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RESEARCH PAPER

Optimisation of CAFE assay for the evaluation of ethanol preference in *Drosophila melanogaster*

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A B S T R A C T:

Alcohol is one of the most harmful psychoactive drugs which can cause various medical, physical, social, financial, and work-related problems.

The fruit fly *Drosophila melanogaster* has been proposed to be a useful model to explore alcohol-induced behavioural, cellular and molecular mechanisms. The capillary feeder assay (CAFE) has been used to measure the development of preference for alcohol that is observable in *Drosophila* as well in mammals including humans.

The aim of this study was to investigate and optimize the CAFE assay to validate further studies using this technique. The parameters that were investigated were: the ethanol concentration and length of exposure, both during pre-exposure and during the preference assay, and whether the sex of the flies affected the outcome. The results indicate that a minimum of two days of pre-exposure of 15% ethanol is required to induce preference for 15% vs 5% ethanol, while there was no significant difference in extending the preference assay beyond two hours and males and females flies behaved in similar manner. Overall these results further validate and better define the usefulness of the CAFE assay for the measurement of alcohol preference in *Drosophila* as a method to explore the mechanisms of preference which may apply also to higher organisms.

KEY WORDS: *Alcohol, ethanol, addiction, Drosophila melanogaster, ethanol preference, CAFE assay.*
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1. INTRODUCTION:

Ethanol, an alcohol produced in natural fermentation, is one of the most commonly consumed psychoactive substances (WHO-alcohol 2022). When consumed in excess, it can lead to behavioural changes that can be harmful to the user potentially leading to organ failure, particularly the kidneys and liver (Varga et al., 2017; Alpert and Heart, 2016). Continued excessive use of ethanol can lead to physiological changes referred to as Alcohol Use Disorder (AUD), these include tolerance, craving, withdrawal and are generally referred to as alcohol addiction. (Witkiewitz et al., 2019). Additionally, AUD has profound impact on the user's immediate family and acquaintances can be harmful to others and is thus a major social-economic cost to society; indeed alcohol has been identified as the most harmful psychoactive drug (Nutt et al., 2010).

The main modes of action alcohol are known to include potentiation at GABA-A receptors, inhibition of glutamate receptor, and additional effects on ion channels resulting in an overall inhibitory effect on neuronal activity (Scaplen and Petrucelli, 2021). Much less is known about the mechanism underlying AUD other than an involvement of the dopaminergic system as is the case of many psychoactive drugs. (Nutt et al., 2015)

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In order to elucidate cellular and molecular mechanism of AUD several animal models of been employed including the fruit fly *Drosophila* melanogaster. *Drosophila* offer several advantages as a model as they have readily observable ethanol-induced behaviours, they have a short life cycle, they have a small well characterized genome which contains 75% of human disease-associated homologous genes, and are relatively inexpensive to maintain (Kaun et al., 2012)

Among the numerous behavioural paradigms that have been used to study alcohol-induced behaviour, the capillary feeder assay (CAFE) has been particularly useful to measure changes in ethanol preference in *Drosophila* (Ja et al., 2007). The fruit flies derive their name from the fact that they are naturally attracted to rotting fruit which contain ethanol. Indeed female fruit flies will preferentially deposit their eggs on organic material containing up to 5% ethanol (Lynch et al., 2017) Experimentally it can be shown that this natural preference for 5% ethanol can be shifted to higher ethanol concentration by continuous or repetitive exposure to higher concentration of ethanol (Devineni and Heberlein, 2009) .This shift in behaviour can be considered an element of the multiple factors that lead to AUD.

In the CAFE assay flies are offered, through capillary tubes, a free choice of food containing different concentrations of alcohol. By measuring the relative consumption of the different foods it is possible to calculate a preference index (Ja et al., 2007). There are some variations within the published protocols, (Ja et al., 2007; Devineni and Heberlein, 2009) and the aim of this study was to establish a reliable protocol for both the induction of alcohol preference (alcohol pre-exposure) and the actual assay. Additionally, it of interest to establish whether there is sex differences in these behaviours. The testing of the range of parameters affecting the assay has allowed better characterization of the assay which can be confidently be used in further studies.

