7

# Zebrafish, a Potential Novel Research Tool for the Analysis and Modeling of Anxiety

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## 1. Introduction

Although numerous medications have been developed for anxiety disorders and related neuropsychiatric conditions including phobias, these diseases still represent a large unmet medical need. This may be because despite the concerted research and drug development efforts by pharmaceutical research companies and academic laboratories alike, the mechanisms of these disorders still remain to be fully elucidated. Animal models have been proposed to accelerate research in this area. The current chapter focuses on a somewhat novel and underutilized laboratory organism, the zebrafish, which may have great utility in anxiety research.

Zebrafish have been successfully utilized in developmental biology, a discipline that often employs molecular biology and genetic methods. As a result of the past three decades of intensive investigation with zebrafish, this species has become one of the favourite model organisms of geneticists. The accumulated genetic knowledge about, and the genetic methods specifically developed for the zebrafish now make this species particularly attractive for several research fields other than developmental biology. One of these fields is behavioural neuroscience. Indeed, the number of zebrafish publications in the latter field has started to exponentially increase. This may be because zebrafish strikes an optimal compromise between system complexity and practical simplicity. On the one hand it is a complex organism with brain anatomy, neurophysiology, and molecular characteristics (e.g. nucleotide sequence of its genes) highly similar to those of other vertebrates including mammals. On the other hand, it is small, easy and cheap to maintain in the laboratory and has been highly amenable to high-throughput screening (e.g. forward genetic or drug screens). The latter is particularly noteworthy for the purposes of unravelling of the genetic (and in general the biological) mechanisms of complex brain functions and the disorders of these functions. High-throughput screens may have the ability to identify a good proportion of the potentially large number of molecular players involved in these functions.

The chapter discusses how the zebrafish may be utilized in the modeling of human anxiety disorders and in the analysis of the mechanisms of these disorders. Admittedly, the zebrafish is rather novel in this research and does not have a proven track record. The chapter is focussed on behavioural test paradigms that may have the capacity to induce anxiety related behavioural responses. The chapter argues that the foundation of research into the mechanisms of anxiety disorders is such behavioural paradigms as they will allow the quantification of functional changes in the brain induced by mutations or drugs and

thus will facilitate the discovery of underlying mechanisms and drug targets. The chapter also argues that the most successful behavioural test paradigms will be those that represent ethological validity, i.e. consider the species-specific characteristics and the ecology and evolutionary history of the zebrafish. The chapter reviews several such recently developed test paradigms and presents data, for example, on the behavioural effects of the natural and synthetic alarm substances, a chemical that is released from the skin of injured fish, as well as on other test methods that utilize visual stimuli, including computer animated (moving) images of sympatric predators of zebrafish. The chapter also provides a detailed description of the behavioural responses these stimuli induce and makes recommendations for further development of these methods and how they may be employed in forward genetic screening for mutations involved in anxiety related phenotypes. The chapter concludes that, although the zebrafish is rather novel in anxiety research, the increasing number of publications with this species suggests a successful future.

## 2. Human anxiety remains a major unmet medical need despite decades of preclinical and clinical research

First let us define some basic terms. I use "fear" to describe the behaviour or internal state of the subject (human or non-human animal) that is elicited by aversive stimuli that can potentially harm the subject and/or signal such harm or forms of danger. For the sake of simplicity, I define anxiety as an abnormally prolonged, exaggerated, or misdirected form of fear. Please note that these definitions do not assume the presence or the absence of consciousness, awareness, or understanding of fear or of the stimuli that induce it and thus are employed equally to human and non-human animals.

Human anxiety is one of the most prevalent neuropsychiatric conditions. Approximately 5% of people living in westernized countries will suffer from general anxiety disorder during their life time and, for example, just in the United States as many as 10 million patients suffer from this disease at any given time point (Weisberg, 2009). The numbers are likely larger for other parts of the world and certainly even more staggering if one considers other types of anxiety disorders such as panic disorders, post-traumatic stress disorders (PTSD), phobias, or less severe forms of anxiety (Garakani et al., 2006; Choy et al., 2007; Klein, 1996). Despite decades of research, the quality of life of individuals suffering from anxiety related disorders is still significantly reduced even in patients with mild forms of the disease (for a review see Mendlowicz & Stein, 2000) because the treatment options, including pharmacological approaches, have been limited, variable or ineffective.

Most agree that the key to the development of appropriate treatment methods is the understanding of the mechanisms of the disease. Unfortunately, the mechanisms of anxiety related disorders have not been fully understood (Matthew et al., 2008). This is not to say that we do not know anything. Clearly a lot of knowledge has been accumulated already. For example, neuroanatomical and neuroimaging studies have confirmed that the amygdala and its reciprocal connections with the prefrontal cortex play a central role (for review see Matthew et al., 2008) but other brain regions, e.g. the periaqueductal gray (Misslin, 2003; Takahashi et al., 2008), have also been implicated in fear responses and anxiety related abnormalities. Progress has been made at levels of investigation other than neuroanatomy too. Numerous neurotransmitter systems, neurochemicals and hormones have been shown to be significantly altered in anxiety disorders (for review see Matthew et al., 2008). For example, the concentration of Corticotropin-Releasing Factor (CRF) has been shown to be

elevated in some anxiety disorders, pharmacological blockade of glucocorticoids and noradrenaline has been proposed for trauma-related anxiety, and the glutamatergic system has been implicated in other forms of anxiety (for review see Matthew et al., 2008). The role the serotoninergic system may play in anxiety disorders has also been extensively studied (e.g. Leonardo & Hen, 2006). The involvement of neuropeptides substance P, neuropeptide Y, oxytocin, orexin, and galanin have also been demonstrated in anxiety (e.g. Matthew et al., 2008). While many of the above mechanisms represent potentially good pharmacological targets allowing the eventual development of drug therapies, the complexity of these disorders and the limited understanding of the mechanisms behind them warrants further detailed inquiries into the neurobiology of the disease.

