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Behavioral Screening of Alcohol Effects and Individual Differences in Zebrafish (*Danio rerio*)

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Abstract

Aim: To better understand the individual differences that make up a population, this study aimed to evaluate the effects of different alcoholic concentrations on the behavioral profiles of zebrafish (*Danio rerio*).

Methods: For this purpose, adult animals were separated into two behavioral profiles: bold and shy, according to the emergence order. Bold and shy fish were individually tested for exploration after exposure to the drug. Acute exposure treatments were alcohol 0.00, 0.10, 0.25 and 0.50%. The behavioral parameters evaluated were speed while moving, maximum speed, total distance traveled and distance from the bottom of the tank.

Results: For the groups that did not receive alcohol, bold animals showed higher speed while moving. Shy 0.00% and shy 0.10% had the highest maximum speed compared with other concentrations and profiles. For the distance from the bottom tank, our results showed that the increase induced by the low acute dose (0.10%) was observed for both profiles.

Conclusions: Our results corroborate with previous findings that alcohol affects the behavioral profiles of zebrafish differently, with bold animals apparently more resistant to these changes.

INTRODUCTION

Natural selection acts on populations making individuals better adapted to cope with pressures imposed by the environment where they evolved (Koolhaas et al. 2010). To survive these pressures, individuals from the same population exhibit different coping strategies and develop behavioral characteristics according to their genotype and phenotype (Koolhaas et al. 1999). As the environmental challenges vary, the behavioral phenotype of an individual also vary through their development, and according to life experiences and social interactions.

Research has been developed in recent years around this theme to better understand the behavioral profile of animal groups (Svartberg 2005; Frost et al. 2007). While in the past, differences in responsiveness between individuals were viewed as noise and outliers discarded, today such variations are understood as differences in individuals' profiles, and help to understand how populations are composed and how they react to environmental pressure. One of the most studied profiles is the bold–shy continuum as it covers animals beyond the primate group (Gosling and John 1999).

Within this continuum, bold individuals correspond to animals that, when placed in an unfamiliar context, tend to be exploitative and take risk, show low sociability, and present less anxious-like behavior (White et al. 2017). Individuals classified as shy, when faced with unknown situations, tend to be risk-averse, do not usually explore the new environment, aggregate to social groups and present increased anxiety response (Moscicki and Hurd 2015). Between the boldest and the shyest individuals, there is a gradient of features that compose the population heterogeneity of behavioral and physiological responses. This continuum has been targeted in the search for answers regarding the susceptibility of certain individuals to develop trends toward drug use and abuse (Ninkovic and Bally-Cuif 2006; Nielsen et al. 2018). For instance, the bold and shy extremes differently respond to social interactions when subjected to alcohol concentrations (Araujo-Silva et al. 2018). However, there are still gaps to be filled when it comes to how alcohol affects the behavioral repertory of these profiles.

Alcohol is one of the most commonly used drugs worldwide (World Health Organization 2018). Being a small molecule extremely soluble in water and fat, it reaches the brain very quickly and causes distraction, changes perception and alters motor patterns, besides causing other symptoms (Hoffman and Tabakoff 1996; Tran and Gerlai 2013). The neurological effects of alcohol intake show a biphasic pattern: low to moderate doses cause stimulant and anxiolytic effects, factors correlated with addiction, while higher doses exert depressive effects such as loss of motor control, disorientation and sedation (Rosenbloom et al. 2004; Carlson et al. 2012; Miller et al. 2013; Quoilin et al. 2013; Tran and Gerlai 2013).

Addictive drugs, such as alcohol, increase brain dopamine and serotonin levels up to 10-fold and alter the normal secretion of these neurotransmitters. In the absence of the drug, the response is the need for more dopamine and serotonin in the synaptic cleft that only the drug can provide (Yoshimoto et al. 1992; Weiss and Porrino 2002). However, not all individuals within a population develop tolerance for this substance and this fact can be explained by the individual differences presented in a population set. From this perspective, this work aims to conduct a behavioral screening of the bold and shy profiles under the effects of alcohol using the zebrafish (*Danio rerio*) as an animal model.

