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## Time-dependent interacting effects of caffeine, diazepam, and ethanol on zebrafish behaviour



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### ABSTRACT

Zebrafish have become a popular animal model for behavioural pharmacology due to their small size, rapid development, and amenability to high throughput behavioural drug screens. Furthermore, water-soluble compounds can be administered via immersion of the fish in the drug solution, which provides a non-invasive drug delivery method. Numerous studies have demonstrated stimulant effects of alcohol. Diazepam and caffeine, on the other hand have been found to have inhibitory effect on locomotor activity in zebrafish. However, the time-dependent changes induced by these psychoactive drugs are rarely reported, and potential drug interactions have not been examined in zebrafish, despite the translational relevance of this question. In the current study, we examine time- and dose-dependent changes in zebrafish following exposure to caffeine, diazepam, and ethanol quantifying four different behavioural parameters over a 30 min recording session. We subsequently analyze potential drug-drug interactions by co-administering the three drugs in different combinations. Our time-course and dose-response analyses for each of the three drugs represent so far the most detailed studies available serving as a foundation for future psychopharmacology experiments with zebrafish. Furthermore, we report significant interactions between the three drugs corroborating findings obtained with rodent models as well as in humans, providing translational relevance for the zebrafish model.

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### 1. Introduction

Alcohol and caffeine are two of the world's most commonly consumed drugs (Beydoun et al., 2014), and the drug cluster of benzodiazepines is among one of the most commonly prescribed medications for treating depression and anxiety disorders (Altamura et al., 2013). The use of psychoactive stimulants such as alcohol and caffeine is likely driven by their stimulant and possibly addictive properties (Budygin and Weiner, 2015; Ferre, 2016). The concurrent consumption of alcohol and caffeine has become increasingly popular due to the common belief that caffeine can counteract the intoxicating effects of alcohol. Although caffeine has been shown to alter the behavioural effects of alcohol, the interactions between these two drugs are poorly understood (López-Cruz et al., 2013). Similarly, long-term use of commonly prescribed

benzodiazepines such as diazepam increases the risk of benzodiazepine abuse and dependence (Janhsen et al., 2015). In addition to treating depression and anxiety-like disorders, benzodiazepines are also prescribed to treat alcohol addiction, especially during the alcohol withdrawal period (Askgaard et al., 2016). However, benzodiazepines have also been reported to increase alcohol consumption (Marczinski et al., 2016) and their combined use has been fatal due to their pharmacological interactions (Koski et al., 2002). Although alcohol, caffeine and benzodiazepines are commonly consumed, their interactions with each other are poorly understood, warranting behavioural pharmacological research studying this question.

Numerous studies have demonstrated that the effects of pharmacological compounds developed for mammals – including rodents and humans – are similar in zebrafish (Stewart et al., 2015; Abreu et al., 2016; Vacaru et al., 2014). For example, alcohol's stimulant and diazepam's sedative effects observed in mammals (Zhou and Kreek, 2014; Fernandes et al., 1999; Zanette et al., 2013) have also been shown in zebrafish (Rosemberg et al., 2012; Nowicki et al., 2015; Bencan et al., 2009). Similarly, caffeine's anxiogenic properties have also been demonstrated in zebrafish (Egan et al., 2009; Maximino et al., 2014; Richendrer et al., 2012).

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Although many studies have revealed that the effects of different pharmacological compounds are evolutionarily conserved from mammals to zebrafish (Rihel et al., 2010), drug interaction effects have remained unexplored in zebrafish. This represents a limitation of the zebrafish model from the perspective of translational relevance because several psychoactive drugs, including alcohol, benzodiazepines (e.g. diazepam), and caffeine are often consumed concurrently among humans (Polak et al., 2016; Koski et al., 2002).

Here we focus on two distinct types of interactions: one, the time-dependent effects of drugs, i.e. time  $\times$  drug interaction; and two, the interaction between different drugs, i.e. drug  $\times$  drug interaction. The behavioural effects of a specific drug are often dependent on the blood and brain concentration of the compound, which is dependent on several factors including absorption, distribution, metabolism, and excretion (ADME) characteristics of the given drug (Cadwell et al., 1995). Detailed ADME characterization of drugs has been rarely performed in zebrafish, partly because it is a labour intensive process, and partly because the zebrafish is a relatively new research species in pharmacology. In this study, we decided to employ an alternative approach, which may shed some light on ADME dependent processes at least from the perspective of functional effects of the drugs under study. We decided to perform a detailed temporal analysis of drug induced behavioural changes. Analysis of the time-course of drug effects is rarely reported in the zebrafish. The quantification of time-dependent behavioural responses may be an effective and relatively simple method with which functional changes induced by the drug in the brain may be characterized. Various drugs are expected to alter zebrafish behavioural responses in a time-dependent manner. For example, caffeine is classified as a psychoactive stimulant and has been shown to increase locomotor activity in rodents in a time-dependent manner (El Yacoubi et al., 2000). Interestingly, zebrafish studies have reported both depressant (Ladu et al., 2015) and stimulant effects (Maximino et al., 2011) following caffeine exposure. These inconsistencies may be partly due to differences in concentration as well as time-related factors idiosyncratic to each study.