2. Materials and Method:

2.1 Materials

Canton S (wild type) Drosophila melanogaster was obtained from the Bloomington *Drosophila* Stock Centre (University of Indiana, USA). Dry fly food reconstituted in water 1:1 w/v was purchased from Phillip Harris (UK). Ethanol (95%), sucrose and yeast extract was purchased from Merck (UK). Fly tubes and cotton plugs(flugs) were purchased from Flystuff (USA).

2.2. Fly maintenance

Flies were maintained in bottles containing 20g of reconstituted food in an incubator at 25°C, relative humidity >70% and 12h light/dark cycle. To separate flies of different sex or to transfer exact numbers to experimental tubes, flies were lightly anaesthetized with CO_2 and allowed to rest at least 24 hours before being used in an experimental setting.

2.3 Capillary Feeder (CAFE) assay

This method was adapted from Devineni and Heberlein (2009). Each assay tube consisted of two chambers separated by cotton plugs. The bottom chamber contained water which was added via a hole perforated on the side of the tube, this is required to maintain humidity in the chamber. The top chamber is where the flies are housed. Four capillary tubes (5ul) are placed through cut-off pipette tips inserted in the top cotton plug. Capillary tubes were loaded with liquid food consisting of 5% sucrose, 5% yeast extract and different concentrations of ethanol. Mineral was oil was added at the top of the capillaries to avoid evaporation. The position of the meniscus of the liquid food in the tube was measured at the beginning and at the end of the experiment to calculate the volume of food consumed by the flies. Flies (8-10) were transferred to the chamber without anaesthesia and allowed to feed for different lengths of time. In the choice assay each two capillary tubes were loaded with liquid food containing different concentrations of ethanol (X and Y respectively). The preference index (PI) for a particular concentration (X) of ethanol was calculated as

PI= ((Volume of X consumed) - (Volume of Y consumed)) / (total volume consumed)

The PI can range from -1 to +1. Positive values indicates preference (for X), whereas a negative value indicates aversion.

2.4 Statistics

Data were analysed using GraphPad prism, ANOVA analysis with Bonferroni multiple comparison was used and statistical significance was accepted when $p<0.05$.

3. Results

3.1 The effect of ethanol concentration and length of exposure during ethanol pre-exposure

Flies have a natural preference for food containing 5% ethanol however this preference can be altered by pre-exposing the flies to higher concentration of ethanol. To optimize the pre-exposure protocol, flies were pre-exposed to 0%, 5 % and 15% ethanol for one or two days. On the final day, a two-hour preference assay was conducted for 15% ethanol vs 0% ethanol. Following a two day pre-exposure with 15% ethanol flies preference for 15% ethanol was significantly increased as compared to flies pre-exposed to 0% or 5% ethanol. One day of pre-exposure was not sufficient for the flies to develop a preference for 15% ethanol (Figure 1).

3.2 The effect of the duration of the preference assay on PI.

To further investigate why one day pre-exposure did not produce significant effect in the previous experiment, the length of the preference assay was extended from 2 to 4 hours. The results indicate that there was no significant effect of increasing the length of the assay. (Figure 2). Similarly, it was of interest to determine whether the length of the assay altered the PI of flies pre-exposed for two days. The results indicate that also for the two day pre-exposure there was no significant difference between the two-hour and four-hour preference assay (Figure 3).

3.3 The effect of the flies'sex on PI

To determine whether the sex of the flies influenced the preference behaviour, male and female *Drosophila* were pre-exposed to 5% and 15% ethanol for two days, , and a preference assay was performed on the following day. The results indicate that there was no significant difference in the PI between the male and female flies (Figure 4)

3.4 Effect of ethanol concentration on PI

Flies were pre-exposed to five different ethanol concentrations over two days: 0%, 5%, 10%, 15%, and 25% ethanol. The preference assay for 15% vs 0% ethanol was then performed on the following day. The results indicate that only 15% ethanol induced a significant ($p < 0.05$) change in PI compared to control flies not exposed to ethanol (Figure 5).

4. Discussion:

4.1. Pre-exposure time has an effect on the preference index:

The aim of this work was to analyse the main parameters of the CAFE assay. In previous work (Devinevi and Heberlein 2009) preference was achieved through multiple preference assays carried out on consecutive days. In this work, to simplify the induction of preference, flies were continuously pre-exposed to ethanol. The results (Figure 1) indicate that exposure for a minimum of two days was required to induce a significant increase in PI for 15% ethanol.