A series of studies involving the effects of alcohol have been exploring the zebrafish (*D. rerio*) as an animal model (Amorim et al. 2017; Chacon and Luchiari 2014; Gerlai et al. 2000). Given its broad application to neuroethological research, this species exhibits a complex behavioral repertoire, exhibits social preference for conspecific individuals and shares 70% of genes with humans, making it a potential translational model (Egan et al. 2009; Kalueff et al. 2014; Norton and Bally-Cuif 2010). To study individual differences, this model was shown to have the necessary characteristics to clarify gaps in the characterization of behavioral profiles and effects of alcohol (Sher 1985; Gerlai et al. 2009), as it responds to the drug in a similar way (Maximino et al. 2014) and presents individual differences (Tudorache et al. 2013) as humans do.

Because alcohol changes the way an individual perceives the physical environment (Samson and Harris 1992; Gilpin and Koob 2008; Nasrallah et al. 2011; Amorim et al. 2017), it is hypothesized that distinct profiles may differ in drug responsiveness. Therefore, individuals characterized as shy under the effect of the drug are expected to explore more the environment and have lower levels of anxiety, while those characterized as bold have greater resistance to the effects of alcohol.

MATERIALS AND METHODS

Animals and housing

In this study, we used zebrafish (*D. rerio*, wild type, both sexes, 6 months, 0.58 ± 0.11 g) obtained from a local farm (Natal, Rio Grande do Norte state) and housed in 50-L tanks with multiphase filtration systems. Temperature, pH and oxygenation were measured regularly (28°C; pH ~ 6.7; O2 ~ 6 mg/L), and the photoperiod adopted was 12 h light/12 h dark. Fish were fed twice daily with pelleted commercial feed (Alcon Basic, 44% protein; 5% fat) and frozen brine shrimp. All procedures were approved by the Animal Use Ethics Committee of the Federal University of Rio Grande do Norte (CEUA 122.055/2018).

Behavioral profile determination

Behavioral profiles were established according to the protocol described by Tudorache et al. (2013), based on the tendency of zebrafish to prefer dark areas and to remain close to the group.

After being housed for 15 days under the stocking conditions described above, the animals were randomly selected and divided into 20 groups of 10 individuals each. For the emergency test, a 15-L (40 × 25 × 20 cm, width × depth × height) tank was divided into two equally sized compartments, with one side covered bottom and walls in black and the other side was white with white walls. In the middle of the tank, a guillotine door was used to control the passage of the fish from black to white side.

A group of 10 fish was placed in the initial zone (black) and allowed to acclimate for 2 min. Then, the door was raised until the first individual passed through it; the door was closed for 30 s and the fish that entered the white compartment was transferred to another tank. The door was reopened until the next individual passed through it and then closed again for 30 s, and so on. The first three animals leaving the dark compartment were considered bold, while the last three animals leaving the initial compartment were classified as shy. The fish were submitted to this procedure only once to avoid habituation and learning. This procedure was repeated for all animals until a stock of 60 bold and 60 shy was obtained. Intermediate animals were excluded from testing and returned to home tanks.

Drug exposure and behavioral registration

Eight groups of fish were formed: four from bold animals ($n = 15$ per group) and four from shy animals ($n = 15$ per group), each group kept in different tanks. After separation of the behavioral profiles, the fish were individually exposed to a 60-min acute treatment with 0.00, 0.10, 0.25 or 0.50% absolute ethyl alcohol 99.8% PA (Dynamics, Contemporary Chemistry Ltd.) and behavior was recorded with a digital camera (Sony Digital Video Camera Recorder; DCR-SX45) in the front array of the fish tank. The videos were analyzed by a tracking software (ZebTrack/UFRN; Pinheiro-da-Silva et al. 2017) developed on MATLAB platform (R2014a; Math Works, Natick, MA). The locomotor parameters evaluated were speed while moving, maximum speed and total distance traveled. Distance from the bottom of the tank was analyzed as a measure of fear and anxiety (Levin et al. 2007; Egan et al. 2009). Following alcohol exposure, the animals were euthanized with clove oil (1 mL/L) according to the protocols established by the Animal Ethics Committee of the Federal University of Rio Grande do Norte.

Statistical analysis

Initially, data were analyzed for homogeneity, normality, collinearity and possible outliers, as suggested by Zuur et al. (2010). Then, we performed a mixed effects model for longitudinal data to considering as dependent variable the behavioral parameters and the independent variables 60 min time and concentrations of alcohol. The mixed model showed random effect factors (represented by behavioral variation within each group) and fixed effect factors (represented by explanatory variables).

