [10,16,18,19,66–68]. However, differently from other authors [22,23], we did not observe an influence of sex on MAP and HR during the stress session. Comparing the responses of males and females under immobilization stress,
Anishchenko et al. [22] observed higher amplitude and duration of MAP elevation in males, and a severe tachycardia in females. Another study, using spontaneously hypertensive rats, showed a greater change in MAP in response to prolonged restraint (60 min) in males, but not in females [23]. Nevertheless, we observed a sex-related difference in the cutaneous vasoconstriction response under stress condition. In this regard, we showed a decrease in the cutaneous temperature only at the beginning of restraint (first five minutes) in females, whereas a sustained decrease throughout the stress session was identified in males. This sex-related effect on sympathetic vasomotor response in cutaneous bed can be related to ovarian hormones. For instance, Zhen et al. [69] showed that under adrenergic nerve stimulation, arteries from female were less responsive than arteries from male rats. They also observed that this sex difference was abolished after ovariectomy of the females but not after orchidectomy of the males [69], suggesting that circulating ovarian hormones inhibits sympathetically mediated vasoconstriction. Besides, vasodilator effect of estrogens has been reported, which seems to be mediated by activation of endothelial nitric oxide synthase (eNOS) and, consequently, an increase of nitric oxide (NO) production [46,70,71]. Considering these effects, estrogen could mediate the sexual differences on hemodynamic adjustments during aversive threats.

Although ethanol exposure did not change the basal MAP in male rats, chronic ethanol vapor inhalation potentiated the effect of acute stress on MAP in males, but not in females. The arterial pressure rise during stress is mediated by an increase in vascular sympathetic tone and activation of α1-adrenoreceptors in vascular smooth muscle [72,73]. In this sense, a sympathoexcitatory effect of ethanol in male rats was described in numerous studies [4,5,7,58,74,75]. For example, Russ and colleagues [5] showed that chronic ethanol increased, via central nervous system modulation, the basal firing rate of sympathetic nerve fibers. They also observed that the sympathetic nervous activity was increased prior to the development of hypertension in males [5]. In addition, higher levels of plasma concentrations of adrenaline and noradrenaline [6,76], as well as enhanced vascular reactivity to α1-adrenoceptor agonists [77–80] was observed in male rats chronically treated with ethanol. In this regard, Stewart & Kennedy [79] showed an ethanol-associated increase in the maximal contractile response to phenylephrine in endothelium-denuded preparations of male, but not of female rats. Thus, it is possible that the initial vasodilatory effect of ethanol [81,82] is completely suppressed by increased sympathetic nervous activity in males [5,83]. Taken together, these results could explain the increase in cardiovascular stress reactivity in male, but not in female rats observed in the present study.

The exacerbated increase in MAP in male rats exposed to chronic ethanol vapor was accompanied by an enhancement in the tachycardia response. Cardiac sympathetic blockers abolish tachycardia response evoked by stress, whereas cardiac parasympathetic blocker increases it [15,68,72,73]. These results demonstrated that both sympathetic and parasympathetic outflows to the heart are activated during stress. Thus, an increase in restraint-evoked HR rise following ethanol exposure may result from a facilitation of cardiac sympathetic response or inhibition of parasympathetic activity in males. In this sense, the sympathoexcitatory effect of ethanol stated above might also mediate the enhanced tachycardia to stress in addition to the change on pressor response. Although previous results from our
group did not indicate changes in parasympathetic activity in male rats subjected to chronic ethanol inhalation [21], we cannot exclude the possibility of an involvement of inhibition of this autonomic branch in facilitation of tachycardia to restraint.

