Original Article

Antipredatory Behavior of Zebrafish: Adaptive Function and a Tool for Translational Research

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Abstract: The zebrafish is gaining popularity in behavioral brain research. It may be a cost-effective tool with which we can improve our understanding of the biological and genetic mechanisms of human brain function and dysfunction. Some, myself and collaborators included, have argued that such translational relevance may be best achieved if one considers the ecology and species-specific characteristics of the study organism. In this review, I focus on our own studies investigating zebrafish fear responses, which may be utilized in analyzing the mechanisms of fear and anxiety, and which may be used for screening anxiolytic drugs. I review how zebrafish respond to their natural and synthetic alarm substance as well as to other fear-inducing stimuli, including sympatric and allopatric predatory fish, sympatric or allopatric harmless fish, moving (animated) images of predatory fish and moving images of abstract shapes. I discuss the behavioral responses these stimuli elicit, summarize the methods of the quantification of the behaviors, and speculate about their possible adaptive nature. Although we utilize complex visual stimuli and do not yet know what key features zebrafish may be sensitive to, our results, together with those published by others, imply that this simple vertebrate may have a bright future in behavioral brain research.

Keywords: antipredatory behavior, anxiety, fear, predators, zebrafish

Introduction

The zebrafish has moved to the forefront of developmental biology and genetics after four decades of research with this species (Grunwald and Eisen, 2003). An increased number of articles recently published attests to the fact that it is also gaining popularity in behavioral brain research (e.g., Gerlai, 2010a, 2011; Sison, Cawker, Buske, and Gerlai, 2006). Many of the papers published on the behavior of this species are motivated by the notion that one may utilize this simple vertebrate as a tool with which the mechanisms of complex human diseases, including brain disorders, may be investigated. The main rationale is that research with zebrafish would be more cost effective than with humans or
with any other vertebrate model organisms, including the rat or the house mouse. I and others (Guo, 2004) have argued that zebrafish strike an optimal compromise between system complexity and practical simplicity (Gerlai, 2010a). Practical simplicity comes from such features of the zebrafish as its small size (4 cm long), prolific nature (a single female can spawn 3-4 times a week, producing 200 eggs each time), and the fact that it is a highly social species (a shoaling fish that prefers and thus can be kept at high density) (Miller and Gerlai, 2007; Saverino and Gerlai, 2008). On the other hand, the zebrafish is a complex organism. It is a vertebrate species with a typical vertebrate brain layout and vertebrate physiology (e.g., Alsop and Vijayan, 2008; Chatterjee and Gerlai, 2009; Tropepe and Sive, 2003). Furthermore, and perhaps most importantly, if a zebrafish gene is identified as being involved in an important biological function, it is likely that a human homolog may be discovered that also serves a similar or related function (e.g., Reimers, Hahn, and Tanguay, 2004; Renier et al., 2007). This is because the nucleotide sequence of zebrafish and human genes has been found highly homologous, around 70% on average. Furthermore, nucleotide sequences corresponding to the functionally relevant binding regions of proteins often exceed 90% homology between these two species (Reimers et al., 2004; Renier et al., 2007 and references therein).

Although our knowledge about the genetics and biology, including neurobiology, of zebrafish is rapidly increasing, the behavioral features of this species remain elusive or underexplored at best (Gerlai, 2011; Sison et al., 2006). Perhaps one reason why it is often difficult to investigate the behavior of a novel model organism such as zebrafish is that each species possesses idiosyncratic characteristics, and previous behavioral test paradigms developed for other species cannot be readily adopted (Gerlai, 2001). Although some have stated that the best way to achieve generalization (i.e., translational relevance) is if one ignores these species-specific characteristics, we (Gerlai, 2001, 2002; Gerlai and Clayton, 1999) and others (Blanchard, Griebel, and Blanchard, 2003 and references therein), have argued the opposite. We think that in order to understand the biological and genetic mechanisms of certain complex features, such as behavior, one needs to know how the behavior may be elicited, how it may manifest, and how it can be quantified under the confines of the laboratory. These elements may be specific to the target species and may be dependent upon its evolutionary past and ecology. Our previous, seemingly trivial, example illustrates this point (Gerlai and Clayton, 1999). We argued that it is not useful to study, for example, learning and motivation across multiple species by using the exact same methods. Imagine how a rat would respond to a stack of 100 dollar bills and what a human subject would think if offered a bowl of tasty rat chow as reward in a comparative learning study. Our argument was simple: The best way to analyze translationally relevant mechanisms is to design experiments in which the natural behavior of the subjects is utilized. Forcing the study species into artificial situations may lead to confusing result and may not allow one to tap into functionally homologous neural mechanisms (Gerlai, 2001).