## 3. Laboratory animals: Efficient tools of discovery

Numerous human neuropsychiatric disorder have been successfully modelled or some of the mechanisms underlying these diseases investigated using laboratory animals (Flint & Shifman, 2008). Anxiety disorders are no exception to this (see e.g. Hohoff, 2009). This is not surprising given that at every level of biological organization, i.e. from behavioural traits to the nucleotide sequence of genes, evolutionary conservation of features has been repeatedly demonstrated. This is of course not to say that there are no species specific characteristics. But if one is interested in the fundamentally important questions, evolutionary conservation allows the experimenter to efficiently utilize model organisms especially if the laboratory species is closely related to human. Most anxiety related studies have been conducted with rats, a mammalian species that shows high DNA nucleotide sequence homologies to human. The interest in this species therefore is not surprising. For example, a medline (PubMed) literature search with keywords “anxiety” and “rat” returns close to 8 thousand publications. Another model organism, the house mouse, which is perhaps even more frequently used in biomedical research, is also well utilized in anxiety research. A medline search with this species also reveals close to 5 thousand published studies. In addition to the rodents utilized in the laboratory, other model organisms, including the dog (almost 5 hundred publications) or non-human primates (62 publications) have also been employed in the analysis of anxiety. Even such evolutionarily distant species to us as the fruit fly (Drosophila melanogaster) has been proposed as a research tool for the understanding of the mechanisms of human anxiety (Iliadi, 2009). The rich literature on anxiety research clearly demonstrates the major effort to utilize model organisms for the analysis and/or modeling of human anxiety.

There are two principal reasons why one would like to use model organisms for the analysis of human disorders. First, is a practical consideration: laboratory organisms represent a compromise. These species can be kept and analyzed more cheaply than humans and they face fewer ethical roadblocks. Second, as argued above the evolutionary relatedness of laboratory organisms to us means that there may be numerous functional, e.g., neurobiological, physiological, biochemical and genetic homologies that the researcher can utilize in her/his quest for the understanding of the mechanisms of human anxiety (for examples see review by Denver (2009).

## 4. Naturalistic (ethological) approaches should be employed when the question concerns the biological mechanisms of behaviour

Many successful lines of investigation into the mechanisms of a broad range of behaviours have (e.g. Gerlai et al., 1999; Lu et al., 1997; Grant et al., 1992; Silva et al., 1992) utilized

behavioural, electrophysiological, neuroanatomical and molecular genetics methods to investigate the mechanisms of brain function and how such mechanisms lead to the behavioral output. But when it comes to the method or approach of behavioural experimentation some controversies may need to be cleared. There may be many ways one can study animal and human behaviour. In the past and especially in North America classical psychologists argued that one has to be nature blind and ignore the unique species specific features of different organisms. This is the only way, went the argument, one could study the common, and thus most important, features of the phenomenon under investigation. This tenet led to an important controversy as to how to measure behaviour (Gerlai, 2001). It has been pointed out that, while not necessarily mutually exclusive, two fundamentally distinct approaches emerged, classical psychology and ethology. The classical psychology approach has emphasized the analysis of species invariant features that cut across multiple species, i.e. allow generalization of findings. The argument was that analysis of species independent features is expected to lead to easier translation from animal to human. On the other hand, the ethological approach has put more weight on naturalistic studies sensitive to species-specific features and the evolutionary and ecological relevance of the methods employed. Many, including I too, have argued that the ethological approach is more appropriate especially when one is interested in the question of biological mechanisms of behaviour (Crusio & Abeelen, 1986; Csányi & Gerlai, 1988; Gerlai & Clayton, 1999; Blanchard et al., 2003). There are two main reasons why this argument is made. One, alleles of genes that influence any trait under investigation have been selected by natural selection and the influence they exert on the phenotype is the result of evolution, the phylogenetic argument (Crusio, 1995). Two, analysis of the mechanisms underlying the phenotypical characteristics can only be conducted appropriately if the characteristics are not artificial constructs but are defined in a biologically meaningful manner. Although the question of what is biologically meaningful is not always easy to answer, in case of behaviour, the above argument translates to choosing methods that allow the quantification of natural, species-specific, responses that are the product of the studied organism and not of the experimenter's subjective bias, the phenogenetic argument (Crusio, 1995). Briefly, one needs to design his/her experiments according to the natural behaviour of the studied species. Notably, results of nature-blind experiments may not be easier to generalize to the human clinic. As I put it previously, "offering a sizeable financial reward to a rat and giving tasty rat chow to humans might not represent 'rigorous laboratory control' of motivation: ignoring species-specific characteristics can lead to less obvious, but similar, mistakes in behavioural research" (Gerlai & Clayton, 1999b).

## 5. Antipredatory behaviour: An ethologically relevant method to study anxiety

Naturalistic approaches thus may have an important place in research whose ultimate goal is to understand the biological mechanisms of abnormal fear responses in vertebrates including our own species (for discussion specific to fear/anxiety see Lister, 1990; Blanchard et al., 2003; Rosen et al., 2008; Gerlai et al., 2009). But behavioral analysis is often deceptively simple (Gerlai, 2001) and this is especially true for anxiety paradigms (Bouwknecht & Paylor, 2008). It is therefore important to consider what approach, behavioral method, has the highest possibility for success. Classical laboratory rodents including the rat and the