Differently from males, no effect of chronic ethanol vapor was observed on MAP or HR values during restraint stress in females. Sexual dimorphism influences the cardiovascular effects promoted by chronic ethanol exposure [21,79], which may impact the autonomic and hemodynamic stress responses. In contrast to sympathoexcitatory effects of ethanol in males, an increase in cardiac parasympathetic dominance has been observed in females following long-term ethanol access. In fact, as stated above, we demonstrated increased cardiac parasympathetic activity in female but not in male rats exposed to chronic ethanol vapor inhalation [21]. Further, other studies observed that this effect on cardiac vagal tone was estrogen-dependent [36,84,85]. For example, El-mas and Abdel-rahman [36] showed that the parasympathetic overactivity induced by chronic ethanol was exacerbated in estrogen-replaced ovariectomized rats, when compared to ovariectomized ones. Corroborating this finding, El-mas and Abdel-rahman [37] observed that ovariectomized rats exposed to ethanol presented enhanced sympathetic activity as indicated by significant increases in plasma norepinephrine levels. In this way, an increase in the cardiac parasympathetic activity might constitute a prominent adaptative mechanism in females that preclude the occurrence of changes in cardiovascular reactivity during stressful situations.

**Perspectives and Significance**

Sexual dimorphism is an important factor affecting the cardiovascular response induced by both stress and chronic ethanol access. To the best of our knowledge, the findings reported here are the first to provide evidence related to the impact of chronic ethanol exposure in cardiovascular reactivity during the acute restraint stress. Our data support the notion that exposure to chronic ethanol potentiates the cardiovascular reactivity to stressful stimuli in males but not in females and open doors for future work aimed at testing the mechanism underlying sex differences in the cardiovascular responses to stress, after a chronic exposure to ethanol.

**Conclusions**

The results reported here showed that chronic ethanol vapor inhalation enhanced both blood pressure and tachycardiac responses to acute restraint stress in males, but not female rats. Furthermore, a sex-related difference was observed in the cutaneous vasoconstriction response during stress since males showed a decrease in the cutaneous temperature that was sustained throughout the stress session, while females presented this reduction only at the beginning of restraint. Finally, more research is necessary to improve the understanding of the impact of prolonged ethanol exposure, and the influence of sexual dimorphisms, in other physiological and behavioral responses to stress.

**Declarations**
Ethics approval and consent to participate

The study has been approved by the Ethical Committee for Use of Animal and Subjects of the Physic Institute of São Carlos-USP (approval# 2014/01), which complies with Brazilian and international guidelines for animal use and welfare.

Consent for publication

Not applicable.

Availability of data and materials

All data are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001, as well as by the São Paulo Research Foundation - FAPESP [2013/24896-2] and by the National Council for Scientific and Technological Development - CNPq [164060/2014-6].

Acknowledgements

The authors wish to thank São Paulo Research Foundation - FAPESP and National Council for Scientific and Technological Development - CNPq for supported the research, and the Physic Institute of São Carlos - University of São Paulo were the study was developed.

Authors' information

Affiliations

Laboratory of Neuropsypharmacology, School of Pharmaceutical Sciences, São Paulo State University (UNESP), Rod. Araraquara-Jaú km 1, 14801-902, Araraquara-SP, Brazil.

Paula C. Bianchi, Lucas Gomes-de-Souza, Willian Costa-Ferreira, Paola Palombo, Cleopatra S. Planeta, Carlos C. Crestani

Joint Graduate Program in Physiological Sciences UFSCar/UNESP, Rod. Washington Luís km 235, 13565-905, São Carlos-SP, Brazil.

Paula C. Bianchi, Lucas Gomes-de-Souza, Willian Costa-Ferreira, Paola Palombo, Cleopatra S. Planeta, Carlos C. Crestani
Laboratory of Psychology, Psychology Department, Universidade Federal de São Carlos - UFSCar, Rod. Washington Luís km 235, 13565-905, São Carlos-SP, Brazil.

Paulo E. Carneiro de Oliveira

Laboratory of Pharmacology, Paulista Medicine School, Universidade Federal de São Paulo – UNIFESP, Leal Prado Building, Botucatu 862 Street, 04024-002, Vila Clementino, São Paulo-SP, Brazil.

Sheila A. Engi, Fabio C. Cruz

Joint Graduate Program in Pharmacology, Pharmacology and Molecular Biology Institute - INFAR, Três de Maio 100 Street, 04044-020, Vila Clementino, São Paulo-SP, Brazil.