It may be easy to see the above point, but it may not be so simple to put it into practice. How do we know which test is ethologically/ecologically appropriate? How do we decide what is natural or what is artificial for our chosen study species? These questions are not trivial to answer, a fact that is also demonstrated by the following review. In this review I discuss our own first attempts to investigate zebrafish fear responses (Gerlai, 2010b). Our
ultimate goal is to develop behavioral test methods with which robust fear can be induced and in a reliable manner. Such paradigms then could be utilized in the analysis of the mechanisms of fear, for example, by searching for interesting mutants in a pool of ENU mutagenized zebrafish. Alternatively, such paradigms may, for example, be used in large scale drug screens for the identification of anxiolytic or anxiogenic compounds.

In the following pages I review our own efforts with olfactory cues, live stimulus species, as well as animated images that are expected to induce fear responses in zebrafish. I discuss why we believe some of our methods may have ethological relevance and I also discuss the difficulties with this notion. But before I start this review, I first present a short overview on the relatively small amount of information known about the ecology and natural habitat of zebrafish.

Zebrafish in Nature

Only very few studies have explored the natural habitat and ecology of zebrafish per se. These have been reviewed in two recent papers (Engeszer, Patterson, Rao, and Parichy, 2007; Spence et al., 2006) that also significantly added to this topic by conducting original field studies. The zebrafish is a freshwater species that inhabits small lakes and ponds, slow-moving streams, and rice paddies in a large geographic range that extends from Pakistan in the west to Myanmar (Burma) in the east, and from Nepal in the north to the Indian state of Karnataka in the south. The abiotic characteristics of waters of this large geographic region vary. Water temperature may range from 15 to 39 °C (including seasonal and daily temperature cycles), pH between 5–8.2 and conductivity between 10-300 µS. Zebrafish may be found in clear or turbid water and waters with silt or gravel/rocky bottom. The biotic environment of zebrafish contains a lot of shoaling species related to zebrafish (e.g., Danio and Devario), similar in body size and behavior to zebrafish. These fish may compete with zebrafish for food or other resources, such as mating sites. Numerous predatory fish coinhabit the water with zebrafish too. For example, Channa (snakehead fish), Xenentodon (needle or garfish), Notopterus (knife fish), Mystus (catfish) species and zebrafish have been caught from the same habitats. Furthermore, the geographic distribution of Nandus nandus (Indian leaf fish) also coincides with that of zebrafish. In addition to predatory fish, zebrafish may also be preyed upon by dragonfly larvae as well as fishing birds (Spence et al., 2006), including the Indian pond heron (Ardeola grayii) and the common kingfisher (Alcedo atthis).

Spence et al. (2006) recorded several features of the zebrafish habitat and conducted a correlation analysis (Principal Component Analysis) to investigate whether certain characteristics correlate with the abundance of zebrafish. They found negative correlations between abundance of zebrafish and water depth, water flow, transparency, and the presence of predators, respectively, among other variables. Under semi-natural conditions, Spence et al. (2006) also investigated the vertical position of zebrafish and found this species to predominantly prefer the upper third water layer in an 84 cm deep tank, but also to spend some time in the bottom third layer, while mostly avoiding the middle part of the tank.