To build the mixed model, we used the glmmPQL command from MASS package (Venables and Ripley 2002) from the R Studio program (R Core Team 2019). The glmmPQL command algorithm was applied according to the abnormal distribution and dispersion displayed by the response variable residues during the exploratory analysis. Speed while moving, maximum speed and total distance traveled response variables were classified as positive continuous quantitative data, not including zero ($Y > 0$), while distance from bottom variable was classified as continuous quantitative data ranging from 0 to 10 cm. According to the exploratory data analysis, the best distribution function for maximum speed and total distance traveled was the Gaussian function while for speed while moving and bottom distance datasets were the gamma function. In all cases, post hoc comparisons between treatments of each model were made using Tukey's post hoc test (lsmeans package) (Lenth 2016). For all comparisons, the significance level was set at $P < 0.05$.

RESULTS

The locomotor parameters obtained from zebrafish behavioral profiles exposed to alcohol at different concentrations for 60 min are shown in Figs 1 and 2. Figure 2 shows behavioral changes for every one throughout the 60-min alcohol exposure. Each point in the graphs represents the average behavior of the group at the given time. Comparisons made by the mixed model showed that speed while moving during acute alcohol exposure was significantly changed by alcohol concentrations (GLMM, $\chi^2 = 38.00$, df = 3, $P < 0.001$) and over time (GLMM, $\chi^2 = 15.08$, df = 1, $P < 0.001$), but no statistical significance was obtained between behavioral profiles (GLMM, $\chi^2 = 0.08$, df = 1, $P = 0.7$) (Figs 1A and 2A and B). In terms of interaction, the parameters that showed statistical significance were treatment vs. time (GLMM, $\chi^2 = 17.50$, df = 3, $P < 0.001$), profile vs. treatment (GLMM, $\chi^2 = 82.97$, df = 3, $P < 0.002$) and profile vs. treatment vs. time (GLMM, $\chi^2 = 14.44$, df = 3, $P < 0.002$) (Figs 1A and 2A and B). The Tukey's post hoc comparison test (lsmeans) between groups indicates that bold 0.00% significantly differed from bold 0.10% and bold 0.50%, as well as shy 0.00% significantly differed from the other shy groups. Lsmeans also demonstrated that shy 0.10% and shy 0.25% had the highest speed while moving, significantly different from other treatments.

For maximum speed, mixed model indicated statistical significance between behavioral profiles (GLMM, $\chi^2 = 14.22$, df = 1, $P < 0.001$), treatments (GLMM, $\chi^2 = 8.71$, df = 3, $P = 0.03$) and time (GLMM, $\chi^2 = 6.88$, df = 1, $P < 0.008$) (Figs 1B and 2C and D). In terms of interaction, there were statistical significance in alcohol concentration vs. time (GLMM, $\chi^2 = 8.97$, df = 3, $P = 0.02$), while the interactions profile vs. treatment vs. time were not significant (GLMM, $\chi^2 = 5.73$, df = 3, $P = 0.12$) The post hoc Tukey's (lsmeans) comparison test showed that shy 0.00% and shy 0.10% showed the highest maximum velocity, while the bold 0.00%, bold 0.50% and shy 0.50% had the lowest maximum velocity (Figs 1B and 2C and D).

The mixed model test for total distance traveled showed statistical significance for behavioral profiles (GLMM, $\chi^2 = 5.83$, df = 1, $P < 0.01$), alcohol concentrations (GLMM, $\chi^2 = 37.98$, df = 3, $P < 0.001$) and time (GLMM, $\chi^2 = 7.41$, df = 1, $P = 0.006$) (Figs 1C and 2E and F). Interactions showed statistical significance for profile vs. treatment (GLMM, $\chi^2 = 51.09$, df = 3, $P < 0.001$), treatment vs. time (GLMM, $\chi^2 = 37.90$, df = 3, $P < 0.001$) and profile vs. treatment vs. time (GLMM, $\chi^2 = 39.74$, df = 3, $P < 0.001$). The post hoc Tukey's test (lsmeans) showed that bold 0.00% and shy 0.25% treatments traveled the shortest distance, while bold 0.50% and shy 0.10% traveled the longest distance (Figs 1C and 2E and F).