Sheila A. Engi, Fabio C. Cruz

Biomedical Sciences Institute, Universidade Federal de Uberlândia, Uberlândia, Minas Gerais, Brazil.

Rodrigo M. Leão

Contributions

Paula C Bianchi, Carlos C Crestani, Cleopatra S Planeta, Rodrigo M Leão, and Fabio C Cruz conceived and designed the experiments. Paula C Bianchi, Paola Palombo, Paulo E C Oliveira, Willian C Ferreira, Lucas G Souza, Rodrigo M Leão, and Fabio C Cruz performed the experiments. Paula C Bianchi, Lucas G Souza, Willian C Ferreira, Sheila A Engi, Carlos C Crestani, and Fabio C Cruz analyzed the data. Paula C Bianchi, Carlos C Crestani and Fabio C Cruz wrote the paper. All authors read, critically reviewed, and approved the final manuscript.

Corresponding author

Paula C. Bianchi

Laboratory of Pharmacology, Paulista Medicine School, Universidade Federal de São Paulo – UNIFESP, Leal Prado Building, 862 Botucatu Street, 04024-002, Vila Clementino, São Paulo-SP, Brazil. Tel.: (+55) (11) 5576-4456. E-mail address: paula.bianchi@unifesp.br.

References

[1] World Health Organization. Global status report on alcohol and health 2018. Geneva: 2018.

[2] Abdel-Rahman AA, Wooles WR. Ethanol-induced hypertension involves impairment of baroreceptors. Hypertension 1987;10:67–73.
[3] Abdel-Rahman AR, Dar MS, Wooles WR. Effect of chronic ethanol administration on arterial baroreceptor function and pressor and depressor responsiveness in rats. J Pharmacol Exp Ther 1985;232:194–201.

[4] Crestani CC, Lopes A, Scopinho AA, Ruginsk SG, Uchoa ET, Correa FMA, et al. Cardiovascular alterations at different stages of hypertension development during ethanol consumption: Time-course of vascular and autonomic changes. Toxicol Appl Pharmacol 2014;280:245–55. https://doi.org/10.1016/j.taap.2014.08.012.

[5] Russ R, Abdel-Rahman a R, Wooles WR. Role of the sympathetic nervous system in ethanol-induced hypertension in rats. Alcohol 1991;8:301–7.

[6] Lopes da Silva A, Ruginsk SG, Uchoa ET, Crestani CC, Scopinho AA, Correa FMA, et al. Time-Course of Neuroendocrine Changes and Its Correlation with Hypertension Induced by Ethanol Consumption. Alcohol Alcohol 2013;48:495–504. https://doi.org/10.1093/alcalc/agt040.

[7] Resstel LBM, Tirapelli CR, Lanchote VL, Uyemura A, Oliveira AM De, Corre FMA. Chronic ethanol consumption alters cardiovascular functions in conscious rats 2006;78:2179–87. https://doi.org/10.1016/j.lfs.2005.09.021.

[8] Huether G. The central adaptation syndrome: Psychosocial stress as a trigger for adaptive modifications of brain structure and brain function. Prog Neurobiol 1996;48:569–612. https://doi.org/10.1016/0301-0082(96)00003-2.

[9] Crestani CC. Emotional Stress and Cardiovascular Complications in Animal Models: A Review of the Influence of Stress Type. Front Physiol 2016;7. https://doi.org/10.3389/fphys.2016.00251.

[10] Dampney RAL, Horiuchi J, McDowall LM. Hypothalamic mechanisms coordinating cardiorespiratory function during exercise and defensive behaviour. Auton Neurosci 2008;142:3–10. https://doi.org/10.1016/j.autneu.2008.07.005.

[11] Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. Nat Rev Neurosci 2009;10:397–409. https://doi.org/10.1038/nrn2647.

[12] Buynitsky T, Mostofsky DI. Restraint stress in biobehavioral research: Recent developments. Neurosci Biobehav Rev 2009;33:1089–98. https://doi.org/10.1016/j.neubiorev.2009.05.004.