Reproductive behavior of zebrafish has not been observed in nature, but Engeszer et
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al. (2007) cites a report stating that the breeding season of zebrafish is between April and August, which coincides with the monsoon season. However, Spence et al. (2006) argue that zebrafish breed all year round, an assertion supported by finding females with mature eggs even in January.

The food preference of zebrafish in nature has also been documented (McClure, McIntyre, and McCune, 2006). The mouth of the zebrafish opens upward, which suggests that this fish catches its food items from below. Usually, this anatomy is found in fish that feed from the surface of the water. The gut of zebrafish has been found to contain mainly insects, but crustaceans, fish scales, algae, and detritus were also identified. Analysis of the insects revealed that their origin was primarily terrestrial and not aquatic (McClure et al., 2006), suggesting that the dominant mode of foraging for zebrafish indeed involves catching insects that fall into the water.

Shoaling behavior of zebrafish has been reported (Engeszer et al., 2007; McClure et al., 2006; Spence et al., 2006) in nature, but quantitative analysis of shoal size or shoal composition has not been performed (Miller and Gerlai, 2011). Nevertheless, given that zebrafish coinhabits its habitat with other Danio species of similar size and body shape as zebrafish, it is possible that zebrafish forms mixed-species shoals, a speculation that is supported by the results of empirical laboratory studies (Saverino and Gerlai, 2008).

Zebrafish in the Laboratory: Inducing Fear Responses with Olfactory Cues

Numerous fish species have been found to respond to their alarm substance, an olfactory cue that is released from epidermal club cells when the fish’s skin is damaged (e.g., for earlier reviews, see Pfeiffer, 1962; Solomon, 1977; for more recent discoveries, see Brown, Adrian, Lewis, and Tower, 2002 and references therein; Brown, Adrian, and Shih, 2001; Brown, Adrian, Smyth, Leet, and Brennan, 2000). The zebrafish is no exception. It has also been found to show significant alarm reactions to its natural alarm substance (Hall and Suboski, 1995; Waldman, 1982). We have studied the effect of this substance too, which may be extracted by cutting the skin of freshly euthanized fish and washing the cuts to collect the substance (Speedie and Gerlai, 2008). Zebrafish respond to the presence of this substance with a variety of behaviors, including erratic movement, a typical zig-zag motion with rapidly changing and short bouts of linear and angular acceleration and deceleration episodes (Speedie and Gerlai, 2008). Although this behavior has not been observed in nature, in the test aquarium it usually occurs near or on the bottom of the tank and thus its function has been hypothesized to be the generation of turbulence and with it the stirring up of debris in waters with sediment rich substrates (Speedie and Gerlai, 2008). This speculation is supported by the observation that erratic movement is almost always followed by complete cessation of movement (i.e., immobility). Motionless prey under a cloud of debris may be hard for predators to detect (Speedie and Gerlai, 2008). In addition to this behavior, the alarm substance was also found to induce changes in shoaling (increased shoal cohesion) as well as increased bottom dwell time (Speedie and Gerlai, 2008). However, the issue with the alarm substance has been that fresh extraction does not allow one to ascertain the precise concentration of the active ingredient, at least not across multiple experiments (within a particular experiment one can create a dilution...
sequence and thus control at least the relative dose). Thus, even if the extraction protocol is precisely followed, between-study error rate is expected to be high.

This issue was addressed by Parra, Adrian, and Gerlai (2009) who utilized a recently developed synthetic alarm substance, Hypoxanthine-3-N-oxide or H3NO (see e.g., Brown et al., 2001), and showed that this substance is also capable of producing strong fear responses. Notably, the synthetic alarm substance has been found to induce alarm reactions in multiple species of fish (Brown et al., 2001; Brown, Adrian, Nabil, Mark, and Jocelyn, 2003; but also see Mathuru et al., 2012; Smith, 1999). Whether the synthetic substance induced alarm reactions are identical in severity and/or qualitative aspects to those induced by the natural alarm substance is controversial at this point. In our own studies (Speedie and Gerlai, 2008), we found the natural alarm substance to increase bottom dwell time, with the strongest response elicited by an intermediate dose (i.e., we obtained an inverted U shaped dose response curve) in zebrafish. Waldman (1982) also reported that zebrafish move close to the substrate of the tank in response to the skin extract he used. Furthermore, we found the distance between shoal members to decrease after the administration of the natural alarm substance, and the frequency and the duration of erratic movement episodes to increase in a linear dose-dependent manner (Speedie and Gerlai, 2008). H3NO, the synthetic alarm substance, also induced increased erratic movement as well as elevated the frequency of single jumps (also called leaping, dashing, or darting in other publications) in zebrafish (Parra et al., 2009). But interestingly, H3NO did not increase bottom dwell time.