For distance from a bottom (Figs 1D and 2G and H), the mixed comparison model showed that there was no statistical significance for behavioral profiles (GLMM, $\chi^2 = 0.0002$, df = 1, $P = 0.98$) or time (GLMM, $\chi^2 = 2.28$, df = 1, $P = 0.13$). However, statistical significance was observed for alcohol concentrations (GLMM, $\chi^2 = 23.21$, df = 3, $P < 0.001$) and the interaction alcohol vs. time (GLMM, $\chi^2 = 18.91$, df = 3, $P < 0.001$) with shy 0.10% being the farthest from the bottom and shy 0.25% and shy 0.50% were closer to the bottom of the tank.

DISCUSSION

In the present study, we observed that alcohol affects locomotor activity, depending on concentration, but also on behavioral profile. Some animals are more susceptible to changes in locomotor activity even at moderate doses, as seen in the speed while moving parameter for shy individuals, who had increased locomotor activity at concentrations 0.10 and 0.25%. However, the 0.50% concentration caused decrease in locomotor activity in shy animals. On the other hand, bold individuals showed greater constancy in speed, presenting only a decrease in activity at 0.10% concentration. In general, acute exposure to alcohol accompanied by increased drug concentration in the blood has led to suppression or inhibitory effects of behavioral activity.

In zebrafish, acutely exposure to alcohol was shown to increase locomotor activity at low to moderate concentrations and decrease it at high doses (Gerlai et al. 2006). In previous studies involving the effects of alcohol on behavioral profiles, our group observed that locomotion presents as a pattern since very early in the individual life (Leite-Ferreira et al. 2019). It was also observed that different individuals presented different behavioral responses at maximum swimming speed in novel environment. This locomotor parameter shows that shy 0.00% had a higher maximum velocity than bold 0.00%, and that in the alcoholic concentrations of 0.10, 0.25 and 0.50%, there was a decrease in velocity, corroborating previous studies involving the effects of alcohol on different behavioral profiles (Araujo-Silva et al. 2018).

Studies regarding early and late hatching profiles indicate that differences in the locomotor parameters may be related to the metabolic rate (Leite-Ferreira et al. 2019). Indeed, the differences between bold and shy profiles may also be linked to metabolism. Studies suggest that metabolic differences between individuals are linked to differences in neuroendocrine system activity (Koolhaas et al. 1999), leading to profiles named proactive and reactive responders. From a behavioral standpoint, proactive individuals are considered bolder, more aggressive, dominant and less flexible to routine change. Physiologically, they are characterized as having lower reactivity of the hypothalamus-pituitary-adrenal axis (HPA) and maintaining high levels of basal cortisol, as well as high brain dopaminergic levels (Thörnqvist et al. 2019). On the contrary, reactive individuals are characterized by exhibiting the opposite profile from the behavioral and physiological point of view (Koolhaas et al. 1999; Koolhaas et al. 2007; Leite-Ferreira et al. 2019). The behavioral characteristics of the proactive and reactive profiles are similar to the bold and shy models, suggesting that high dopamine release is typical of the stress coping demonstrated by proactive and bold animals, while the passive stress response exhibited by reactive and shy animals seems more associated with an inhibition of dopamine release (Cabib and Puglisi-Allegra 2012; Thörnqvist et al. 2019).

Previous studies have found that bold and shy zebrafish differ in alcohol responsiveness and social stimulation (Araujo-Silva et al. 2018; Goodman and Wong 2019). However, the present study is the first to evaluate locomotor and anxiety-like behavior over time

