Individual differences in risk-taking behavior and effects of exposure to alcohol in zebrafish

Camilla Abrahamsson
2013-08-19

Undergraduate Thesis - 30 hp - Biomedicine Programme

Department of Neuroscience

Supervisor: Svante Winberg
Abstract

Background - It has been extensively debated if there is a relationship between personality types and alcohol use disorders (AUD). Is there an association between individual capacity to cope in a stressful and challenging situation and vulnerability to addictive behavior? Understanding the mechanisms involved in AUD is challenging, and in search of this knowledge, it is essential to use valid animal experimental models. The zebrafish has been proposed as a novel tool in alcohol research, to reveal a large number of behavioral and physiological effects as a response to acute and chronic alcohol exposure. Objective - This paper will argue the association between individual differences in risk-taking behavior, aggression and acute alcohol exposure. Methods – First, risk-taking behavior was assessed by monitoring individual zebrafish behavior in a novel tank. Zebrafish were classified according to total time spent on the bottom into high risk-taking (short duration), intermediate risk-taking (intermediate duration) and low risk-taking (long duration). Next, intermediate risk-takers were excluded and high and low risk-taking zebrafish were exposed to acute alcohol exposure (30 min in 0.00% and 0.25% ethanol), and risk-taking behavior and aggression of the fish was determined. Results – High, intermediate and low risk-taking zebrafish were classified using the novel tank diving test, since large individual differences are shown it is proved that novel tank diving test represent a useful tool for classifying risk-taking behavior based on time spent at bottom. After alcohol exposure, a significant main effect at the bottom of a novel tank was shown in short and long zebrafish, but no effect of treatment was found. No significant interactions between group and treatment were found either in the novel tank test or in the aggression test. Conclusions – The differences in duration of time spent on the bottom support that the novel tank diving test can be used to sort zebrafish based on individual risk-taking behavior. The study gave little support for an association between risk-taking and effects of alcohol exposure. Further studies are required to get a deeper knowledge about the relationship between individual differences in risk-taking behavior and the mechanisms involved in excessive alcohol intake and AUD.
Contents

Introduction ........................................................................................................................................ 3
    Background .................................................................................................................................... 3
    Animal model ............................................................................................................................... 4
    Behavior ....................................................................................................................................... 4
    Coping styles .............................................................................................................................. 5
    Zebrafish in experimental alcohol research .................................................................................. 6
        Novel tank diving test ............................................................................................................. 7
        Aggression test ......................................................................................................................... 7
    Project focus ............................................................................................................................... 7
        Aim of the study ....................................................................................................................... 7

Materials and methods ..................................................................................................................... 8
    Subject ........................................................................................................................................ 8
    Novel tank diving test ................................................................................................................ 8
    Voluntary alcohol intake ........................................................................................................... 9
    Acute alcohol exposure in the novel tank diving test ................................................................. 10
    The mirror test .......................................................................................................................... 10
    Statistical analysis .................................................................................................................... 11

Results ........................................................................................................................................... 12
    Novel tank diving test ................................................................................................................ 12
    Acute alcohol exposure in the novel tank diving test ............................................................... 13
    The mirror test .......................................................................................................................... 13

Discussion ..................................................................................................................................... 16

References ....................................................................................................................................... 17
Introduction

Background

Alcohol consumption has become an increasing worldwide health issue. Complications of excessive drinking and alcohol use disorders (AUD) pose enormous problems, both at an individual level and for the society, alcohol does not just affect the abuser, both caregivers, relatives, coworkers, friends and also health care costs and indirect costs are highly affected (NIAAA 2013). AUD includes both alcohol abuse and alcohol addiction, of which alcohol addiction represents a chronic, relapsing disease characterized by compulsive intake and loss of control (Grant et al. 2004; NIAAA 2013)

The prevalence of AUD is high. In Sweden about 780 000 persons are estimated to abuse alcohol while the number of people who are addicted to alcohol is around 330 000 people (CAN 2013).

It has been extensively debated if there is a relationship between personality types and vulnerability for AUD (Mulder 2002). There are many risk factors that have been identified, including some personality traits (Leggio et al 2009). Even if several definitions like novelty-seeking, impulsive decision-making, and extravagant behavior can be given, the general view implies that they all relate to risk-taking behavior, which is associated with a predisposition to rewarding and addictive behaviors (Laviola et al 1999; Leggio et al 2009) Since some of these risk factors vary among individuals as dimensions of normal behavior, individual differences in vulnerability still remain an important phenomenon to understand (Hasin and Kilcoyne 2012).

Clearly, AUD and alcohol related diseases are subjects for medical need. AUD are a polygenetic disorder affected by multiple genetic, epigenetic and environmental factors. (Morozova 2012; De Bellis 2002). As alcohol acts through numerous of biochemical pathways potentially affecting several biological targets the mechanism of action is challenging to understand (Vengeliene 2008). In search of understanding the mechanism involved in individual vulnerability for AUD, it is essential to use well-developed animal experimental models (Stephens et al 2013).