[13] Campos AC, Fogaca M V., Aguiar DC, Guimaraes FS. Animal models of anxiety disorders and stress. Rev Bras Psiquiatr 2013;35:S101–11. https://doi.org/10.1590/1516-4446-2013-1139.

[14] Bali A, Jaggi AS. Preclinical experimental stress studies: Protocols, assessment and comparison. Eur J Pharmacol 2015;746:282–92. https://doi.org/10.1016/j.ejphar.2014.10.017.
[15] Crestani CC, Tavares RF, Alves FHF, Resstel LBM, Correa FMA. Effect of acute restraint stress on the tachycardiac and bradycardiac responses of the baroreflex in rats. Stress 2010;13:61–72. https://doi.org/10.3109/10253890902927950.

[16] Busnardo C, Alves FHF, Crestani CC, Scopinho AA, Resstel LBM, Correa FMA. Paraventricular nucleus of the hypothalamus glutamate neurotransmission modulates autonomic, neuroendocrine and behavioral responses to acute restraint stress in rats. Eur Neuropsychopharmacol 2013;23:1611–22. https://doi.org/10.1016/j.euroneuro.2012.11.002.

[17] Herborn KA, Graves JL, Jerem P, Evans NP, Nager R, McCafferty DJ, et al. Skin temperature reveals the intensity of acute stress. Physiol Behav 2015;152:225–30. https://doi.org/10.1016/j.physbeh.2015.09.032.

[18] Vianna DML, Carrive P. Changes in cutaneous and body temperature during and after conditioned fear to context in the rat. Eur J Neurosci 2005;21:2505–12. https://doi.org/10.1111/j.1460-9568.2005.04073.x.

[19] Gomes-de-Souza L, Oliveira LA, Benini R, Rodella P, Costa-Ferreira W, Crestani CC. Involvement of endocannabinoid neurotransmission in the bed nucleus of stria terminalis in cardiovascular responses to acute restraint stress in rats. Br J Pharmacol 2016:2833–44. https://doi.org/10.1111/bph.13560.

[20] Reckelhoff JF. Gender Differences in the Regulation of Blood Pressure. Hypertension 2001;37:1199–208. https://doi.org/10.1161/01.HYP.37.5.1199.

[21] Bianchi PC, Costa Ferreira W, Antonagi Engi S, Palombo P, Carneiro de Oliveira PE, Gomes de Souza L, et al. Prolonged Exposure to Alcohol Vapor Causes Change in Cardiovascular Function in Female but not in Male Rats. Alcohol Clin Exp Res 2019;43. https://doi.org/10.1111/acer.14035.

[22] Anishchenko TG, Glushkovskaya-Semyachkina O V., Berdnikova VA, Sindyakova TA. Sex-related differences in cardiovascular stress reactivity in healthy and hypertensive rats. Bull Exp Biol Med 2007;143:178–81. https://doi.org/10.1007/s10517-007-0043-9.

[23] Azar T, Sharp J, Lawson D. Stress-like cardiovascular responses to common procedures in male versus female spontaneously hypertensive rats. Contemp Top Lab Anim Sci 2005;44:25–30.

[24] Hammer JH, Parent MC, Spiker DA, World Health Organization. Global status report on alcohol and health 2018. vol. 65. 2018. https://doi.org/10.1037/cou0000248.

[25] Vieira JO, Duarte JO, Costa-Ferreira W, Morais-Silva G, Marin MT, Crestani CC. Sex differences in cardiovascular, neuroendocrine and behavioral changes evoked by chronic stressors in rats. Prog Neuropsychopharmacology Biol Psychiatry 2018;81:426–37. https://doi.org/10.1016/j.pnpbp.2017.08.014.

[26] Anishchenko TG, Mamontov BN, Shorina LN. Sex differences in the cholinergic status of albino rats. Bull Exp Biol Med 1992;114:1408–11. https://doi.org/10.1007/BF00841578.
[27] Leinwand LA. Sex is a potent modifier of the cardiovascular system. J Clin Invest 2003;112:302–7. https://doi.org/10.1172/JCI19429.