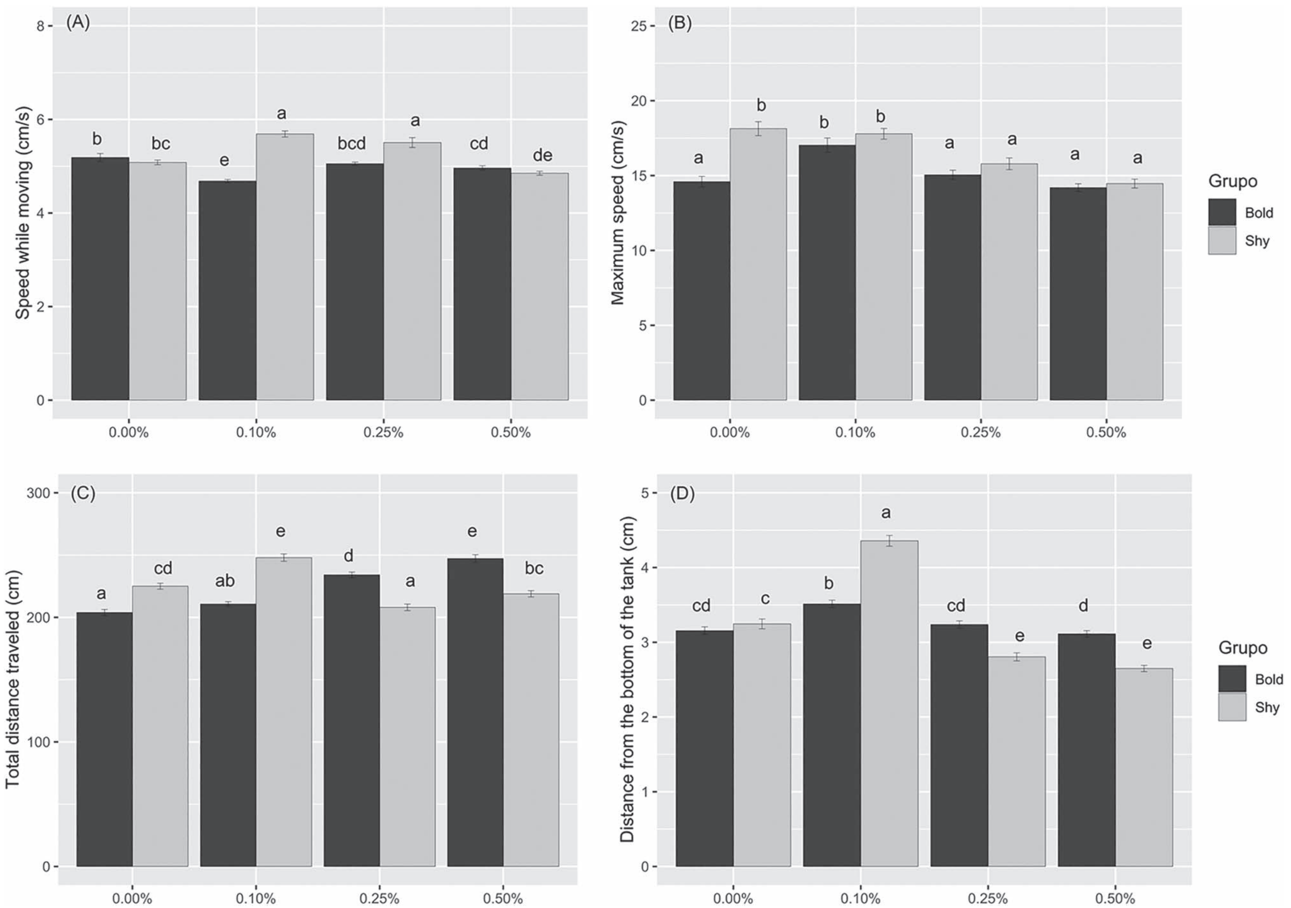

Fig. 1. Behavioral response of zebrafish profiles under acute alcohol exposure. Bold and shy profiles were defined based on the emergency test: the first three fish to leave the dark and shoaling area were considered bold and the last three to leave were considered shy. Profiles were exposed to one out of four alcohol concentrations (0.00, 0.10, 0.25 or 0.50%) for 60 min and behavior was recorded. (**A**) speed while moving, (**B**) maximum speed, (**C**) total distance traveled, (**D**) distance from the bottom of the tank. GLMM modeling test was performed for comparisons between groups. Dark gray bars represent bold profile and light gray bars represent shy profile (Mean ± SD). Different letters indicate statistical significance between groups.

comparing bold and shy profiles under the effects of alcohol. When analyzed individually, bold 0.00% animals show shorter total distance traveled compared to shy 0.00% individuals. The reduced total distance traveled is also maintained at the 0.10% dose, but at 0.25 and 0.50% doses, there is an inversion, and bold individuals explore the environment more than shy ones. These findings differ from those previously found when different profiles were confronted with a social stimulus (Araujo-Silva et al. 2018). A likely explanation for this event is that zebrafish, when exposed to alcohol and tested for group cohesion, exhibits higher levels of locomotion than when tested in isolation (novel tank) (Ladu et al. 2014). According to other studies, alcohol administration altered individual locomotion (Gerlai et al. 2000; Mathur and Guo 2011; Maximino et al. 2011), but this effect was lessened when the fish were in group.