mouse have been successfully employed in anxiety research using antipredatory paradigms (e.g. Hendrie et al., 1996). In these tests the subject is exposed to stimuli specific to its natural predator, and the species-typical antipredatory responses of the subject (e.g. freezing) are quantified. Rosen et al. (2008), for example, use trimethylthiazoline, a chemical that is present in the fox's urine and is known to be effective for rodents. Barros et al. (2008), who studied the marmoset, used a cat (a taxidermied wild oncilla cat, the natural predator of the marmoset) as a predator stimulus. Apfelbach et al. (2005) review a large variety of predator odors and their fear inducing effects in different prey species, including cat odor induced antipredatory responses in the rat. Others have utilized eye spots or eye like structures placed on objects mimicking the appearance of predators, an approach that has been effective in a variety of species including rodents, birds and fish (e.g. Gerlai et al., 2000; Miklosi et al., 1997 and references therein). The argument for using ethologically relevant stimuli and measuring species-specific responses in laboratory model organisms is principally based upon the notion that human anxiety disorders are likely to develop as a result of abnormal functioning of neurobiological mechanisms (brain areas, circuits and/or molecular mechanisms) that have evolved to subserve avoidance of predators or other harmful or dangerous agents in nature during our evolutionary past. Given that our species shares its evolutionary past with those of others this approach may have translational relevance. For example, Denver (2009) reviews the structural and functional evolution of vertebrate neuroendocrine stress systems and explains that "Recent findings suggest that the proteins, gene structures, and signaling pathways of the HPA [hypothalamus-pituitary-adrenal] axis were present in the earliest vertebrates and have been maintained by natural selection owing to their critical adaptive roles". This author also concludes that numerous neurotransmitters and neuromodulators influencing stress-related behaviors, such as anxiety and fear, are evolutionarily conserved. Others also argue that the basic neuronal mechanisms are shared across mammalian species, and, for example, the same set of genes may regulate critical aspects of anxiety in humans and in lower species (e.g. Hovatta and Barlow, 2008). Briefly, the translational relevance of fear/anxiety paradigms is expected to be high as long as the mechanisms that evolved in the brain to subserve these behaviors are properly engaged by the experimental set up.

## 6. Zebrafish in the analysis of fear and anxiety

As outlined above there have been a large number of studies devoted to the analysis of the biological mechanisms of anxiety and a considerable amount of effort has been invested in the development of pharmacological treatments of anxiety related disorders For preclinical research most of these studies used rodents. As we have accumulated a large amount of data on these rodent species, it may be logical to think that building upon this excellent foundation may be the only way to proceed. In the subsequent pages, however, I will try to persuade the reader that although abandonment of rodent research is certainly not to be recommended, utilization of another vertebrate, the zebrafish may be a good idea.

### 6.1 Practical simplicity meets system complexity: zebrafish as an optimal compromise for research

A commonplace in research known to many scientists is shown by the following equation: C = E x T, where E is a measure of the ease of use of a research species in the laboratory, T is

the translational relevance of this species, and C is a constant. In other words, the more translationally relevant a species is for human, the less easy it is to use in the lab, and vica versa. Importantly, however, C, as defined above, may not be a universal constant: for some species C may be higher than for others. I argue that zebrafish represents an optimal compromise between practical simplicity and system complexity, i.e. its C is large. It is a small (4 cm long) freshwater fish which is easy to maintain and breed in the laboratory. Due to its highly social nature (shoaling) and its small size, a large number of zebrafish can be housed in small fish tanks. A single female may lay 2-300 eggs at every spawning and may spawn 2-3 times a week. Briefly, a large number of experimental subjects can be obtained quickly and utilized for research in a cost effective manner. These features make the zebrafish particularly appropriate for high-throughput screening applications including forward genetic mutagenesis screens or large scale drug (pharmacological compound) screens. But there are other important features of zebrafish one needs to consider for translational research.

Notably, zebrafish possess high nucleotide sequence homology (60-80%) with that of human genes. Importantly, this sequence homology is functionally relevant as the amino acid sequence of zebrafish proteins (60-90% sequence homology) especially at the functionally relevant catalytic or ligand binding domains of the proteins (approaching 100% sequence homology), has been found highly similar between zebrafish and human (Renier et al., 2007; Reimers et al., 2004), which demonstrates evolutionary conservation of function and allows one to use this relatively distantly related species for translational research. It is notable that evolutionary conservation, i.e. functional and structural homologies, do not end at the nucleotide or amino acid sequence levels, but have been demonstrated at numerous other levels of the biological organization of zebrafish, including, for example, its neurotransmitter systems (Mueller et al., 2004; Panula et al., 2006; also see Chatterjee & Gerlai, 2009; Gerlai et al., 2009) and its neuroendocrine responses to stress (Alsop & Vijayan, 2008). Conservation of function (at the gene expression level) has been found in zebrafish even in such responses as neuro-adaptation to drugs of abuse (Kily et al., 2008). Therefore I and others (Shin & Fishman, 2002) have argued that the zebrafish is an appropriate model organism for the analysis of a range of human diseases.

### 6.2 Genetics: the strength of zebrafish

A strength of zebrafish as a research tool is that by now an arsenal of genetic tools have been developed for this species and the amount of information on the zebrafish genome has also become substantial. For example, a large number of genetic markers crucial for the localization and identification of randomly induced mutations have been established. These include rapid amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLP) (Donovan et al., 2000; Guo et al., 2000; Zhang et al., 1998), polymorphic microsatellite markers and radiation hybrid maps with microsatellite markers and expressed sequence tags (ESTs) (Geisler et al., 1999) as well as single nucleotide polymorphisms (SNPs) (Stickney et al., 2002). The latter study also utilized oligonucleotide microarrays, the gene chip technology that is rapidly spreading in zebrafish research (Sipe & Saha, 2007). A viral infection-based mutagenesis technique has been established for the generation of insertional mutations that could be rapidly cloned due to the presence of the viral tag in the genome (Amsterdam et al., 1999). An entire company was formed to use this methodology and by now a large library of mutants has been generated (see e.g.

http://www.znomics.com/; also see Wang et al., 2007). A gene-breaking transposon-based method to generate mutations has also been developed for zebrafish (Sivasubbu et al., 2006). In addition to forward genetic approaches, reverse genetic methods have been implemented. Morpholino antisense knockdown allows the inactivation of known genes in embryos (Nasevicius & Ekker, 2000; also see Bill et al. 2009 for more recent review). Targeted-induced local lesions in genomes (TILLING) has been successfully adapted to zebrafish (Wienholds et al., 2002). Targeted gene disruption has also been achieved with the use of zinc-finger nucleases (Doyon et al., 2008). More recently, a Gal4/Upstream Activating Sequence approach has been employed for the flexible deployment of transgenes in the analysis of expression patterns of target genes (Scott, 2009), and a transposon-based genetic approach has been proposed for zebrafish (Ni et al., 2009). Importantly, all these tools and pieces of information are in the public domain (e.g., GenBank, Sanger Center website, and ZFIN, see Sprague et al., 2001). Briefly, the zebrafish has become one of the most preferred laboratory animal species of geneticists.