[28] Matthews KA, Stoney CM. Influences of sex and age on cardiovascular responses during stress. Psychosom Med 1988;50:46–56. https://doi.org/10.1097/00006842-198801000-00006.

[29] Corrao G, Bagnardi V, Zambon A, Arico S. Exploring the dose-response relationship between alcohol consumption and the risk of several alcohol-related conditions: a meta-analysis. Addiction 1999;94:1551–73.

[30] Corrao G, Bagnardi V, Zambon A, La Vecchia C. A meta-analysis of alcohol consumption and the risk of 15 diseases. Prev Med (Baltim) 2004;38:613–9. https://doi.org/10.1016/j.ypmed.2003.11.027.

[31] Puddey IB, Rakic V, Dimmitt SB, Beilin LJ. Influence of pattern of drinking on cardiovascular disease and cardiovascular risk factors—a review. Addiction 1999;94:649–63.

[32] Sesso HD, Cook NR, Buring JE, Manson JE, Gaziano JM. Alcohol Consumption and the Risk of Hypertension in Women and Men 2008. https://doi.org/10.1161/HYPERTENSIONAHA.107.104968.

[33] Taylor B, Irving HM, Baliunas D, Roerecke M, Patra J, Mohapatra S, et al. Alcohol and hypertension: gender differences in dose-response relationships determined through systematic review and meta-analysis. Addiction 2009;104:1981–90. https://doi.org/10.1111/j.1360-0443.2009.02694.x.

[34] Wakabayashi I. Influence of Gender on the Association of Alcohol Drinking With Blood Pressure 2017;21. https://doi.org/10.1038/ajh.2008.299.

[35] El-mas MM, Abdel-rahman AA. An association between the estrogen-dependent hypotensive effect of ethanol and an elevated brainstem c - jun mRNA in female rats 2001;912:79–88.

[36] El-mas MM, Abdel-rahman AA. Exacerbation of myocardial dysfunction and autonomic imbalance contributes to the estrogen-dependent chronic hypotensive effect of ethanol in female rats. Eur J Pharmacol 2012;679:95–100. https://doi.org/10.1016/j.ejphar.2012.01.008.

[37] El-mas MM, Abdel-rahman AA. OVARIECTOMY ALTERS THE CHRONIC HEMODYNAMIC AND SYMPATHETIC EFFECTS OF ETHANOL IN RADIOTELEMETERED FEMALE RATS 2000;22:109–26.

[38] El-Mas MM, Fan M, Abdel-Rahman AA. Upregulation of cardiac NOS due to endotoxemia and vagal overactivity contributes to the hypotensive effect of chronic ethanol in female rats. Eur J Pharmacol 2011;650:317–23. https://doi.org/10.1016/j.ejphar.2010.10.032.

[39] Sparrow MG, Roggendorf H, Vogel WH. Effect of ethanol on heart rate and blood pressure in nonstressed and stressed rats. Life Sci 1987;40:2551–9. https://doi.org/10.1016/0024-3205(87)90078-6.
[40] Abdel-Rahman a a. Gender difference in baroreflex-mediated bradycardia in young rats: role of cardiac sympathetic and parasympathetic components. Can J Physiol Pharmacol 1999;77:358–66. https://doi.org/10.1139/cjpp-77-5-358.

[41] Ajayi A., Hercule H, Cory J, Hayes B., Oyekan A. Gender difference in vascular and platelet reactivity to thromboxane A2-mimetic U46619 and to endothelial dependent vasodilation in Zucker fatty (hypertensive, hyperinsulinemic) diabetic rats. Diabetes Res Clin Pract 2003;59:11–24. https://doi.org/10.1016/S0168-8227(02)00180-8.

[42] Blizard DA, Peterson WN, Iskandar SS, Shihabi ZK, Adams N. The Effect of a High Salt Diet and Gender on Blood Pressure, Urinary Protein Excretion and Renal Pathology in Shr Rats. Clin Exp Hypertens Part A Theory Pract 1991;13:687–97. https://doi.org/10.3109/10641969109042072.