In line with this finding, Mathuru et al. (2012) also found H3NO not to induce the full fear repertoire of effects elicited by the skin extract of zebrafish. In this latter paper, the authors not only performed a detailed analysis of the behavioral effects of skin extract but also investigated the different components of the extract as to their potential identity and phenotypical effects. They found that chondroitin, a glycosaminoglycan purified from the zebrafish skin extract, induced significant fear responses in zebrafish (Mathuru et al., 2012). It is also notable that there may be several other reasons why the effects of synthetic and natural (skin extracted) alarm substances may differ. There are numerous methodological differences among the cited studies and there may also be significant population or strain differences among the zebrafish used in these papers. Also, importantly, and as mentioned before, the exact dose of the skin extract is most often unknown. Thus, although a within-experiment dilution sequence may give one the ability to conduct a dose response analysis, such an analysis is useless across experiments unless the exact identity and concentration of the active ingredients of the skin extract solution are identified. This is particularly problematic if one considers the found inverse U-shaped dose response profile of the skin extract.

Although not without controversies, H3NO and most recently chondroitin have been identified as important alarm substances that induce robust alarm reactions in zebrafish (Brown et al., 2000, 2001, 2002, 2003; Mathuru et al., 2012). It has been argued that a range of species may respond to the same alarm substances. This lack of species-specificity is not surprising. It is likely that natural selection has favored those individuals that were not highly selective and responded to alarm substances of multiple prey species (Parra et al., 2009). After all, the ability of the prey to sense the presence of a dangerous predator should not depend upon what prey species the predator has just captured.

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The Sight of Predators as Fear Inducing Stimuli

A common problem with all of the alarm substance studies has been that olfactory cues are highly difficult to work with. The onset and offset (particularly the latter) of their administration is difficult to control precisely and may require labor-intensive and time-consuming water changes and thorough cleaning of the experimental tank. Some olfactory cues, including the synthetic alarm substance H3NO, may not be very stable, may decay over time and lose their potency if not stored or handled properly (personal observation), and may also loose efficacy depending on water chemistry, particularly pH (Brown et al., 2002). For these reasons, we have decided to explore alternative ways of inducing fear in zebrafish. Zebrafish is a diurnal species which has excellent vision. The zebrafish visual system is tetrachromatic (e.g., Connaughton and Nelson, 2010). This fish has four different cones that are sensitive to particular wave-lengths of light. Zebrafish may use it to detect the presence of mates, competitors, food items, as well as predators. Indeed, we have been able to induce robust fear responses by exposing zebrafish to live predatory fish (Bass and Gerlai, 2008). Zebrafish responded strongly irrespective of whether the predator was in the same tank as the test subject or if it was outside of it, as long as the test subject could see the predator (Bass and Gerlai, 2008). The responses of zebrafish included moving away from the location where the predator was shown, as well as increased number of erratic movement episodes and leaping (a single acceleration bout achieved by the use of the caudal fin). In addition, zebrafish also distinguished predatory and harmless fish species as they responded with increased shoal cohesion (smaller inter-individual distance within the shoal) only to predatory, but not to harmless, fish species shown.