An animal model has the advantage of allowing the experimenter to control the factors one wants to investigate and therefore perhaps faster and more efficient discovery of how the studied factors affect the chosen phenomena (Gerlai 2013). Rodent models have been used extensively in alcohol research, as they are mammals that self-administer most drugs of abuse, including ethanol (Crabbe et al 1994). Many different outbred and inbred strains or strains obtained through selective breeding are available. The Wistar rat strain is one example of a commonly used outbred strain that also exert foundation stock for several rat lines selectively bred for high and low voluntary ethanol intake (Crabbe et al 1994). Animal models used for alcohol research cannot precisely replicate situations in humans, since numerous complex factors influence alcohol consumption and AUD in the human species (Gerlai 2013). However, analysis of diverse and distantly related vertebrate species may allow the discovery of fundamental similarities in alcohol-mediated mechanisms, which then may lead to better translation to humans. More recently findings point towards another promising model organism, novel in alcohol research: the zebrafish, Danio rerio (Gerlai 2013)
Animal model
The zebrafish is one of the most important vertebrate model organisms and has risen to prominence because of important findings in genetics, developmental biology, neurophysiology and biomedicine (Vascotto 1997; Grunwald & Eisen 2002; Rubinstein 2003; Amsterdam & Hopkins 2006). Being a vertebrate, the zebrafish is more comparable to humans than invertebrate model species such as Drosophila melanogaster or C. elegans (Postlethwait et al 1998; Barbazuk et al 2000). It is a genetic model possessing biological characteristics basic to all vertebrates, from neuroanatomy, neurochemistry to the nucleotide sequence of DNA (Gerlai 2013). Most importantly it is a small, robust fish, so large numbers can easily and cheaply be kept in captivity (Westerfield 2000). Its generation time is short, it breeds all year round and a single spawning can produce hundreds of offspring (Detrisch et al 1999).

Several genes that have been discovered in the zebrafish are evolutionarily conserved and have homologs in mammals (Cerda et al 1998). Neurotransmitter systems as the dopaminergic, GABAergic (gamma-aminobutyric acid) and serotonergic system seen in fish resemble those found in mammalian species, including humans (Winberg 1993). These favorable attributes make zebrafish an appropriate experimental vertebrate and a model organism that hold great promise for analysis of drug-induced behavior and in alcohol research (Gerlai 2013).

Worldwide over 800 laboratories now routinely use the zebrafish in research (ZFIN 2013) and recently there is an increasing interest in its use as a model for understanding the genetic basis of behavior (Gerlai 2003). It has been argued that one of the best and most objective methods to investigate functions of the brain and thus discover mutations or drug-induced changes in the brain is by using behavioral analysis (Blaser and Gerlai, 2006).

Behavior
The zebrafish behavioral repertoire is complex and has allowed a large development of behavioral models (Gerlai 2000). Zebrafish is a cyprinid shoaling fish (McCann 1971) and forming a group or a shoal is a behavior that appears to be innate and it seems to be a strategy that is effective against predators in several fish species (Lim 1979). Another also known behavior of zebrafish is to establish dominance hierarchies (Spence 2006). Zebrafish of both sexes can establish dominance hierarchies, and the main factor in displaying dominance status is to show aggressive behavior (Spence 2007).

Aggression is prevalent throughout the animal kingdom and it facilitates access to resources such as food, shelter and reproduction. Dominance hierarchies are occurring in many group-living animal species and can affect the lifelong success of individuals (Van Staaden 2011). During the formation, hierarchies can be unstable and all individuals involved are often stressed with increased levels of glucocorticoids (Winberg 1993). When hierarchies become stable, the dominant individuals usually show no signs of stress whereas the subordinates respond with a chronic stress response (Overli 1999, Winberg 1993). Larson et al. (2006) demonstrated that zebrafish also establish dominant-subordinate relationships and that aggressive interactions still occur in shoals of fish, but once dominance relationships become determined aggression becomes less intense. It has been established that serotonin (5-hydroxytryptamine, 5-HT) has an inhibitory effect on aggressive behavior, and this seems to be conserved across the vertebrates (Popova 2006; Winberg 1993) Since 5-HT has been shown to exert inhibitory modulation of the dopaminergic (DA) system, with different 5-HT receptors acting in opposite direction on dopaminergic activity, low levels of 5-HT may
contribute to enhanced dopamine transmission (Seo 2008). Despite this, studies have also showed facilitation of DA release in the presence of 5-HT agonists (Ennis et al 1981). Therefore, the existing evidence of association between 5-HT and DA neurons in the brain is controversial. Low aggressive behavior is associated with increased brain 5-HT activity and subordinate social status, while increased DA activity is associated with aggressive, competitive behavior and dominance (Winberg 1993). Population density, social stability, food availability and structure of the habitat all have strong effects on the presence of aggressive behavior (Ashley 2007). When fish are housed in pairs, the subordinates often appear paler and occupy a small area, while the dominant individual are darker and utilises the entire aquarium (Larson et al 2006). Interaction between fish may involve both social preference and the opposite dominant-subordinate relationships. (Benus 1991; Schjolden 2005).