### 6.3 Behaviour: The weakness of zebrafish

An important drawback one has to face when using zebrafish is that the behaviour of this species is not well characterized. This is not to say that there are no behavioural studies on zebrafish or that these behavioural studies are unimportant or inappropriate. On the contrary, there is an increasing number of behavioural neuroscience studies published on zebrafish. Nevertheless, compared to classical laboratory study species such as the rat, mouse, or even the fruit fly zebrafish behavioural research is in its infancy, the number of studies, and with it the amount of information on the behaviour of this species is orders of magnitude less than what is available for classical laboratory model organisms (Sison et al., 2006). Without proper behavioural tests, and without thorough understanding of the behavioural features of zebrafish, it is not possible to utilize behavioural phenotyping of mutation or drug effects, and how these manipulations may influence brain function becomes difficult to investigate (Gerlai 2002). Briefly, behavioural analysis is s a major bottleneck in zebrafish research. A simple literature search in Medline with the keyword "behavior" and "rat" reveals over 100 thousand papers, and another with keywords "behavior" and "mouse" returns about 50 thousand papers. But even for the fruit fly one finds about 5 thousand publications in this area of investigation while for zebrafish this number is less than 100. Although this number is indeed orders of magnitude less than the above, it is notable that the majority of these zebrafish publications were published only recently demonstrating a clear upsurge of interest in this species. It appears that, behavioural brain research and behaviour genetics have discovered the utility of zebrafish. Perhaps one of the best studied of the behaviour of zebrafish is their fear responses. Below I briefly discuss what we know about zebrafish fear and its quantification with an emphasis on how screening applications may be developed and utilized.

### 6.4 Induction of fear in zebrafish using ecologically relevant stimuli: The effect of the natural alarm substance

Predator-prey encounters have not been documented for zebrafish in nature but numerous piscivores have been found to inhabit the slowly moving creeks and small lakes of India and Nepal where zebrafish have been found (Engeszer et al., 2007). The zebrafish belongs to the

Osterophisan superorder of fishes, and numerous species of this superorder have been demonstrated to respond to alarm substances, natural "pheromones" first described by von Frisch (1938; 1941). These substances are released from epidermal club cells of the fish upon infury of the skin (e.g. Pfeiffer, 1972). The zebrafish was known to respond to its natural alarm substance (Schutz, 1956; Pfeiffer, 1963) and later a number of other fish species were also found to exhibit such a response (for a review see Pfeiffer, 1977). The range of behavioural reactions exhibited by fish in response to the alarm substance was also described in a detailed manner (Pfeiffer, 1977) and it was concluded that these responses may significantly differ from species to species but may include "A) fish swimming excitedly with their heads against the bottom and with their bodies at an angle of about 60$^{o}$ to the floor; B) becoming motionless and showing no movement for several minutes; C) sinking to the bottom and spitting gas for a considerable time; D) fleeing to the surface when they are alarmed, crowding together there and swimming hastily, frequently jumping out of the water; or E) fleeing towards the depth where they form a dense school". Waldman (1982) analyzed the effect of alarm substance on shoaling as well as the position of zebrafish in the vertical column of the water and found that it induces fish staying closer to each other and closer to the bottom tank he used. Waldman also described his personal observation regarding a potential developmental trajectory of the alarm substance induced behavioural reactions and theorized that zebrafish may only start exhibiting the alarm reaction after their age of 50 days post-hatching. Pfeiffer and Waldman had no access to technologically advanced video-recording and analysis methods such as tracking systems, thus many of their observations may only be regarded as working hypotheses. By now the technology allows us to precisely track the location as well as movement pattern of fish (see e.g. Blaser & Gerlai, 2006; Miller & Gerlai, 2007; 2008), which has enabled us to confirm many of the intuitions of the above authors. Furthermore, these behaviour quantification methods now allow automated measuring of behaviour, a prerequisite for high-throughput screening.

Another important factor one has to discuss is the origin of the fish used in the behavioural studies. In the early studies with the alarm substance, the zebrafish studied were purchased from local pet-stores and thus numerous factors potentially influencing the behaviour of the experimental fish could not be controlled. For example, the age, potential exposure to other fish species, housing density, type and amount of food prior to experimentation, temperature and water chemistry were all among the environmental conditioned that remained uncontrolled prior to arrival of the fish to the laboratory. The first paper in which the effect of alarm substabce was analyzed with all these factors rigorously monitored and experimentally controlled was conducted by Speedie & Gerlai (2008). This study confirmed that zebrafish not previously exposed to any predatory, harmful, or aversive stimuli would still show a robust alarm reaction to the natural alarm substance, i.e. the alarm response to the substance is innate and represents a genetic predisposition. Speedie & Gerlai (2008) found a significant increase of shoal cohesion, i.e. a decrease of distance between members of the zebrafish group being tested in response to administration of the alarm substance. These authors also found the duration and the number of episodes of erratic movement (zig-zagging) to increase. Freezing (complete immobility) and bottom dwell time also appeared to increase as a result of exposure to the substance. Notably, the alarm substance induced behavioural changes were observed independently of whether the experimental zebrafish were or were not exposed to a live predator during the experiment. In other words, the alarm substance alone could elicit the full repertoire of alarm reactions (Speedie & Gerlai, 2008).