An interesting aspect of these findings was that the two predatory species used (the Indian leaf fish, \textit{Nandus nandus} and the Compressed African cichlid, \textit{Dimidochromis compressiceps}) induced different behavioral responses (Bass and Gerlai, 2008). Presentation of the leaf fish led to increased erratic movement and leaping frequency, whereas the African cichlid was mainly ineffective. This difference was observed despite the fact that the African cichlid performed a significantly higher frequency of predatory attacks. Given that the experimental zebrafish used in these studies were bred and raised in the laboratory and had never been exposed to predators of any kind, we concluded that zebrafish possess genetic predispositions that allow them to respond to aversive cues without any prior exposure to such cues. That is, the possibility of zebrafish being able to innately recognize its natural (sympatric) predator was raised (Bass and Gerlai, 2008).

Most recently, however, this notion was put under scrutiny (Ahmed, Fernandes, and Gerlai, 2012). The issue with the previous study that demonstrated the differential effect of the sympatric leaf fish vs. the allopatric compressed cichlid was that only one sympatric and one allopatric predator was compared; i.e., the results may have been idiosyncratic to the pair of predatory stimulus fish used. It is possible that other sympatric predators may not induce strong antipredatory reactions or, alternatively, allopatric predators other than the compressed cichlid do. We have started to address this question by exposing zebrafish to animated images of a series of sympatric predators (Ahmed, Fernandes, and Gerlai, 2012).

Our results partially confirmed that zebrafish are capable of responding to fear...
inducing stimuli without any prior exposure to such stimuli. But the answer to the question of whether zebrafish are able to recognize their natural (sympatric) predators was further complicated by our results. This is what we found: Four sympatric predator species, the Indian leaf fish \((\textit{Nandus nandus})\), the blotched snakehead fish \((\textit{Channa maculata})\), a catfish \((\textit{Mystus bleekeri})\), and the clown knife fish \((\textit{Chitala chitala})\) were found to be highly effective. In fact, the knife fish and the catfish appeared even more effective in inducing the fear responses than the leaf fish was. Interestingly, the fifth predator, the freshwater needle fish \((\textit{Xenentodon cancila})\), whose image we used, did not induce the expected fear responses and in fact, instead of an avoidance reaction, it elicited approach from zebrafish. This was quite surprising to us as this predator species has been repeatedly identified as one that inhabits the same waters as zebrafish, and it is also known to be a dedicated piscivore \((\text{Engeszer et al., 2007; Spence et al., 2006})\). Also notably, the size of the image of the needle fish we used was large enough for a live individual to be able to capture and eat a fully grown adult zebrafish. Furthermore, in another independent study, we also found the needle fish to be ineffective in inducing fear reactions in zebrafish \((\text{Luca and Gerlai, 2011})\). We speculated that perhaps the manner in which the image of the needle fish was presented did not mimic the natural behavior of this predator and that may have been the reason why zebrafish explored this stimulus rather than avoiding it. For example, we utilized a standard presentation method that included making the image of the given predator move with a set speed of 0.3 cm/sec. Also, the images moved across the entire surface area of the side wall of the test tank; i.e., they were not localized to any particular layer of the tank. The advantage of this computer animation was that it allowed us to present the stimuli in a consistent manner across all types of stimuli and sessions. The disadvantage may have been, however, that particular predators may exhibit different motor patterns or may occupy different layers in the water column in nature. For example, while the knife fish, leaf fish, and the snakehead fish are ambush predators and are expected to stay in position or move only slowly until the predatory strike, the needle fish is a fast moving and actively hunting predator \((\text{Gerlai, personal observation; also see Foster, 1973})\). Also, the knife fish and the leaf fish tend to be either on the bottom or the mid layers of the water column whereas the needlefish is usually found near the water surface. In summary, it is likely that the needlefish was presented in an artificial manner that did not recapitulate the natural behavior of this piscivore.

This latter speculation brings up an important point. We know practically nothing about what makes a predator a predator for zebrafish. We do not know what stimuli zebrafish may be sensitive to and how certain stimuli define danger for zebrafish. Such stimuli may include the color and pattern, the size and body proportions, and/or the motor patterns of the stimulus fish or any combination of such features. In fact, it is possible that zebrafish may not need to be presented with a realistic image of a sympatric predator in order to respond to the stimulus with a full-fledged fear reaction, a possibility that is now supported by empirical findings \((\text{Ahmed, Fernandes, and Gerlai, 2012})\).