Coping styles
Studies both in humans and in animals demonstrate that individuals may differ in their ability to cope with stressful situations (Ursin 1998; Ursin 1995). Behavioral studies in vertebrates (including teleost fish) have also shown that individuals differ in behavioral traits much in the same way as humans differ in personality (Gosling 2001). It seems that stress coping styles have been shaped to form general adaptive responses in reaction to everyday challenges in the natural habitat (Bell 2007).

As early as 1915 Cannon described the fight-flight response (Cannon 1915) and 1972 Engel and Schmale described the conservation-withdrawal response (Engel and Schmale 1972) and this started the thinking of difference in how to master a situation. Jim Henry (1977) suggested that stress could be distinguished in two stress response patterns and he started the current thoughts of coping style, and these ideas has led to a growing interest in the biological basis of individual variation within species (Bell 2007).

Coping is characterized by individual differences in behavioral and physiological efforts to master a challenging situation, individual variation that are both stable over time and across situations (Koolhaas et al 1999). This stable individual variation has now been showed in a variety of species (Koolhaas et al 1999; Bell 2007). Studies, predominantly on rodents, have focused on both behavioral and neuroendocrine parameters and suggest the existence of at least two distinct stress coping strategies, usually referred to as proactive and reactive stress coping styles (Koolhaas et al 1999). Animals that react actively to threatening situations by fleeing or attacking are considered to be a proactive type, and an animal that are more careful and prefer to wait passively for a threat to pass are a reactive type (Benus 1991; Koolhaas 1999).

Because the proactive coping style is characterized by preparation for action, physiologically it is usually associated with high sympathetic response in terms of plasma levels of adrenalin and noradrenalin, heart rate, blood pressure but a moderate hypothalamic-pituitary-adrenal (HPA)/interrenal (HPI, the interrenal being the teleostean homologue of the mammalian adrenal) axis response (Koolhaas et al 1999; Overli et al 2007; Mommsen et al 1999). The proactive individuals are adapted to highly predictable environments with increased survival in stable environmental conditions. (Benus et al 1987; Benus et al 1991). A reactive coping style, on the other hand, involves the opposite responses; a higher parasympathetic reactivity, strong bradycardic response, and higher HPI/HPA-axis reactivity, leading to higher plasma ACTH and glucocorticoid levels (Koolhaas 1999; Overli et al 2007). Individuals with a reactive coping style appear to have an advantage under variable and unpredictable environmental conditions (Koolhaas et al 1999; Overli et al 2007). By acting passively
reactive individuals seem to be more adaptive and flexible and surviving by staying away from danger (Benus 1991). Overview of characteristics is summarized in Table 1.

<table>
<thead>
<tr>
<th>Table 1. Characteristics of proactive and reactive animals.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proactive</strong></td>
</tr>
<tr>
<td><strong>Behavioral characteristics</strong></td>
</tr>
<tr>
<td>Attack latency</td>
</tr>
<tr>
<td>Active avoidance</td>
</tr>
<tr>
<td>Routine formation</td>
</tr>
<tr>
<td>Flexibility</td>
</tr>
<tr>
<td><strong>Physiological and neuroendocrine characteristics</strong></td>
</tr>
<tr>
<td>HPA axis activity</td>
</tr>
<tr>
<td>HPA axis reactivity</td>
</tr>
<tr>
<td>Sympathetic reactivity</td>
</tr>
<tr>
<td>Parasympathetic reactivity</td>
</tr>
</tbody>
</table>

Modified from Koolhaas (1999) and Overli et al (2007)

Moreover, there are studies in teleost fish that suggest the existence of different stress coping styles similar to those in mammals (Overli et al 2007). Two strains of rainbow trout, selected for consistently high or low cortisol responses to stress, high responsive (HR) and low responsive strain (LR), have been established through selective breeding (Pottinger and Carrick 1999). These two strains differ in behavioral traits in a way that suggest that they display divergent stress coping strategies. The low responsive (LR) strain tend to show proactive behaviors such as enhanced aggression, social dominance and differences in brain monoamine neurochemistry have also been reported in these lines (Overli et al 2007).