In summary, the induction of fear responses in zebrafish was found possible under controlled laboratory conditions. This is an important step foreward but the difficulty with the studies employing the natural alarm substance has been that the exact concentration of the substance cannot be determined. The dose response analysis in the above cited zebrafish studies was based upon relative doses only, i.e. the experimenters utilized a dilution sequence but could not really ascertain what and how much was in the starting solution. This may not be an important issue as long as the relative doses are compared WITHIN a study. The absolute amount of alarm substance to be extracted from the skin of zebrafish almost certainly varied from study to study no matter how precisely the extraction protocol was followed and thus comparison of effects BETWEEN experiments was impossible to make. Without establishing the exact chemical identity of the alarm substance and without precisely measuring its concentration it was impossible to establish identical doses across different studies. This is a major issue for large scale behavioural screens.

### 6.5 H3NO, the synthetic alarm substance

The above problem was successfully addressed recently (Parra et al., 2009): a synthetic alarm substance was found just as effective in inducing fear in zebrafish as the natural alarm substance. Alarm substances of the Osteriophysan superorder of fishes were identified from numerous species in the past (Pfeiffer, 1977; Pfeiffer et al., 1985). A common chemical structure shared across these multiple species was found (Kelly et al., 2006; Brown et al., 2000; 2003). Based upon this discovery, a compound mimicking this common chemical element was synthesized. The compound is called hypoxanthine 3-N-oxide, or H3NO, a purine derivative oxidized at the 3-position. Hypoxanthine 3-N-oxide has now been shown to induce alarm responses in numerous fish species including the ones that belong to the Osteriophysan superorder (Pfeiffer, 1977; Pfeiffer et al., 1985; Brown et al., 2003; 2002; 2001; 2000). Zebrafish also belong to this superorder and thus it was hoped that this species too would respond to the synthetic alarm substance with species specific alarm reactions. This is what Parra et al. (2009) have now demonstrated. Their findings are not surprising from an evolutionary stand point. If a prey species is too selective about the taxonomic origin of the odour cue that signals danger, members of such a species would be in a disadvantage as they would not be able to recognize imminent danger, the presence of a hunting predator. It should really not matter what prey species the predator catches, and thus being selective about the alarm cue would be an evolutionary failure. Indeed as Parra et al. (2009) found, the synthetic alarm substance did induce a full fledged alarm reaction in zebrafish. These reactions included erratic movements and jumps, typically observed in response to the natural alarm substance. Thus, now we have a compound whose concentration can be precisely determined and thus its alarm inducing effects are no longer dependent upon the method of alarm substance extraction. Briefly, now we can expect high replicability across laboratories or across different independent experiments or across a large number of experimental subjects, prerequisites for high throughput screening.

### 6.6 Visual cues as fear inducing stimuli: The sight of the sympatric predator

It is important to realize that although the use of alarm substance in anxiety research is highly promising, the utility of olfactory cues such as this may be limited, or at least complicated from a practical standpoint. For example, although such an olfactory cue is clearly ethologically relevant and induces robust and species specific fear responses, odour

cues are not easy to control in terms of the timing of their delivery, their removal, and their spatial localization. Briefly, its hard to precisely control when and where they are perceived. For example, although one may think the precise delivery time is easy to establish, it must be noted that it may take time for the substance to diffuse well enough to reach the target subject. It is also notable that removal of such an odour cue is also complicated. For example, residual odour cues left behind from a prior session may influence the behaviour of subsequent subjects. Cleaning the test tanks is labour intensive, and one may not be entirely certain whether the cleaning indeed removed all cues. Ascertaining that the substance used remains active also requires some attention. For example, in our hand even when stored in dry powder format at -20 $^{o}$C, H3NO did deteriorate over a period of several months. Also, as explained above, the on-set and offset of the administration of the odour cue is not precise and for example, multiple on and off time periods are next to impossible to accomplish. Therefore it is likely that cues of other modalities, particularly visual cues, may hold better practical utility (Bass & Gerlai, 2008).

Sympatric predators may induce alarm responses as prey species that coinhabit and thus co-evolved with them may have developed genetic predisposition to innately "recognize" such predators. This was, for example, shown with another fish species, paradise fish (*Macropodus opercularis*), which was found to respond both to the sight and the smell of the snakehead fish (*Chana*) without any prior exposure to this predatory fish (Gerlai, 1993). Paradise fish were also found to exhibit some flexibility and learn to associate otherwise harmless visual stimuli with aversive stimuli (pain or predators), a response that was dependent upon genetic factors (Miklosi et al., 1997 and references therein). These results suggest that innate predator recognition and plastic learning-based antipredatory responses are not mutually exclusive features. Zebrafish have also been found to exhibit learning based alarm reactions (Hall & Suboski, 1995) and more recently they were also shown to respond to their sympatric predator without prior learning (Bass & Gerlai, 2008). The latter authors found zebrafish to exhibit elevated number of jumps in response to the sight of the Indian leaf fish (*Nandus nandus*), a sympatric predator that lives in the same geographical region where zebrafish are found. Importantly, the antipredatory response was elicited by the Indian leaf fish the very first time the experimental subjects were presented with it, demonstrating the lack of need to learn. Also importantly, when zebrafish were exposed to an allopatric predator or to non-predatory fish species, they did not exhibit the antipredatory reactions, which demonstrates that the Indian leaf fish induced responses were indeed specific to this sympatric predator. It is also notable that the Indian leaf fish was not presented in the same water where the experimental zebrafish were swimming, that is the predatory fish was physically isolated from the zebrafish subjects (Bass & Gerlai, 2008). Thus, the only modality the experimental zebrafish could utilize was visual. Admittedly predator-prey interaction between the Indian leaf fish and zebrafish has not been observed in nature (Engeszer et al., 2007). Nevertheless, the above results imply that the zebrafish may have a genetic predisposition to be sensitive to the visual cues that characterize its sympatric predators. From a practical perspective this is great news for the experimenter. Visual cues are easy to control and thus perhaps high throughput behavioural screening may be more feasible using such cues. In the above studies, however, live stimulus fish were presented. This poses a problem. The live predatory fish may change its behaviour from trial to trial. That is, consistent stimulus presentation across multiple experimental zebrafish subjects is difficult