[43] Khan IY, Taylor PD, Dekou V, Seed PT, Lakasing L, Graham D, et al. Gender-Linked Hypertension in Offspring of Lard-Fed Pregnant Rats. Hypertension 2003;41:168–75. https://doi.org/10.1161/01.HYP.0000047511.97879.FC.

[44] Weinstock M, Razin M, Schorer-apelbaum D, Men D, McCarty R. Gender differences in sympathoadrenal activity in rats at rest and in response to footshock stress. Int J Dev Neurosci 1998;16:289–95. https://doi.org/10.1016/S0736-5748(98)00021-5.

[45] Taylor TA, Gariepy CE, Pollock DM, Pollock JS. Gender Differences in ET and NOS Systems in ET B Receptor–Deficient Rats. Hypertension 2003;41:657–62. https://doi.org/10.1161/01.HYP.0000048193.85814.78.

[46] Tostes RC, Nigro D, Fortes ZB, Carvalho MHC. Effects of estrogen on the vascular system. Brazilian J Med Biol Res 2003;36:1143–58. https://doi.org/10.1590/S0100-879X2003000900002.

[47] Gilpin NW, Richardson HN, Cole M, Koob GF. Vapor inhalation of alcohol in rats. Curr Protoc Neurosci 2008. https://doi.org/10.1002/0471142301.ns0929s44.

[48] Gilpin NW, Smith AD, Cole M, Weiss F, Koob GF, Richardson HN. Operant Behavior and Alcohol Levels in Blood and Brain of Alcohol-Dependent Rats. Alcohol Clin Exp Res 2009;33:2113–23. https://doi.org/10.1111/j.1530-0277.2009.01051.x.

[49] Vendruscolo LF, Roberts AJ. Operant alcohol self-administration in dependent rats: focus on the vapor model. Alcohol 2014;48:277–86. https://doi.org/10.1016/j.alcohol.2013.08.006.

[50] Roberts DCS, Morgan D, Liu Y. How to make a rat addicted to cocaine. Prog Neuro-Psychopharmacology Biol Psychiatry 2007;31:1614–24. https://doi.org/10.1016/j.pnpbp.2007.08.028.

[51] Roberts AJ, Cole M, Koob GF. Intra-amygdala muscimol decreases operant ethanol self-administration in dependent rats. Alcohol Clin Exp Res 1996;20:1289–98.
[52] Meinhardt MW, Sommer WH. Postdependent state in rats as a model for medication development in alcoholism. Addict Biol 2015;20:1–21. https://doi.org/10.1111/adb.12187.

[53] Leão RM, Cruz FC, Vendruscolo LF, de Guglielmo G, Logrip ML, Planeta CS, et al. Chronic Nicotine Activates Stress/Reward-Related Brain Regions and Facilitates the Transition to Compulsive Alcohol Drinking. J Neurosci 2015;35:6241–53. https://doi.org/10.1523/JNEUROSCI.3302-14.2015.

[54] Cruz FC, Engi SA, Leão RM, Planeta CS, Crestani CC. Influence of the single or combined administration of cocaine and testosterone in autonomic and neuroendocrine responses to acute restraint stress. J Psychopharmacol 2012;26:1366–74. https://doi.org/10.1177/0269881112453210.

[55] Oliveira LA, Gomes-de-Souza L, Benini R, Crestani CC. Control of cardiovascular responses to stress by CRF in the bed nucleus of stria terminalis is mediated by local NMDA/nNOS/sGC/PKG signaling. Psychoneuroendocrinology 2018;89:168–76. https://doi.org/10.1016/j.psyneuen.2018.01.010.

[56] Benini R, Oliveira LA, Gomes-de-Souza L, Rodrigues B, Crestani CC. Habituation of the cardiovascular response to restraint stress is inhibited by exposure to other stressor stimuli and exercise training. J Exp Biol 2020;223:jeb219501. https://doi.org/10.1242/jeb.219501.

[57] Benini R, Oliveira LA, Gomes-de-Souza L, Crestani CC. Habituation of the cardiovascular responses to restraint stress in male rats: influence of length, frequency and number of aversive sessions. Stress 2019;22:151–61. https://doi.org/10.1080/10253890.2018.1532992.