Blanchard (2009) is reviewing that rodents responding with high activity in a novel environment, and sensation seeking humans share a number of similarities. Sensation seekers are individuals characterized by a willingness to take risks in order to attain stimulation (Zuckerman 1979) and there are studies suggesting that sensation seeking may increase an individuals vulnerability to drug abuse (Howard et al 1997). Evidence of common behavioral tendencies, physiological responses and gene expression patterns suggest that rats responding with high activity in a novel environment also have different physiological responses to stress, which indicates similarities with both proactive/reactive animals and risk-taking behavior in humans. It has been demonstrated that high responders display a much higher propensity to self-administer psychostimulants and develop operant self-administration of central stimulants compared to low responder rats (Bardo et al 1996; Blanchard et al 2009). Based on that studies have been made comparing voluntary alcohol intake in high risk-taking and low risk-taking rats (Momeni 2013).

Zebrafish in experimental alcohol research

Studies have showed that levels of aggression and social coherence could be modulated by exposure to alcohol (Gerlai 2000) and the zebrafish is a very suitable model organism in alcohol research (Gerlai 2000; Gerlai 2003). Alcohol delivery to the zebrafish is simple because it is a substance that can be delivered from the water in which they swim and alcohol is easily absorbed by the blood vessels of the gill and the skin of the fish (Ryback 1969; Ryback 1970).
Novel tank diving test
To investigate risk-taking behavior, a novel tank diving test has been developed (Gerlai 2000), conceptually similar to the rodents’ choices of walled arms in an elevated plus maze and positions near the walls of an open field (OF) test (Levin 2007). The innate behavior of zebrafish in an unfamiliar environment is to seek protection by diving and spend the majority of time at the bottom, until they feel safe enough to explore (Egan 2009)

By using this model individual difference in risk-taking behavior can be classified according to their activity, the duration of time the experimental fish is spending in the top, middle or bottom zone and the latency to enter each zone (Gerlai 2000).

Aggression test
Studies have also shown that a low dose of alcohol affects the zebrafish aggressive behavior towards a mirror (Gerlai 2003). Based on that, aggressive behavior can be registered quantifying the responses of an individual experimental fish to its mirror image (Gerlai 2000). Attack behavior is characterized by short bouts of fast swimming directed towards the opponent (the mirror) and is sometimes accompanied by opening the mouth and biting (Gerlai 2000). In addition, a commonly studied behavior that is associated with a reactive behavioral profile and increased anxiety in zebrafish is increased erratic movement, fast zigzagging, with sharp turning angles (Levin 2007), a behavior which also can be analysed in the mirror test.

Project focus
To understand individual differences in behavior, coping capacity and risk factors to addictive behavior, the association between coping styles, risk-taking and vulnerability are important to identify and an active area of investigation.

Recently studies have been made to compare voluntary ethanol intake in different rat strains and findings have shown that although all rats were of Wistar origin, they display profound differences in voluntary ethanol consumption and behavior (Palm 2011). So far, studies on the association between individual differences in risk-taking behavior and voluntary alcohol intake are limited in the literature.

Aim of the study
Few previous studies on alcohol intake at individual level are reported in zebrafish. The aim of this study is to address individual differences in risk-taking behavior and associations between risk-taking behavior, aggression and acute alcohol treatment. Furthermore, we will determine whether zebrafish can be used for studies of voluntary alcohol intake.
Materials and Methods

Subjects
Adult AB zebrafish (*Danio rerio*) were kept at approximately 27 °C on a 14:10-h light/dark cycle with lights on at 7.00 am to 9.00 pm. Behavioral testing took place during the light phase between 8.00 am and 5.00 pm. The tank water used was Uppsala municipality tap water (pH 7.2-7.6) of which 15% was exchanged daily. Fish were sorted by sex (male, n = 56) and housed individually in 2.8 l tanks for at least one week before any experiments started. Main feed was Sera San tropical flake food (Gibbon, Sweden), which was provided once daily. The experimental outline is illustrated in Figure 1. The experimental protocols and use of animals in this research was approved by the Uppsala Ethical Committee and followed the guidelines of the Swedish Legislation on Animal Experimentation.

Novel tank diving test
Fish were housed in isolation for at least one week before being transferred from their home aquarium to the experimental arena. Zebrafish were placed individually in a 1.99 l experimental tank (length: 24.2 x height: 19.6 x width: 4.2 cm) maximally filled with aquarium treated water. Four tanks rested on a level stable surface (Figure 2A). The test was performed for a total of 30 min. To minimize disturbance from the neighbour fish a paper wall was placed between the arenas. After 60 s of acclimation in the novel tank, swimming behavior was recorded by using Ethovision 9.0 (Noldus, The Netherlands). In the analysis, each tank was divided into three zones, a bottom, middle and top zone (Figure 2B). The following parameters were recorded: Angular velocity, distance moved (cm), latency (LAT, s) to first visiting a zone, duration (DUR, s) of time spent in a certain zone and velocity (cm/s).

After each fish tested, the arena was cleaned with tap water, sprayed with 50% ethanol, wiped and cleaned with system water before next fish was studied. After the novel tank diving test, fish were isolated for at least one week before next test started. Based on performance, fish was divided by tertiary split into long, intermediate and short duration at the bottom.
Voluntary alcohol intake
A shuttle tank is a tank in which an animal can shuttle between two compartments via a short passage. Water is slowly rotating inside each compartment, preventing any mixing of water between the two zones (Figure 3). The idea was to have fish move freely between zones with different concentrations of alcohol, and allow them to choose which zone to stay in as a way of measuring voluntary alcohol intake.