According to distance from the bottom of the tank, parameter used to indicate anxiety-like behavior (Stewart et al. 2011); in this study, bold and shy 0.00% individuals showed similar response. Usually, it is expected that shy individuals present higher levels of anxiety; however, boldness/shyness dimension seems to present two categories of behavior related to fear/anxiety response: the first that involves risky or dangerous stimuli response and the second that involves unfamiliar situations (Conrad et al. 2011). Although the two dimensions are distinguishable, individuals that present high score in one not always respond similarly to the other (Maximino et al. 2012). Thus, a group of anxiety-related tests and behaviors should be used to thoroughly evaluate anxiety response (Maximino et al. 2012). Among the behavioral analysis are freezing, latency to the upper region of the tank, reduced exploration, geotaxis (close distance to the bottom), thigmotaxis, scototaxis, opercular movements, body color change and erratic movement (Kalueff et al. 2013). As we have used a single behavioral indicator of anxiety for the novel tank test, our bold and shy 0.00% zebrafish showed similar distance to the bottom. However, alcohol exposure leaded to different responses in each profile. Alcohol at 0.10% concentration caused shy animals to be more active and even farther from the bottom compared to bold animals, while higher alcohol concentrations caused the opposite effect. Being an anxiolytic drug alcohol acts on the nervous system reducing the risk aversion behavior. When it reaches the central nervous system, it stimulates neurons to release serotonin and dopamine, neurotransmitters that regulate anxious behavior (Banerjee et al. 2014). In addition, this substance may affect two other neurotransmitters by inhibiting the glutamatergic and stimulating the GABAergic transmission (Morrisett and Swartzwelder 1993; Zaleski et al. 2004), initially inducing to a calming and less inhibited behavior effect. Other studies have shown that low doses of alcohol decrease bottom tank permanence, an indicator of anxiety, while higher doses