[58] Chan TC, Wall RA, Sutter MC. Chronic ethanol consumption, stress, and hypertension. Hypertension 1985;7:519–24. https://doi.org/10.1161/01.HYP.7.4.519.

[59] Chan TCK, Sutter MC. Ethanol consumption and blood pressure. Life Sci 1983;33:1965–73. https://doi.org/10.1016/0024-3205(83)90734-8.

[60] Engi SA, Planeta CS, Crestani CC. Effect of Voluntary Ethanol Consumption Combined with Testosterone Treatment on Cardiovascular Function in Rats: Influence of Exercise Training 2016:1–18. https://doi.org/10.1371/journal.pone.0146974.

[61] El-Mas MM, Fan M, Abdel-Rahman AA. Endotoxemia-mediated induction of cardiac inducible nitric-oxide synthase expression accounts for the hypotensive effect of ethanol in female rats. J Pharmacol Exp Ther 2008;324:368–75. https://doi.org/10.1124/jpet.107.127498.

[62] El-Mas MM, Fan M, Abdel-Rahman AA. Facilitation of myocardial PI3K/Akt/nNOS signaling contributes to ethanol-evoked hypotension in female rats. Alcohol Clin Exp Res 2009;33:1158–68. https://doi.org/10.1111/j.1530-0277.2009.00939.x.

[63] Duan J, Esberg LB, Ye G, Borgerding AJ, Ren BH. Influence of gender on ethanol-induced ventricular myocyte contractile depression in transgenic mice with cardiac overexpression of alcohol dehydrogenase 2003;134:607–14.
[64] Takase B, Kurita A, Noritake M, Uehata A, Maruyama T, Nagayoshi H, et al. Heart rate variability in patients with diabetes mellitus, ischemic heart disease, and congestive heart failure. J Electrocardiol 1992;25:79–88. https://doi.org/10.1016/0022-0736(92)90112-D.

[65] Tsuji H, Larson MG, Venditti FJ, Manders ES, Evans JC, Feldman CL, et al. Impact of reduced heart rate variability on risk for cardiac events. The Framingham Heart Study. Circulation 1996;94:2850–5. https://doi.org/8941112.

[66] Crestani CC, Alves FHF, Busnardo C, Resstel LBM, Correa FMA. N-Methyl-d-aspartate glutamate receptors in the hypothalamic paraventricular nucleus modulate cardiac component of the baroreflex in unanesthetized rats. Neurosci Res 2010;67:317–26. https://doi.org/10.1016/j.neures.2010.05.001.

[67] Zhang Z-Q, Julien C, Barrès C. Baroreceptor modulation of regional haemodynamic responses to acute stress in rat. J Auton Nerv Syst 1996;60:23–30. https://doi.org/10.1016/0165-1838(96)00023-9.

[68] Carrive P. DUAL ACTIVATION OF CARDIAC SYMPATHETIC AND PARASYMPATHETIC COMPONENTS DURING CONDITIONED FEAR TO CONTEXT IN THE RAT. Clin Exp Pharmacol Physiol 2006;33:1251–4. https://doi.org/10.1111/j.1440-1681.2006.04519.x.

[69] Zhen LI, Krause DN, Doolen S, Piper Duckles SUE. Ovariectomy eliminates sex differences in rat tail artery response to adrenergic nerve stimulation. Am J Physiol - Hear Circ Physiol 1997;41. https://doi.org/10.1152/ajpheart.1997.272.4.h1819.

[70] Mendelsohn ME. Protective effects of estrogen on the cardiovascular system. Am J Cardiol 2002;89:12–7. https://doi.org/10.1016/S0002-9149(02)02405-0.

[71] Hodgin JB, Knowles JW, Kim HS, Smithies O, Maeda N. Interactions between endothelial nitric oxide synthase and sex hormones in vascular protection in mice. J Clin Invest 2002;109:541–8. https://doi.org/10.1172/JCI0214066.