4.2. The impact of the preference assay length on the preference index.

In the original work by Ja et al (2007) flies were observed to consume food from the capillary tubes for up to 5 days and it was noted that the flies consumed food at relatively constant rate. In this work, it was important to establish whether the flies were given sufficient time during the preference assay for significant changes in PI to be measured. A comparison of different duration of the preference assay (figure 2 and 3) indicate that there is no significant difference in the PI values between 2 and 4 hours duration of the preference assay. It can be concluded that the important parameter is the length of the preexposure as opposed the length of the preference assay in order to be able to measure a shift in preference from 5% to 15% ethanol.

4.3: The impact of the flies' sex on the preference index.

In previous work some authors state that they segregate flies by sex and use only one sex (Ja et al,. 2007) and some authors do not specify this point (Devinevi and Heberlein 2009) and are thus presumably using both sexes together. In this work the impact of the flies' sex was specifically assessed and the data (Figure 4) indicated that there was no significant difference in the response of males and females. Therefore, it is acceptable to use both sexes in the same experiment as it is case for this study for all figures except for figure 4.

4.4. The effect of ethanol concentration during pre-exposure

The CAFE is designed to measure a shift of the natural preference of flies for 5% ethanol to a higher concentration. Most researchers measure the shift to 15% ethanol (Devinevi and Heberlein 2009). It was of interest to determine whether different doses of ethanol would have a similar effect. The results indicate that while 10% showed an apparent increase in PI the results were not statistically significant. Similarly, 25% ethanol produced a non-significant increase in PI while 15% ethanol produced a significant increase in PI and it thus the most useful concentration to use in the CAFE assay.

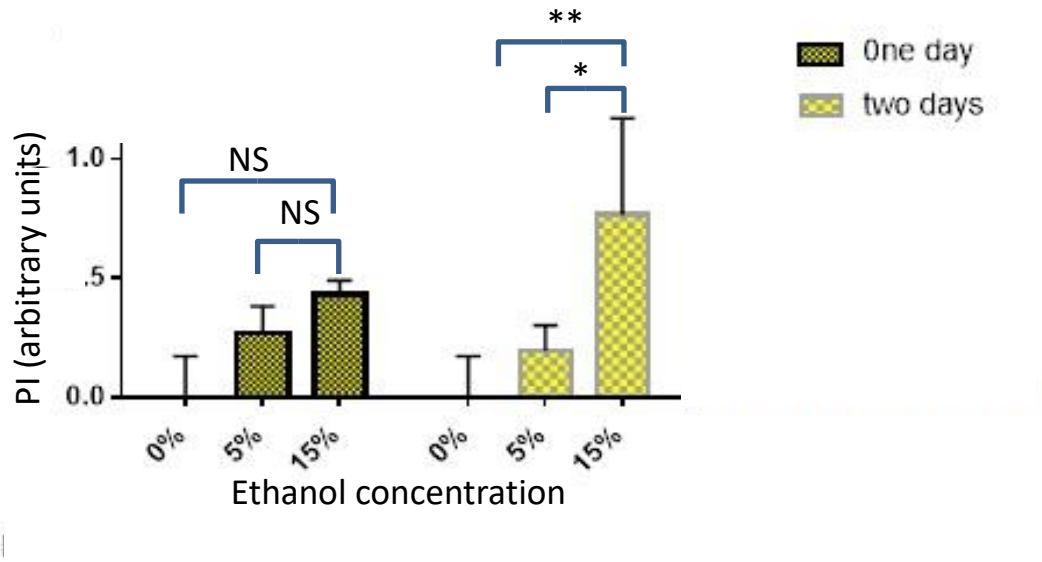

Figure 1: The effect of pre-exposure on PI. Flies were pre-exposed for one or two days to the different concentrations of ethanol indicated on the x axis, The preference assay was carried out for two hours (0% vs 15% ethanol). The preference index (PI) was calculated as described. Each column represents average values of multiple assay vials with six flies in each vial (n=3, error bars SD). Two-way analysis of variance (ANOVA) and Bonferroni's post analysis, showed a significant difference (* $p < 0.05$, ** $p < 0.01$) between 15% vs 0%, ($p=0.009$) and 15% vs 5% ($p=0.0154$), this effect was not significant when flies were pre-exposed for only one day.