to ascertain. This may be a crucial issue for high throughput screening where thousands of zebrafish may need top be tested in a consistent and highly controlled manner before a behavioural outlier, presumably a mutant, may be identified. One way to address the issue of experimental control and consistent stimulus delivery is to walk away from the presentation of live stimulus fish and use instead computerized images. Could the image of a sympatric predator induce alarm reactions?

### 6.7 The computerized predator: Animated image to induce fear

To answer the above question, Gerlai et al. (2009) experimented with using computerized image presentation to induce fear responses in zebrafish. The authors presented animated (moving) images of the Indian leaf fish to zebrafish and demonstrated that this stimulus elicited erratic movement and jumping from zebrafish, behaviours that were found to be also induced by the alarm substance or by the live Indian leaf fish. At this point it is not known what feature(s) of the computerized image induced the fear responses. In other words, we do not know what makes a good predator for zebrafish. Possible visual properties of the predator zebrafish may respond to include color and pattern (brown patches and markings on a silver background), size (about 10-12 cm long), body proportions (relatively large head and mouth), and/or movement pattern (slow or stationary ambush predator) or any combination of these features. It is possible that when certain key features of a sympatric predator are exaggerated one could induce a further elevated fear response in zebrafish. And clearly, many parameters of the fear paradigm may need to be optimized. Since the publication of the Gerlai et al. (2009) paper, we have already completed another study in which all we did was to lengthen the test tank. The slight change in the dimension of the test apparatus resulted in a robust behavioural change in the zebrafish. In this apparatus, the image of the Indian leaf fish now induced a robust avoidance reaction (increased distance from the image) as well as an increased bottom dwell time. This is noteworthy for two reasons. One concerns the different strategies prey may engage in under specific circumstances. When the prey is within striking distance from the appearing predator swimming away may not be an optimal antipredatory response. Thus in a small test tank other behaviours may be seen, which in our case included erratic movement and jumping. However, if a larger (longer) tank is used, the natural behavioural response of zebrafish to the approaching predator is escape, i.e. increase of the distance between the predator and the prey presumably because this larger tank placed the zebrafish subject outside of the striking distance of the (image of the) predator. The second point concerns the practical aspect of this finding. Measuring distance is much easier and can be better automated than measuring a complex motor pattern like erratic movement. Thus, the longer tank offers the ability to automate the behavioural test. But other modifications beyond changing the dimensions of the tank may also enhance its ability to induce and record fear responses. For example, freezing may be more robustly induced if the tank provides hiding places (e.g. artificial plants). Again, quantification of immobility is achievable using video-tracking systems and thus this behaviour, similarly to measuring distance, can be precisely quantified using automated methods. In summary, the computerized presentation of visual cues have already been shown to induce robust fear and the induced fear responses have been shown to be quantifiable using also computerized video-tracking methods. Thus, the fear paradigm presented above is fully automatable and is ready for high throughput screening of mutations or compounds that alter such fear responses.

### 6.8 Automation is the key for high throughput screening

As explained above, automated stimulus delivery and automated quantification of behaviour are the two important components of high throughput behavioural tests because such automation allows one to run the tests in parallel, i.e. scale up. In this section I explore further the question of automation. Undoubtedly, so far the most sophisticated pattern detection device has been the human brain: the experimenter can identify complex motor and posture patterns as he/she observes the behaving fish (e.g. Gerlai & Csányi, 1990). This is what classical ethologists have been advocating for decades: observe your subject and measure the elements of the ethogram, i.e. how much time the animal spends doing certain things and how often these things (behaviours) occur. A notable drawback of this method, however, is that it is painstakingly slow and extremely labour intensive. The experimenter has to watch video-recordings and key in his/her observations. This is definitely not high throughput! Observation-based behaviour analysis thus have no place in large scale screens. But this method does have merits and certainly makes sense at the earliest phases of characterization of behavioural responses. This is because it allows one to obtain highly detailed information about animal behaviour and perhaps unexpected changes in the behaviour. But once this preliminary idea-generating pilot work is complete and once the experimenter established how the animal responds in the given behavioural task the next step must be to develop automated behaviour quantification techniques. As we have seen, automated induction and quantification of fear responses is already a reality for zebrafish research.

Numerous commercially produced video-tracking systems are available for the researcher and the utility of video-tracking as compared to other behavioural quantification methods has already been systematically analyzed specifically for zebrafish (e.g. Blaser & Gerlai, 2006). But other automated behaviour measuring methods including the force transducer method may also be considered (e.g. Fitch et al. (2002) that allow automated quantification of behaviour. Some commercially available force transducer based methods are claimed to be able to detect particular "force-prints" that correspond to specific motor and posture patterns. If indeed these force prints correspond to motor patterns, the force transducer method could in principle replace the labour intensive observation-based method of ethologists. For aquatic organisms, however, force transducer-based detection is inappropriate because in the water environment the test subject will not generate acceleration forces detectable by the system. Video-image or video-tracking analysis systems have not been successfully employed in motor pattern quantification, although the claim has been made that they are capable of doing so at least for the house mouse (for a review see e.g. Gerlai, 2002). Despite the infrequent use of zebrafish in behavioural studies, we already have evidence showing that videotracking-based automated quantification of fear responses of zebrafish can reveal significant changes in complex motor patterns (Gerlai et al., 2009). For example, reduced swimming speed, increased within individual temporal variability of swimming speed, increased turn angle and increased within individual temporal variability of turn angle upon presentation of the predator image correlated well with certain complex motor patterns, such as erratic movement and jumping, one would expect to be able to quantify only using observation based methods. The close correlation between the above video-tracking and observation based parameters is not surprising: erratic movement is associated with rapid changes in the direction and speed of swimming, i.e. increased

variability of swim speed and increased turn angle and increased variability of turn angle. Similarly, jumping is a rapid and transient increase of swim speed which is expected to translate to increased swim speed variability. In summary, quantification of fear responses has already been automated using videotracking so has the induction of fear responses thus, as we already argued above, a high-throughput fear paradigm is now available for zebrafish (Gerlai et al., 2009).