Due to technical difficulties, this test could not be performed, and this is something the lab will continue to explore further. Instead behavioral assessments after acute ethanol exposure were performed.

Figure 2. Novel tank diving test. Four tanks rested on a level stable surface (A). Each tank was divided into three zones, a bottom, middle and top zone (B). Behavior was recorded by using Ethovision 9.0 (Noldus, The Netherlands)

Figure 3. Shuttle tank setup.
**Acute alcohol exposure in the novel tank diving test**

For this experiment fish initially characterized as having an intermediate duration at the bottom was excluded and only the extremes, fish with short and long duration at the bottom were further characterized. The zebrafish were assigned to four groups, two for each concentration: Long EtOH 0.00%; n=10, Long EtOH 0.25%; n=10 Short EtOH 0.00%; n=10, Short EtOH 0.25%; n=10. The ethanol concentrations given represents the corresponding alcohol concentration (volume percentage) in the holding tank in which experimental fish were held for 30 min before the behavioral tests. The alcohol concentrations were chosen on the basis of previous reports in the literature (Gerlai 2013). The alcohol-treated and the control fish were tested subsequently in the novel tank diving test as described above. During these tests, the alcohol concentration in the test tank was kept identical to that of the pre-test holding tank. Fish were treated and tested in an order randomized across treatment groups.

**The mirror test**

Fish were individually netted into one zone of a tournament-tank, which was divided in two zones, length; 9.1 x height; 19.3 x width; 6.7 cm (Figure 4). A mirror was placed toward the sidewall of the tank, so that when the experimental fish swam to the side of the tank their mirror image appeared to them (Figure 4). Before the mirror was displayed the fish were housed in isolation for at least 12 hours, and an empty tank was placed on each side of the tournament-tank. The tank water used was Uppsala municipality tap water (pH 7.2-7.6) and EtOH at 0.25% was pipetted into the water 30 min prior to behavioral testing. After the mirror was displayed the fish were video-recorded for 10 min. The amount of time the experimental fish spent with aggressive display or attack behavior against the mirror (aggression) was registered. In addition, the amount of time the fish spent zigzagging was also measured. Manual scoring of the behavior was performed by an observer using the program Score 3.3 (Copyright Soldis, Uppsala, Sweden). Latency (LAT, s), frequency (FRQ) and duration (DUR, s) of the registered behaviors were analysed.

![Figure 4. Aggression studied using the mirror test. Tournament-tank divided in two zones. Zebrafish were screened for aggressive behavior against a mirror.](image-url)
**Statistical analysis**

**Novel tank diving test**
Individual differences in the novel tank diving test were investigated. Zebrafish (n=56) were divided by a tertiary split into short (n=20), intermediate (n=16) and long (n=20) based on the proportion of the total time spent on the bottom of a novel tank. To compare the three groups in the novel tank diving test, the non-parametric Kruskal-Wallis was used, followed by the Mann-Whitney U-test. Non-parametric statistics were used since most of the data did not show a normal distribution.

**Alcohol exposure**
For analysis of the effects of alcohol on performance in the novel tank diving test and the aggression test only the short and long groups were used. Despite the fact that data did not show a normal distribution, parametric statistics was used for investigation of interaction effects. Using repeated measures analysis of variance (ANOVA), the effects of group (short, long), treatment (EtOH, H2O) and time were analysed.

Differences were considered statistically significant at p<0.05. Statistica 10.0 (StatSoft Inc., USA) was used for the statistical analyses.

In addition, to give an overview of individuals and variables, the multivariate data analysis method principal component analysis (PCA) was performed. For multivariate data analyses the software Simca-P+ 12.0 (Umetrics AB, Sweden) was used.
Results

Novel tank diving test

The zebrafish were divided by a tertiary split into short (n=19), intermediate (n=18) and long (n=19) animals based on the duration of time spent at the bottom of a novel tank (Figure 5). The mean percentage time spent on the bottom of the tank was 14.7%, 30.3% and 51.4% for short, intermediate and long zebrafish, respectively. The descriptive results of the 30 min session in the novel tank test are shown in Table 2, and was in general statistically significant comparing short, intermediate and long zebrafish.