[72] Baudrie V, Tulen JHM, Blanc J, Elghozi J-L. Autonomic components of the cardiovascular responses to an acoustic startle stimulus in rats. J Auton Pharmacol 1997;17:303–9. https://doi.org/10.1046/j.1365-2680.1997.00465.x.

[73] dos Reis DG, Fortaleza EAT, Tavares RF, Corrêa FMA. Role of the autonomic nervous system and baroreflex in stress-evoked cardiovascular responses in rats. Stress 2014;17:362–72. https://doi.org/10.3109/10253890.2014.930429.

[74] Tirapelli CR, Leone AFC, Coelho EB, Resstel LBM, Corrêa FMA, Lanchote VL, et al. Effect of ethanol consumption on blood pressure and rat mesenteric arterial bed, aorta and carotid responsiveness. J Pharm Pharmacol 2007;59:985–93. https://doi.org/10.1211/jpp.59.7.0011.

[75] Resstel LBM, Scopinho AA, Lopes da Silva A, Rodrigues JA, Correia FMA. Increased Circulating Vasopressin May Account for Ethanol-induced Hypertension in Rats. Am J Hypertens 2008;21:930–5.
[76] Vasconcelos SMM, Cavalcante RA, Aguiar LMV, Sousa FCF, Fonteles MMF, Viana GSB. Effects of chronic ethanol treatment on monoamine levels in rat hippocampus and striatum. Brazilian J Med Biol Res 2004;37:1839–46. https://doi.org/10.1590/S0100-879X2004001200009.

[77] Ladipo C, Adigun S, Nwaigwe C, Adegunloye B. Chronic Ethanol Consumption Alters Vascular Smooth Muscle Responses In Rats. Clin Exp Pharmacol Physiol 2002;29:707–9. https://doi.org/10.1046/j.1440-1681.2002.03721.x.

[78] Marchi KC, Muniz JJ, Tirapelli CR. Hypertension and chronic ethanol consumption: What do we know after a century of study? World J Cardiol 2014;6:283–94. https://doi.org/10.4330/wjc.v6.i5.283.

[79] Stewart CW, Kennedy RH. Effects of chronic ethanol consumption on aortic constriction in male and female rats. Eur J Pharmacol 1999;366:55–60. https://doi.org/10.1016/S0014-2999(98)00900-5.

[80] Tirapelli CR, Al-Khoury J, Bkaily G, D’Orléans-Juste P, Lanchote VL, Uyemura SA, et al. Chronic Ethanol Consumption Enhances Phenylephrine-Induced Contraction in the Isolated Rat Aorta. J Pharmacol Exp Ther 2006;316:233–41. https://doi.org/10.1124/jpet.105.092999.

[81] Liu J, Tian Z, Gao B, Kunos G. Dose-dependent Activation of Antiapoptotic and Proapoptotic Pathways by Ethanol Treatment in Human Vascular Endothelial Cells. J Biol Chem 2002;277:20927–33. https://doi.org/10.1074/jbc.M110712200.

[82] Toda N, Ayajiki K. Vascular Actions of Nitric Oxide as Affected by Exposure to Alcohol. Alcohol Alcohol 2010;45:347–55. https://doi.org/10.1093/alcalc/agq028.

[83] Randin D, Vollenweider P, Tappy L, Jéquier E, Nicod P, Scherrer U. Suppression of Alcohol-Induced Hypertension by Dexamethasone. N Engl J Med 1995;332:1733–8. https://doi.org/10.1056/NEJM199506293322601.

[84] Ibrahim BM, Fan M, Abdel-rahman AA. Oxidative Stress and Autonomic Dysregulation Contribute to the Acute Time-Dependent Myocardial Depressant Effect of Ethanol in Conscious Female Rats 2014;38:1205–15. https://doi.org/10.1111/acer.12363.

[85] El-Mas MM, Abdel-Rahman AA. Endothelial and neuronal nitric oxide synthases variably modulate the oestrogen-mediated control of blood pressure and cardiovascular autonomic control. Clin Exp Pharmacol Physiol 2014;41:246–54. https://doi.org/10.1111/1440-1681.12207.