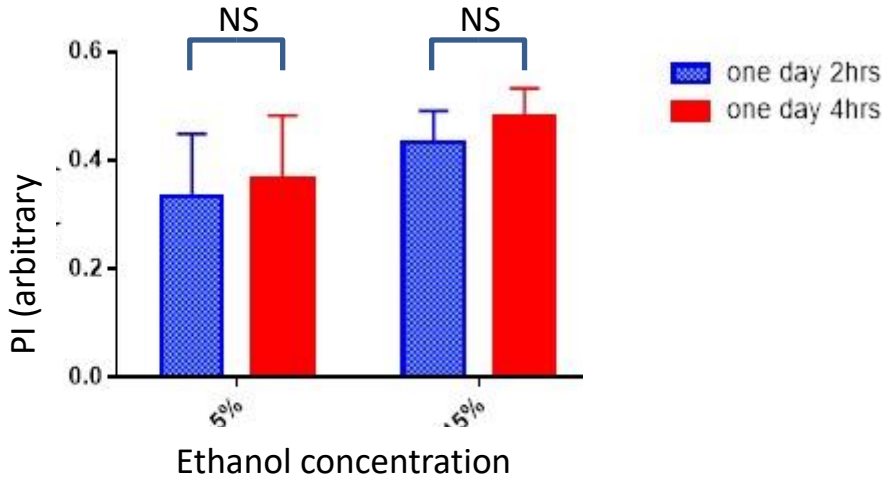

Figure 2: The effect of the duration of preference assay on PI in one day pre-exposure. Flies were pre-exposed for one day to 5% ethanol or 15% ethanol as indicated on the x axis. On the next day, the

preference assay was carried out for two hours or four hours. Each column represents average values of multiple assay vials with six flies in each vial (n=3, error bars = SD). Analysis with two way ANOVA and Bonferroni's post analysis shows no significant difference between two hours (p = 0.4264) and four hours (p = 0.3277) for 5% and 15% ethanol pre-exposure respectively.

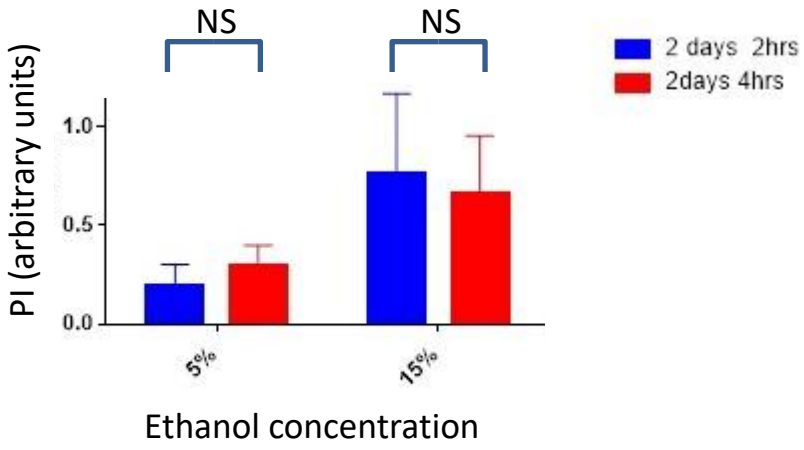

Figure 3: The effect of the duration of the preference assay on PI in two days pre-exposure. Flies were pre-exposed for two days to 5% ethanol or 15% ethanol. On the next day, the preference assay was carried out for two hours or four hours. Each column represents average values of multiple assay vials with six flies in each vial (n=3, error bars = SD). Analysis with two way ANOVA Bonferroni's test shows no significant difference between two hours (p=0.0543) versus four hours (p value=0.2416).