![Novel tank diving test](image)

**Figure 5.** The total duration (s) spent on the bottom in the novel tank diving test by male AB zebrafish. Values represent mean±SEM. ****p<0.0001 comparing short, intermediate and long zebrafish (Mann-Whitney U-test). Kruskal–Wallis H=48.89 p<0.0001.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Short</th>
<th>Intermediate</th>
<th>Long</th>
<th>Kruskal-Wallis test</th>
</tr>
</thead>
<tbody>
<tr>
<td>AV</td>
<td>1.7±3.8</td>
<td>14.2±7.9</td>
<td>11.5±7.6</td>
<td>n.s.</td>
</tr>
<tr>
<td>DM</td>
<td>11144.3±975.3 a,b,c</td>
<td>10195.3±832.2</td>
<td>8035.8±771.3 ab</td>
<td>H=9.76, p=0.0076</td>
</tr>
<tr>
<td>DURB</td>
<td>264.2±24.3 b,b,c</td>
<td>544.5±18.3 a,a,b,c</td>
<td>925.4±50.4 a,a,b,b</td>
<td>H=48.89, p&lt;0.0001</td>
</tr>
<tr>
<td>DURT</td>
<td>894.5±49.9 b,b,c</td>
<td>581.4±30.2 a,a,c</td>
<td>327.6±43.6 a,a,b,b</td>
<td>H=38.08, p&lt;0.0001</td>
</tr>
<tr>
<td>V</td>
<td>6.2±0.5 a,c</td>
<td>5.7±0.5</td>
<td>4.5±0.4 ab</td>
<td>H=10.20, p=0.0061</td>
</tr>
</tbody>
</table>

Table 2. Results from the first trial (risk-taking behavior) in the novel tank diving test in adult male AB zebrafish.

Parameters recorded during the 30-min trial of the first novel tank diving test. Values represent mean±SEM. a,p<0.01 a,b,p<0.001 compared to short. b,p<0.01 b,b,p<0.001 compared to intermediate and c,p<0.01 c,a,b,c,p<0.001 compared to long (Mann-Whitney U-test).

Abbreviations: AV, angular velocity; DM, distance moved; DURB, duration bottom (s); DURT, duration top; V, velocity.

The principal component analysis (PCA) was used to illustrate the relationship between short, intermediate and long zebrafish when the parameters duration at bottom (DURB), distance moved (DM) and velocity (V) were analysed. The results are shown in Figure 6. As illustrated in the score plot, a clear separation between the three groups was found, which is supported...
by the conventional statistical analysis. Two principal components explained 100% of the variance ($R^2_{X(cum)}=1.00$, $Q^2_{X(cum)}=0.917$).

**Acute alcohol exposure in the novel tank diving test**

Only the long and short groups were exposed to ethanol. After randomising the groups into ethanol and control, a statistical analysis was performed in order to exclude basal group differences in long and short groups respectively. The results from the acute ethanol exposure in the novel tank diving test are shown in Table 3. Group differences comparing long and short groups were found for distance moved, velocity and duration at the top. However, no effects due to treatment, interactions or effects over time were revealed.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Short</th>
<th>Long</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.00% EtOH</td>
<td>0.25% EtOH</td>
</tr>
<tr>
<td>AV</td>
<td>Diving test 1 3.6±4.9</td>
<td>0.7±5.8</td>
</tr>
<tr>
<td></td>
<td>Diving test 2 5.2±3</td>
<td>2.2±5.3</td>
</tr>
<tr>
<td>DM****</td>
<td>Diving test 1 11621.2±1398.4</td>
<td>11424.1±1503.9</td>
</tr>
<tr>
<td></td>
<td>Diving test 2 11458±632.6</td>
<td>11079.6±1035.1</td>
</tr>
<tr>
<td>DURT****</td>
<td>Diving test 1 891.5±73.5</td>
<td>880.6±65.9</td>
</tr>
<tr>
<td></td>
<td>Diving test 2 676.7±86.53</td>
<td>818.5±72.6</td>
</tr>
<tr>
<td>V****</td>
<td>Diving test 1 271.8±0.8</td>
<td>6.4±0.845</td>
</tr>
<tr>
<td></td>
<td>Diving test 2 6.37±0.4</td>
<td>0.74±0.6</td>
</tr>
</tbody>
</table>

**Table 3.** Comparing short and long zebrafish using repeated measures ANOVA in diving test 1 and after exposure to EtOH in diving test 2. Values are expressed as mean±SEM ****p<0.0001 comparing short and long zebrafish.
As shown in Figure 7, a significant main effect at the bottom of a novel tank was shown, but there was no effect of treatment and no interaction between group and treatment. However, a significant effect of time ($p=0.042$) was noted independent of treatment. The short group spent longer time at the bottom of the tank in the second test relative to the first test.

**Novel tank diving test**

![Graph showing mean duration at the bottom of a novel tank](image)

**Figure 7.** Mean duration at the bottom of a novel tank compared to mean duration (s) after acute alcohol exposure in the novel tank diving test. Values represent mean±SEM ****$p<0.0001$ repeated measures ANOVA.

The results from PCA confirm the results of significant main effect of groups but no effect of treatment and no interaction between group and treatment (Figure 8).