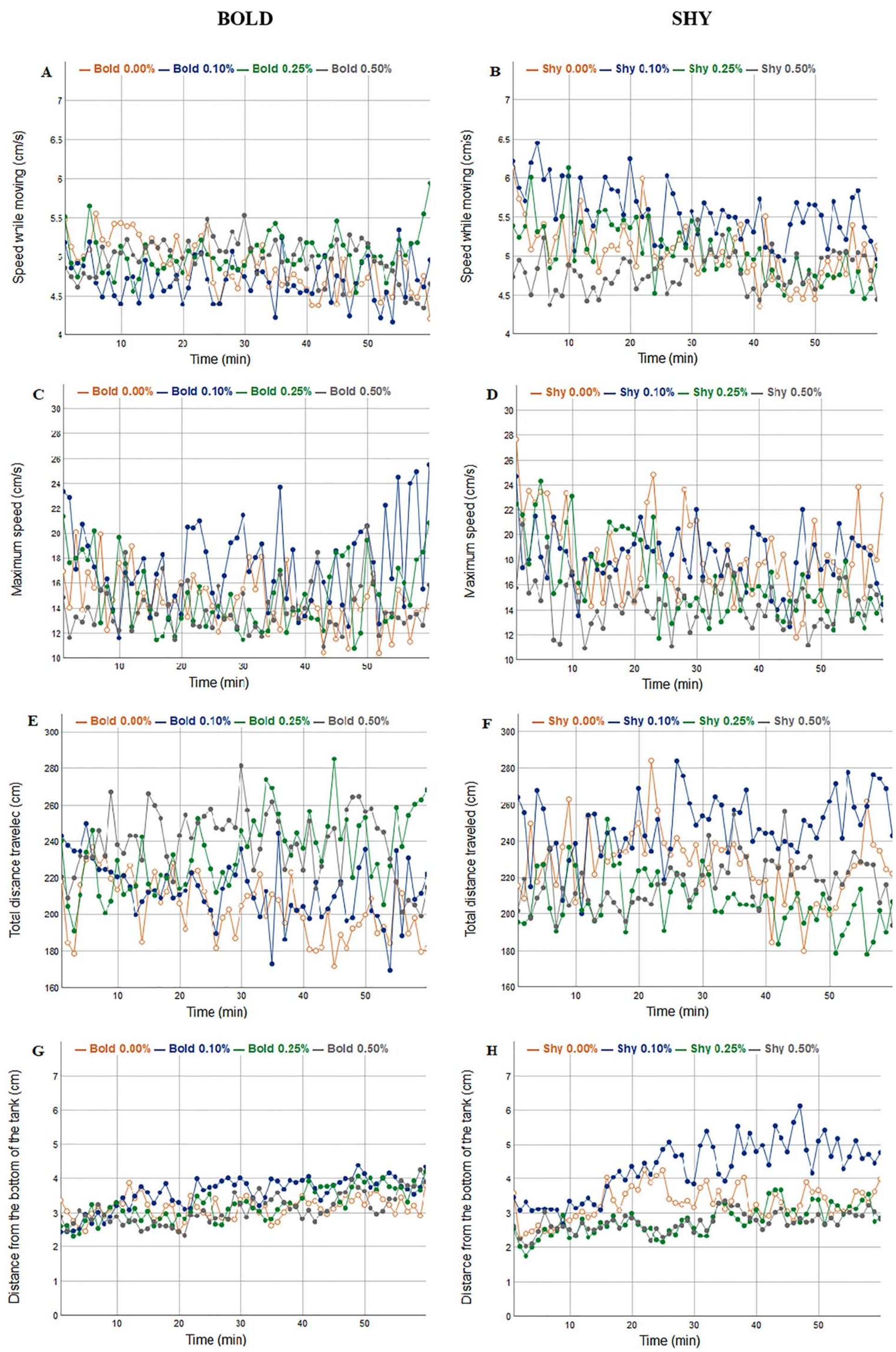

Fig. 2. Behavioral changes over time during 60 min alcohol exposure. Fish were separated into bold and shy profiles and exposed to alcohol concentrations of 0.00, 0.10, 0.25 or 0.50% for 60 min during which the behavior was recorded. The average is shown for each 1-min interval the total 60 min of recording. Left column graphs show locomotion and anxiety-like parameters recorded for bold fish (A, C, E, G) and right column graphs show behavioral parameters registered from shy fish. For statistical results see 'results section'.

increase anxiety-like behaviors such as bottom dwelling and freezing (Mathur and Guo 2011), corroborating our findings. The observed changes in shy fish behavior seem to be related to decreased anxiety due to alcohol exposure, which is supposed to affect shy profile more than bold profile at 0.10% concentration, and thus suggesting that bold individuals have a higher resistance to the effects of alcohol than shy ones.

The higher resistance of bold profile to the effects of alcohol may also be related to the effects of this drug on the reward system guided by dopamine. According to Coppens et al. (2010), proactive and bold individuals have low serotonin and high dopamine levels, while those classified as reactive and shy have high serotonin and low dopamine levels. Dahlbom et al. (2011) showed that zebrafish males are bolder than females and that the behaviors exhibited could be used to predict which fish became dominant or subordinate, positively correlating the dominance of the animal with higher dopamine release in the central nervous system. These findings corroborate the hypothesis that there is probably greater susceptibility of shy individuals and greater resistance of bold individuals to alcohol search. The propensity for alcoholism seems to have characteristics related to the individual's genotype, thus being a critical hereditary factor to be taken into account. Shreds of evidence from genetic studies in humans indicate that 50–60% of the risk of alcohol dependence can be inherited (Goldman, Oroszi, & Ducci 2005; Stacey, Clarke, & Schumann 2009). At the same time, impulsiveness is a personality trait linked to a predisposition to drug addiction (Gullo and Potenza 2014) and can be explored by the shy-bold dimension (Wilson et al. 1994). In this respect, concepts overlap, and personality should be considered when studying alcoholism and possible treatments for this disease.

In conclusion, although our study presents some limitations in terms of the number of anxiety/fear protocols used to test individuals (we used only novel tank) and the range of behavioral parameters analyzed, the results obtained are robust and confirm that there are differences between bold and shy profiles from the behavioral point of view, and when subjected to low and moderate doses of alcohol, the responses are also divergent. To better elucidate the effects of alcohol on the neurophysiology of individuals and further suggesting differences in alcoholism treatment related to the individual profile, new studies correlating dopamine and serotonin levels are needed to close the gaps that permeate studies involving behavioral profiles and drug effects on the nervous system.

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CONFLICT OF INTEREST STATEMENT

The authors have declared that no competing interests exist.

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