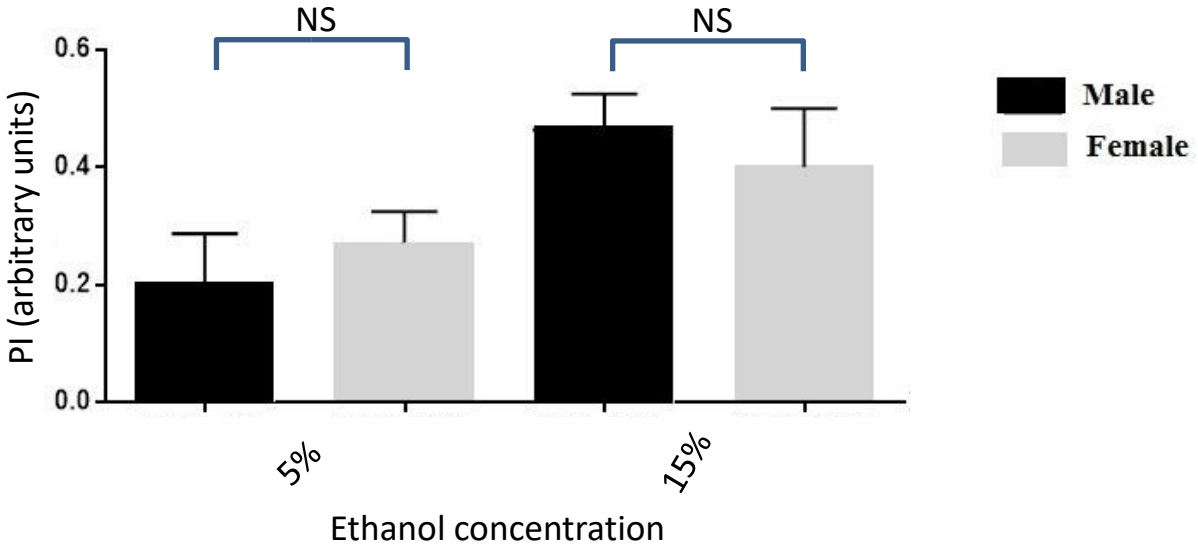

Figure 4. Effect of flies' sex on PI. Male and female flies were pre-exposed for one day to 5% ethanol and 15% ethanol. On the next day, the preference assay was for carried out for two hours. Each column represents average values of multiple assay vials with six flies in each vial (n=3, error bars = SD). Two way

ANOVA analyses with Bonferroni's analysis showed no significant difference between male vs. female for both 5% and 15% ethanol ($p > 0.05$)

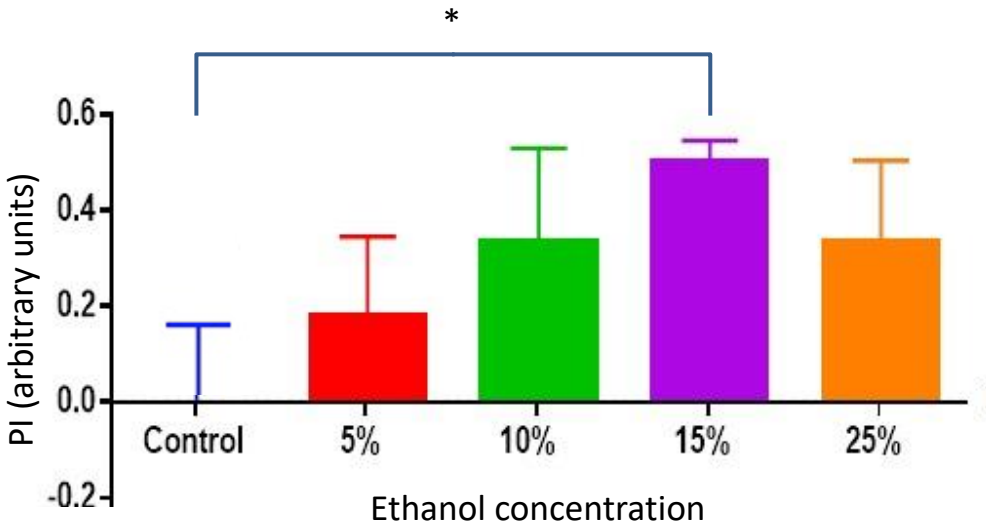

Figure 5: Effect of ethanol concentration during pre-exposure on PI. Flies were pre-exposed for two days to different ethanol concentrations; the next day preference assay was carried out for two hours Each column represents average values of multiple assay vials with six flies in each vial (n=3, error bars = SD). ANOVA with Bonferroni's analysis indicate that only 15% ethanol pre-exposure led to a significantly increase in PI as compared to unexposed control flies.