In this latter study, zebrafish were presented with abstract shapes in a manner identical to how the images of sympatric predators were shown. Interestingly, one of the shapes, a rectangle whose speed of movement, size, “body proportions” (12 x 3.8 cm, length x height) and color (light greenish brown) were similar to corresponding features of
the presented predator images, induced fear responses that were statistically indistinguishable from those induced by the most effective sympatric predator images (e.g., that of the knife fish, catfish, snakehead fish, and leaf fish). That is, although similar in some characteristics to the images, the otherwise featureless (no pattern, no eyes, no mouth, no fins) “predator,” the slowly moving greenish-brown rectangle, was just as effective as the images of real piscivores. Based on this finding, one could argue that any object of a certain size moving in a certain speed may induce the fear responses in zebrafish. However, we have evidence that this is not true (Ahmed, Fernandes, and Gerlai, 2012). Another geometric shape (a flattened star), whose size, speed, color, and overall proportions were all identical to those of the rectangle, was completely ineffective as it induced no fear reactions in zebrafish (Ahmed, Fernandes, and Gerlai, 2012). In summary, the above findings clearly demonstrate our lack of understanding of what stimuli and in what combination define a predator for zebrafish. Systematic dissection of features of predators will need to be conducted. These studies are now quite feasible with the use of computer animation software. It is clear, however, that realistic images of sympatric predators are not necessary for the induction of fear responses in zebrafish.

This notion is also supported by another study in which we presented computer animated objects, including an expanding dot and the silhouette of a bird of prey, to zebrafish and measured the fear reactions of the experimental zebrafish (Luca and Gerlai, 2011). The main goal of this study was to explore whether the location of presentation of these stimuli matters and whether the fear responses are uniform or idiosyncratic to the context in which they are induced. The results indicated that the expanding dot induced a strong fear response, especially when presented from above. We speculated that this stimulus may resemble a rapidly approaching aerial predator (birds pull their wings close to their body when striking with a dive from above and thus may appear cylindrical when viewed head-on). Importantly, the very same stimulus (expanding dot) when presented on the side induced much less robust reactions as compared to when it was shown from above, but it still did lead to avoidance of the location of the stimulus (Luca and Gerlai, 2011). Surprisingly, the bird silhouette was effective both when shown from above and from the side (Luca and Gerlai, 2011). It led to significant reduction of distance swum and increased the number of erratic movement episodes. But in other behaviors the side versus top presentation of this stimulus induced different responses. When shown on the side the bird silhouette increased the number of leaps (called jumps in that paper), but such change was not observed when the stimulus was shown from the top. The percent of time the fish remained completely immobile was affected the opposite way by the different location of stimulus presentation. When shown from the top the bird silhouette increased immobility, but when shown from the side it decreased it. Similarly, when shown from the top the stimulus increased bottom dwell time, but when shown from the side it decreased it.

Taken together, these findings and the ones obtained in a series of experiments with different predator images and other abstract shapes suggest that zebrafish have a complex antipredatory repertoire, and how this species responds to a particular aversive stimulus is dependent upon both the type and the location of the stimulus. It is thus likely that one needs to measure a range of behaviors to capture the changes in fear response induced by, for example, a mutation, a novel drug, or the particular aversive stimulus employed. This
brings us to the next question: How can one quantify these responses and detect changes in fear-related reactions?