In addition to the time-dependent effects of drugs, drug  $\times$  drug interactions have also received little attention in zebrafish. Examining drug  $\times$  drug interactions in zebrafish will help us elucidate mechanistic questions, e.g. how different biochemical pathways may interact in the zebrafish brain. Drug interaction studies with model organisms such as the zebrafish are also relevant from a human health perspective, because they mimic what often happens in everyday life resulting from the concurrent use of multiple drugs. Alcohol and diazepam are two psychoactive drugs whose actions on the central nervous system have been thoroughly investigated with mammals (Pietrzak and Czarnecka, 2005). Concurrent consumption of these drugs is common with adverse side effects due to their interactions (Koski et al., 2002). Caffeine is a psychoactive stimulant and can act antagonistically against both alcohol and diazepam (López-Cruz et al., 2013), and is also often consumed in combination with one or both of the above drugs (Polak et al., 2016). Although the separate effects of alcohol, diazepam, and caffeine on zebrafish motor behavioural responses have been investigated, the effects of co-administration of these compounds, and the potential interaction among them have not been systematically examined.

In the current study, we provide the most detailed temporal and dose-response analysis reported for caffeine, diazepam, and ethanol, and examine both time  $\times$  drug and drug  $\times$  drug interactions in zebrafish. To examine time  $\times$  drug interactions, we first conducted multiple dose-responses and time-course analyses for acutely administered alcohol, caffeine, and diazepam respectively. We quantified different motor responses in zebrafish over a 30 min drug exposure session. We subsequently examined drug  $\times$  drug interactions by characterizing the time-dependent behavioural effects of drugs co-administered in all possible different combinations.

## 2. Methods

### 2.1. Animals and housing

641 adult AB strain zebrafish (12–14 months old) of mixed sexes were used for behavioural testing. Zebrafish were raised and housed in 37 L tanks ( $n = 20$  per tank) with mechanical, chemical, and biological filtration. The progenitors of this strain originated from the ZFIN Center (Eugene, Oregon, USA) and have undergone many generations of inbreeding in the laboratory. Water quality was maintained at optimal parameters (pH: 6.5–7.5, conductivity: 150–300  $\mu$ S, temperature: 26.5–28.5  $^{\circ}$ C). Zebrafish were fed twice a day with dry flake food (2:1 ratio of TetraMin:Spirulina) and once a week with nauplii of brine shrimp (*Artemia salina*). Zebrafish were raised on a 14:10 light:dark cycle with lights turning on at 08:00. Behavioural tests were conducted between 10:00 and 17:00.

### 2.2. Behavioural testing procedure and experimental design

Zebrafish were individually netted from their 37 L housing tanks and placed singly into a 1.5 L trapezoidal testing tank containing 1 L of water from their housing tanks and the corresponding concentration of caffeine, diazepam, or alcohol for 30 min as described below. We chose 30 min as the duration of exposure since we were interested in the total time-course of behavioural effects, and all three drugs have previously been reported to alter behavioural changes within 3–10 min of exposure (Cachat et al., 2010; Bencan et al., 2009; Wong et al., 2010). The sides, back, and bottom of the testing tank were covered with white corrugated plastic sheets to block external cues and to provide a clear and consistent testing background for video-tracking. A video camera was placed in front of each testing tank and recorded the entire 30 min drug exposure session.

Our goal was to conduct a detailed dose-response and time-course analysis for caffeine, diazepam, and ethanol on zebrafish behavioural responses to serve as a reference for future studies to determine effect sizes and conduct power analyses. However, rather than conducting one large experiment with different doses of each of the 3 drugs (which would significantly reduce statistical power due to the number of groups and increased error variation due to the increased length of such a study), we conducted multiple smaller dose-response experiments for each drug. We limited the number of groups in each experiment to 4–8 concentrations, which is more typical of the number of doses employed in zebrafish pharmacology studies. Furthermore, we utilized sample sizes ( $n = 14$ –20) which have been commonly employed in the past to make our current study more comparable with previous psychopharmacology studies conducted with zebrafish (Cachat et al., 2010; Bencan et al., 2009; Maximino et al., 2014; Nowicki et al., 2014; Richendrfer et al., 2012; Tran et al., 2016).

### 2.3. Drug administration

Caffeine (Sigma-Aldrich, Cat #C0750) was added directly to the testing water to make a final concentration of 0, 1, 5, and 10  $\mu$ M in experiment 1a ( $n = 14$  per group), 0, 1, 10, and 100  $\mu$ M in experiment 1b ( $n = 14$  per group), and 0, 20, 40, 80, 160, 320, 640, and 1280  $\mu$ M in experiment 1c ( $n = 15$  per group). Diazepam (Toronto Research Chemicals Inc., Cat D416855) was added directly to the testing water to achieve a final concentration of 0, 0.1, 0.5, and 2.5  $\mu$ M in experiment 2a ( $n = 24$  per group) and a final concentration of 0, 5, 10, and 20  $\mu$ M in experiment 2b ( $n = 14$  per group). Since diazepam has low water solubility, it was dissolved using dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Cat #D-8779) as a vehicle. The final concentration of DMSO in all tanks for experiment 2 was 0.01% v/v. The concentration of DMSO used in this experiment has previously been shown not to alter behavioural response in zebrafish of the AB strain (Tran et al., 2015a, 2015b). Ethanol (Commercial Alcohols, Brampton, ON, CA) was mixed directly

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