![Graphs showing principal component analysis](image)

**Figure 8.** The principal component analysis (PCA) comparing short and long zebrafish in diving test 1 and after exposure to EtOH in diving test 2. The score plot (A) illustrates the individuals and separation of the groups. There was no separation due to treatment. The loading plot (B) illustrates the novel diving test variables that were included in the analysis. Variables located further away from the origin are most important for differentiation between groups.

Abbreviations: LE, Long 0.25% ETOH; LW, Long 0.00% ETOH; SE, Short 0.25% ETOH; SW, Short 0.00% ETOH; DM, distance moved; DURB, duration bottom; DURY, duration top; $V$, velocity. 1, diving test 1; 2, diving test 2. Two principal components explained 100% of the variance ($R^2_X$($\text{cum}$)=0.755, $Q^2_X$($\text{cum}$)=0.364).
The mirror test
During the mirror test, two fish died, leaving the following number of animals: Long EtOH 0.00%; n=10, Long EtOH 0.25%; n=9, Short ETOH 0.00%; n=9, Short ETOH 0.25%; n=10. There were no significant main effects nor interactions between groups and treatment in mean number of aggressive acts performed during 10 min in zebrafish paired with their mirror imagine (Table 4). The findings from the conventional statistical analysis was confirmed also in the PCA (Figure 9).

Table 4. Results from the mirror test with adult zebrafish male divided by time spent on bottom of a novel tank and treatment (EtOH).

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Short 0.00% EtOH</th>
<th>Short 0.25% EtOH</th>
<th>Long 0.00% EtOH</th>
<th>Long 0.25% EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Attack mirror FRQ</td>
<td>11.9±4.1</td>
<td>18.1±9.5</td>
<td>34.7±12.1</td>
<td>16.9±4.4</td>
</tr>
<tr>
<td>LAT</td>
<td>116.9±58.1</td>
<td>214.9±66.7</td>
<td>189.5±53.3</td>
<td>188.4±50.3</td>
</tr>
<tr>
<td>DUR</td>
<td>3.7±1.6</td>
<td>6.1±3.5</td>
<td>36.5±25.7</td>
<td>9.1±5.3</td>
</tr>
<tr>
<td>Zigzagging FRQ</td>
<td>24.4±5.3</td>
<td>27.2±6.6</td>
<td>32.7±5.9</td>
<td>30.5±5.1</td>
</tr>
<tr>
<td>LAT</td>
<td>0.9±0.6</td>
<td>0.8±0.5</td>
<td>1±0.5</td>
<td>1.9±0.8</td>
</tr>
<tr>
<td>DUR</td>
<td>55.4±10.5</td>
<td>67.4±16.4</td>
<td>84.2±21.3</td>
<td>65.4±11.2</td>
</tr>
</tbody>
</table>

Behavioral parameters recorded during a 10-min trial in the mirror test. Values represent mean±SEM. Abbreviations: FRQ, frequency; LAT, latency (s) DUR, duration (s).

Figure 9. The principal component analysis (PCA) from the trial using the mirror test on male short and long zebrafish. The score plot (A) illustrate the individuals and separation of the groups. The loading plot (B) illustrate the mirror test variables that were included in the analysis. Variables located further away from the origin are most important for differentiation between groups. Abbreviations: LE; Long 0.25% ETOH, LW; Long 0.00% ETOH, SE; Short 0.25% ETOH, SW; Short 0.00% ETOH, MedFrqAtta; mean frequency attack, MedLatAtta, mean latency (s) attack, MedDurAtta, mean duration (s) attack, MedFrqZigz, mean frequency zigzagging, MedLatZigz, mean latency (s) zigzagging, MedDurZigz, mean duration (s) zigzagging.
Discussion

In this study the association between risk-taking behavior and alcohol exposure was investigated. Zebrafish were screened for individual differences in risk-taking behavior, defined by time spent at the bottom of a novel tank, in line with rats' choices of positions near the walls of a novel circular open field arena in agreement with previous studies e.g. Levin (2007). The tertiary split of animals into short, intermediate and long were based on the scoring in the novel tank diving test. High risk-taking short zebrafish spent less time at the bottom of a novel tank than intermediate and long zebrafish.

Despite the fact that novel tank diving test is based on forced exploration, large individual differences are shown, which made it possible to divide animals into three distinct groups based on performance. With this great distribution in the novel tank diving test, it has been proven to represent a useful and valid tool for classifying risk-taking behavior based on time spent on bottom.

It was found that individuals with short duration at bottom were displayed higher locomotor activity (distance moved and velocity) in the novel tank diving test. This implies that risk-taking and high activity form a behavioral syndrome. Suggesting that the present classification of animals based on risk-taking behavior (short/long) seems to share some common traits with animals selected for a proactive/reactive stress-coping style, as defined by Koolhaas et al (1999). Similar results were found by Pottinger and Carrick (2001) in rainbow trout selected for high (HR) or low (LR) stress responses. In their study, the HR animals were typically located on the base of the aquarium, usually immediately adjacent to the aquarium wall, exhibiting little swimming activity, compared to the LR fish. However, it should also be pointed out that the behavior of the HR-LR lines of fish is highly context dependent, and these fish are influenced by factors such as novel environments and group size (Schjolden et al 2005).