### 6.9 Light vs. dark, novel vs. familiar places: Other methods to measure fear responses

There may be several ways one can induce and study the effect of fear. Novelty has long been known to induce fear responses in a variety of species including humans. For example, the open field task has been extensively used with rodents (e.g. Prut & Belzung, 2003; Crusio & Abeelen, 1984) or other animals including fish (Egan et al., 2009; Csányi & Gerlai, 1988). In this task, the subject is exposed to an unfamiliar environment. The response to this novel environment is believed to arise as a result of a compromise between opposing forces or tendencies: exploration, which is believed to be associated with active responses, and fear, which is often associated with passive responses. Exploratory activity is considered adaptive as it may lead to finding food, mates and escape routes, for example, while passive fear induced responses (immobility) is argued to reduce predation risk (Crusio & Abeelen, 1986). The adaptive aspect of these responses may seem speculative but quantitative genetic analyses did confirm ambidirectional selection force underlying open field behaviour. That is, in the evolutionary past of the house mouse individuals that performed at intermediate levels (not too active but not too passive either) had been favoured (Crusio & Abeelen, 1986), a finding that extends to other vertebrates including fish (Gerlai et al., 1990). It is likely that the evolutionary past of zebrafish is similar and this species too has been under ambidirectional selection as far as novelty induced behavioural responses are concerned. Therefore, exposing this fish to a novel environment is expected to induce moderate levels of fear reactions. Importantly, behavioural experimentation always includes at least some level of handling of animals by humans, which is also expected to induce fear.

Novelty induced fear responses have been analyzed in zebrafish by Levin et al. (2007) who showed an initially low level of exploratory activity of zebrafish that gradually increased with time. These authors also described a "diving" response, i.e. increased amount of time spent on the bottom of the test tank, a response that slowly habituated as the fish got accustomed to their novel environment. Egan et al. (2009) also reported similar findings. Levin et al. (2007) showed nicotine had anxiolytic properties as this drug reduced novelty induced fear responses. In addition to novelty, different levels of illumination have also been explored to induce and test fear responses.

The light-dark preference paradigm has been often utilized especially with rodents (e.g. Hascöett et al., 2001; Belzung & Griebel, 2001), but more recently with zebrafish too (see below). The assumption underlying this paradigm is that the nocturnal rodent is expected to prefer, i.e. hide in dark places and avoid well illuminated areas. The evolutionary, i.e. adaptive significance of this behaviour is believed to be associated with predator avoidance. A well illuminated rodent can be easily picked up by an aerial or terrestrial predator. Whether zebrafish prefer well illuminated or dark places, has been somewhat controversial in the literature. The starting assumption was that as a result of the zebrafish being diurnal,

active during the day and sleeping during the dark phase of the photoperiod, this species should prefer well illuminated areas where it can visually detect approaching predators more easily. And indeed, this was exactly what was found in a light dark preference task: zebrafish avoided the dark compartment and preferred a well illuminated compartment of a two compartment shuttle box (Gerlai et al., 2000). However, Serra et al. (1999) showed that zebrafish prefer areas of the test environment with a dark background. The difference in the results of these two studies is not easy to explain but may be due to numerous methodological differences. For example, Serra et al (1999) used a dark background but the compartment was well illuminated. Whereas Gerlai et al. (2000) used a dark compartment that was truly dark as it was covered on all sides and the top. One may argue that a dark background, however well illuminated it may be, allows zebrafish to camouflage well (zebrafish has a dark olive-brown back), but a dark cave may harbour predators that remain undetectable for the diurnal zebrafish that uses vision as one of its primary senses.

### 6.10 Pharmacological analysis of fear responses of zebrafish: The first pioneering studies

Many pharmaceutical research companies have been searching for anxiolytic compounds. This is despite that there are numerous prescription medications already available for anxiety and related disorders. The reason for the continued search for better drugs is that we do not really have a complete understanding of how anxiety develops and what biological mechanisms may underlie this disease cluster. The other reason is that the currently available, however numerous, drugs are often not efficacious or do not work for all patients. Briefly, there is still a large unmet medical need for anxiety related disorders. One way zebrafish may be beneficial for such research is to speed up discovery of the biological mechanisms. This may be achieved using, for example forward genetic screens that identify mutations leading to the isolation of underlying genes. Another completely different approach has been to search for compounds, small molecules as they are called in pharmaceutical research jargon, which may alter fear responses. It is thus important to consider what is known about the psychopharmacological properties of zebrafish in the context of fear and anxiety. For example, could one detect the efficacy of known anxiolytic drugs using zebrafish. That is does the zebrafish model have predictive validity. Predictive validity is an important question for the use of novel model organisms. The main point with regard to the translational relevance of laboratory model organisms concerns the notion "evolutionary of homology", i.e. conservation of biological function across previously utilized species (e.g. rodents), the novel laboratory species (e.g. zebrafish), and humans. Admittedly, zebrafish have been used very infrequently in psychopharmacological analyses. Nevertheless, the few studies that have been completed suggest a possibly bright future for drug development with the use of zebrafish.