5. Conclusion

In summary this work has investigated the main parameters of the CAFE assay, establishing that the concentration (15% ethanol) and the length of pre-exposure (2 days) are the key factors required to induce a shift in the preference for food containing different level of ethanol. Other parameters such as the duration of the preference assay the assay and flies sex do not seem to have an impact on the measurement of preference. It should be considered that in this work, wild type Canton S flies were used. It would be of interest to see whether flies with different genetic background behave differently. This would of course be of importance if the CAFE assay were to be used to look at the effect of gene knock-outs or knock-down where controls with similar genetic background would have to be used. The CAFE assay also offers the possibility to test pharmacologically the mechanisms of AUD (Koyyada et al., 2018), it is thus important to establish the conditions at which the assay should be carried out to allow a comparison between results from different investigators. The work described here will hopefully contribute to the standardisation of the CAFE assay and to further advances in the understanding of the mechanisms of AUD.

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Conflict of interest

There is no conflict of interest in publishing this data

6. References

Alpert L, Hart J. The Pathology of Alcoholic Liver Disease. Clin Liver Dis. 2016 Aug;20(3):473-89. doi: 10.1016/j.cld.2016.02.006. Epub 2016 Apr 9. PMID: 27373610.

Devineni AV, Heberlein U. Preferential ethanol consumption in *Drosophila* models features of addiction. Curr Biol. 2009 Dec 29;19(24):2126-32.

doi: 10.1016/j.cub.2009.10.070. Epub 2009 Dec 10. PMID: 20005106; PMCID: PMC2805771.

Ja WW, Carvalho GB, Mak EM, de la Rosa NN, Fang AY, Liong JC, Brummel T, Benzer S. Prandiology of *Drosophila* and the CAFE assay. Proc Natl Acad Sci U S A. 2007 May 15;104(20):8253-6. doi: 10.1073/pnas.0702726104. Epub 2007 May 9. PMID: 17494737; PMCID: PMC1899109.

Kaun KR, Devineni AV, Heberlein U. *Drosophila* melanogaster as a model to study drug addiction. Hum Genet. 2012 Jun;131(6):959-75. doi: 10.1007/s00439-012-1146-6. Epub 2012 Feb 17. PMID: 22350798; PMCID: PMC3351628.

Koyyada R, Latchooman N, Jonaitis J, Ayoub SS, Corcoran O, Casalotti SO. Naltrexone Reverses Ethanol Preference and Protein Kinase C Activation in *Drosophila* melanogaster. Front Physiol. 2018 Mar 14:9:175. doi: 10.3389/fphys.2018.00175. PMID: 29593550; PMCID: PMC5861182.

Lynch ZR, Schlenke TA, Morran LT, de Roode JC. Ethanol confers differential protection against generalist and specialist parasitoids of *Drosophila* melanogaster. PLoS One. 2017 Jul 12; 12(7):e0180182. doi: 10.1371/journal.pone.0180182. PMID: 28700600; PMCID: PMC5507509.

Nutt DJ, King LA, Phillips LD; Independent Scientific Committee on Drugs. Drug harms in the UK: a multicriteria decision analysis. Lancet. 2010 Nov 6;376(9752):1558-65. doi: 10.1016/S0140-6736(10)61462-6. Epub 2010 Oct 29. PMID: 21036393.

Nutt DJ, Lingford-Hughes A, Erritzoe D, Stokes PR. The dopamine theory of addiction: 40 years of highs and lows. Nat Rev Neurosci. 2015 May;16(5):305-12. doi: 10.1038/nrn3939. Epub 2015 Apr 15. PMID: 25873042.

Scaplen KM, Petruccelli E. Receptors and Channels Associated with Alcohol Use: Contributions from *Drosophila*. Neurosci Insights. 2021 Mar 30;16:26331055211007441. doi: 10.1177/26331055211007441. PMID: 33870197; PMCID: PMC8020223.

Varga ZV, Matyas C, Paloczi J, Pacher P. Alcohol Misuse and Kidney Injury: Epidemiological Evidence and Potential Mechanisms. Alcohol Res. 2017;38(2):283-288. PMID: 28988579; PMCID: PMC5513691.

Witkiewitz K, Litten RZ, Leggio L. Advances in the science and treatment of alcohol use disorder. Sci Adv. 2019 Sep 25; 5 (9):eaax4043. doi: 10.1126/sciadv.aax4043. PMID: 31579824; PMCID: PMC6760932.

WHO-alcohol . Last accessed 15/7/2022 https://www.who.int/health-topics/alcohol#tab=tab_1