The short and long, respectively, groups based on the novel tank diving test were then selected for further studies on the effects of acute alcohol exposure, in the novel tank diving test and in a mirror test. To our knowledge, this is the first alcohol exposure study using zebrafish sorted by performance in the novel tank. The present findings gave no support for an association between risk-taking behavior and effects of alcohol exposure since zebrafish classified as short and long did not present a change in profile after acute alcohol exposure in novel tank diving test or the mirror test that would be interpreted as a change in risk-taking behavior.

Using rats selected for high and low locomotor responses to novelty, it has been demonstrated that high responders (HRs) display a much higher tendency to self-administer psychostimulants and develop operant self-administration of central stimulants compared to low responder rats (Bardo et al 1996; Blanchard et al 2009). Based on that studies have been made comparing voluntary alcohol intake in high risk-taking and low risk-taking rats (Momeni 2013). The present findings is in agreement with Momeni (2013), that could not demonstrate any evident association between individual differences in risk-taking behavior and voluntary alcohol intake in rats, but in contrast to others studies bases on findings between novelty seeking/risk-taking to rewarding and addictive behaviors in humans (Laviola et al 1999; Leggio et al 2009) and rodents (Blanchard et al 2009).

The contradictory outcome in the HR-LR line regarding effect of psychostimulants and alcohol consumption may be explained by the fact that alcohol is known to have a very complex
pharmacological profile, acting through numerous biochemical pathways potentially affecting several biological targets (Vengeliene 2008). As psychostimulants on the other hand are known to exhibit elevated mesolimbic dopamine activity, which has been implicated in central reward signaling and drug addiction (Robbins et al 1996).

We used the novel tank diving test in which it was assumed that decreased duration at bottom and higher activity would be good predictors of high risk-taking behavior, and that risk-taking individuals would be more physiologically prepared for higher activity. Although activity in a novel environment is commonly used to model traits of sensation seeking or novelty in humans (Blanchard et al 2009), studies in social status closely linked to coping styles have showed that activity may not be a good measurement in all situations. Dahlbom (2011) showed that both distance moved in the novel object test and the roof test correlated negatively with distance moved in the open field test. Similar contradictions to the expected results were also seen by Champagne et al (2010) who showed that zebrafish stressed from confinement had a higher activity than controls in an open field, both in an inner and outer zone. It has been suggested that locomotor response in a novel open field reflects escape behavior rather than novelty-seeking (Bardo et al 1996) which must be considered also in the novel tank diving test.

Earlier studies have found that alcohol proved to be an appropriate drug with which aspects of behavior relevant for aggression could be modulated (Gerlai 2000; Gerlai 2003). Induced hyperactivity at intermediate doses (0.25 and 0.50%) and enhanced aggressive display has been demonstrated (Gerlai 2000). In contrast to previous studies our results showed no significant interaction between groups and treatment in the number of aggressive acts performed during the ten minutes of behavioral monitoring, although aggressive acts were displayed against the mirror.

The number of aggressive acts decreases with stable hierarchies, where the subordinate accept their low rank and stop challenging the dominant. A consequence of zebrafish fighting their own mirror image is that in mirror fights the opponent (i.e. its own image in the mirror) never displays submissive behaviors, and the dominate/subordinate relationship will never occur which can affect aggressive display and that must be considered in this model of aggression.

Reasons for the negative outcome in the present studies can only be speculated upon. One reason could be the use of tertile split for classification of animals as the use of quartile split has been shown to generate more extreme groups (Cain 2005). The total number of animals used may have been too small since large groups of animals may reduce variability within each group. It may also be that a different outcome had been achieved using a different concentration of ethanol or a different exposure time although the concentration and the exposure time in this study were based on reports in the literature (Gerlai 2013).

In conclusion, the short/long classification based on duration of time at the bottom in the novel tank diving test is a useful test for separating zebrafish based on individual differences in risk-taking behavior. It is possible that associations between individual risk-taking and alcohol-induced behavioral effects would be more pronounced using voluntary alcohol intake. The present findings therefore warrants further studies regarding individual differences in risk-taking behavior of relevance to excessive alcohol intake and AUD.
References


Gosling SD (2001) From mice to men: what can we learn about personality from animal research? Psychol Bull 127:45-86.


Palm S, Roman E, Nylander I (2011) Differences in voluntary ethanol consumption in Wistar rats from five different suppliers. Alcohol 45:607-14


Øverli Ø, Sørensen C, Pulman KG, Pottinger TG, Korzan WJ, Summers CH, Nilsson GE (2007) Evolutionary background for stress-coping styles: relationships between physiological,