Alcohol (ethanol, ethyl alcohol or EtOH) is one of the best studied drugs in zebrafish research. For example, the effect of developmental alcohol exposure was shown to be strain dependent (Loucks & Carvan, 2004), early embryonic alcohol exposure were found to exert significant behavioural effects in the adult (Fernandes & Gerlai, 2009), adaptation (tolerance) after chronic alcohol exposure as well as alcohol withdrawal induced behavioural responses were all demonstrated (Gerlai et al., 2009; 2006), and numerous changes induced by acute alcohol administration have also been revealed (Gerlai et al., 2000). Importantly, alcohol has both anxiolytic (for the effects of lower doses of alcohol in zebrafish see Gerlai et al., 2000,

also see Egan et al., 2009) as well as anxiogenic properties (for the effects of prolonged exposure to alcohol and during withdrawal in zebrafish see Gerlai et al., 2009, also see Egan et al., 2009) depending on concentration and mode or regime of its administration. Other drugs of abuse have also been shown to exert significant behavioural effects in zebrafish. For example, the rewarding properties of cocaine have been shown and mutants with altered cocaine reinforced place preference have already been identified in forward genetic screens (Darland & Dowling, 2001). The reinforcing properties of drugs of abuse have also been analyzed (Ninkovic & Bally-Cuif, 2006). Drugs of abuse, similarly to alcohol, often have anxiety altering properties again depending on concentration and dosing regimen employed. For example, a cocaine withdrawal induces anxiety responses in zebarfish (Lopez-Patino et al., 2008). Some classical anti-anxiety drugs have also been tested using zebrafish, e.g. α-fluromethylhistidine exhibited an anxiolytic profile (Peitsaro et al., 2003), diazepam reversed cocaine withdrawal induced anxiety, and the benzodiazepine inverse agonist FG-7142 induced anxiety in zebrafish (Lopez-Patino et al., 2008). Also, acute administration of caffeine, known to induce anxiety in humans (e.g. Childs et al., 2008) and rodents (e.g. El Yacoubi et al., 2000), also led to increased anxiety responses, e.g. reduced frequency of visits to the upper water layer and increased erratic movements in zebrafish (Egan et al., 2009).

Levels of stress hormones have also been analyzed in zebrafish (Alsop & Vijayan, 2008a) and numerous similarities between zebrafish and human stress responses have been revealed, which strengthen the translational relevance of zebrafish in fear and anxiety research. For example, the sight of a predator elevates cortisol levels in zebrafish (Barcellos et al., 2007). It is important to note that cortisol, as in zebrafish, is also the primary stress hormone of the HPA axis in human but not in rodents. In the latter corticosterone plays a more important role instead. Last, treatment with the widely prescribed antidepressant Prozac, i.e. fluoxetine (a selective serotonin reuptake inhibitor) zebrafish reduced their fear responses and spent more time in the top portion of a novel tank and also performed fewer erratic movements. Interestingly, these behavioural changes were accompanied by reduced whole-body cortisol levels (Egan et al., 2009), responses that parallel those seen in rodents (Dulawa et al., 2004).

## 7. Outlook to the future

It is difficult to forecast how useful zebrafish may become in the modeling and analysis of the biological mechanisms of human fear and anxiety. At this point, however, it seems that the main components necessary for such a research to be successful in the future already exist. While only distantly related to human, the zebrafish has already proven its translational relevance. But perhaps the most important advantage of this species as a laboratory tool may be best described with one word: numbers. Complex biological phenomena are associated with large number of mechanisms. These may be discovered using broad screens, genetic or pharmacological. Zebrafish have been proven to be ideal for large scale screens due to several of its features, but mainly to the fact that a large number of these little fish can be produced fast and can be maintained and now tested efficiently in the laboratory. Given the complexity of the mechanisms of fear and anxiety, one may expect the need to identify a large number of molecular players, i.e. genes and their protein products and the biochemical interactions between the proteins. I argue that this complexity may be best tackled, at least initially, using large scale screens for mutations and drugs. These

screens are the key to the identification of potential targets and leads that may subsequently be followed up on by more targeted hypothesis driven analyses. These big "fishing" experiments may be best conducted using the easy to keep and highly prolific zebrafish. It is important to note that I am not advocating the screening approach as the only possible or only potentially fruitful one. Obviously as our knowledge accumulates, increasingly directed and hypothesis driven in depth analyses become possible. But what I am arguing is that the notion of hypotheses having to drive our research somewhat clouded our judgement and we perhaps started the in depth analyses too soon. There expected to be a large number of unknown mechanisms waiting to be discovered and their discovery may be significantly facilitated by "blind", i.e. unbiased, screening applications. And this is exactly where zebrafish has a major advantage over other laboratory organisms.

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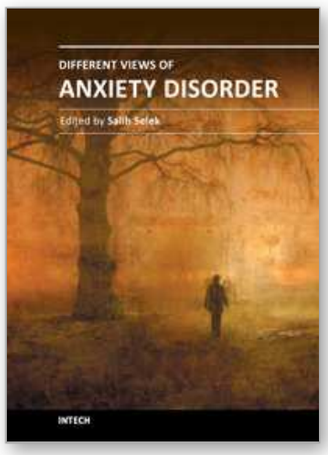


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Anxiety, whether an illness or emotion, is a term with historical roots even in the Bible, but it was not popular until the modern age. Today, we can group, diagnose and treat several anxiety disorders to an extent, but the assessment of symptoms and severity, dealing with resistant conditions, new treatment modalities and specific patient population, such as children, are still the challenging aspects of anxiety disorders. This book intends to present anxiety disorders from a different view and discuss a wide variety of topics in anxiety from a multidimensional approach. This Open Access book addresses not only psychiatrists but also a broad range of specialists, including psychologists, neuroscientists and other mental health professionals.

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