**Quantification of Fear Responses**

Quantification of behavior in general is a whole scientific field to itself (Martin and Bateson, 1993; also see Gerlai, 2002). Here, I will focus on two principally different methods: observation based and video-tracking based applications as they pertain to the analysis of zebrafish behavior (Blas er and Gerlai, 2006). Each has its own merits and disadvantages. Observation-based behavioral quantification is time-consuming and labor-intensive (e.g., Gerlai, Crusio, and Csányi, 1990). The observer needs to view the animals performing in the task and needs to identify the behaviors, record the amount of time for which the behavior occurred, and/or record the number of occurrences of the behavior. The advantage of this approach is that even with sophisticated computer technologies of the 21st century, the human brain is still perhaps the best pattern detection device and thus observation-based approaches allow the experimenter to quantify often rather complex motor and posture patterns that would escape detection by automated techniques such as video-tracking (Gerlai, 2002). In the case of fear, one of the most typical responses to a variety of fear-inducing stimuli has been erratic movement or zig-zagging (e.g., Gerlai, 1993; Gerlai, Lahav, Guo, and Rosenthal, 2000; Bass and Gerlai, 2008). This characteristic fear reaction is difficult to capture with video-tracking (but see Gerlai, Fernandes, and Pereira, 2009). Another behavior that is similarly problematic to quantify using video-tracking is leaping (or jumping). It is also notable that in response to novel treatments (drugs, induced mutations, or other new experimental manipulations) one may see unexpected or previously undescribed motor and posture patterns that would remain undetectable if one used traditional video-tracking parameters (see below). Thus, it is recommended that analysis of the effects of such novel treatments start with observation-based methods (e.g., Gerlai and Clayton, 1999). It is also notable that, unlike common wisdom, observation-based quantification methods do have high inter- and intra-rater reproducibility, and if the studies are conducted blind and experimental design includes proper randomization, then experimenter bias is also not a problem. Furthermore, nowadays the experimenter using observation-based methods does not have to rely on his/her stopwatch. These approaches move beyond the old-fashioned paper-pencil method and now employ sophisticated software aids, applications that turn the computer into a practically limitless number of stopwatches that can allow one to measure both mutually exclusive and overlapping behaviors of multiple subjects (players) at the same time.

The alternative to observation-based methods is, for example, the automated computerized video-tracking, which has also been successfully employed in the analysis of zebrafish behavior (Blaser and Gerlai, 2006). This method allows one to record the xy (or in more advanced cases the xyz 3D) coordinates of the location of the target subject(s) in high temporal resolution (e.g., 10-40 video-frames per second). From these coordinates, one can extract a large number of important behavioral measures, including exact location of the fish, the distance of the fish from a predetermined area, line or point, total distance moved, speed, turn angle, and several other characteristics of the movement (swim) path of
the subject. The main advantage of this approach is that it is highly quantitative. A human observer may be able to distinguish fast swim speed from slow, but he/she will definitely not be able to precisely measure how much or how fast the subject was moving. Similarly, although one can record how much time the test subject spent in the bottom third of the tank, for example, the exact distance from the bottom can only be quantified using the video-tracking software. This is an important drawback of observation-based methods, as quantitative (e.g., exact distance measures) variables tend to offer better statistical power and less error variation compared to discrete variables (e.g., variables that depend upon thresholds or categories). Another major advantage of video-tracking is that it does not require the presence of the observer. One can video-tape the behavioral sessions of multiple subjects in a large number of experimental set-ups and quantify the behavioral responses in parallel. The ability to scale up behavioral research this way allows the experimenter to utilize video-tracking in high throughput screening applications, the major promise of zebrafish research (Gerlai, 2010a and references therein; Grunwald and Eisen, 2003). It may even be possible to capture some aspects of complex motor patterns using video-tracking. For example, we have found that erratic movement significantly correlates with within-individual temporal variance of speed (because erratic movement is associated with episodes of very fast and very slow swim bouts) as well as turn angle (because this behavior is associated with a series of quick direction changes), and with the within-individual temporal variance of turn angle (Gerlai et al., 2009), parameters that standard video-tracking software applications routinely extract. Furthermore, one can easily see how combining certain classical video-tracking parameters and setting up certain filters (criteria) may allow one to define more complex behavioral variables. For example, leaping (jumping) may be defined by swim speed reaching a minimum threshold (e.g., 5 cm/sec) and at the same time bout length not exceeding an upper limit (e.g., 2 sec). In summary, although observation-based methods are recommended at the early discovery/development phase of the research study, video-tracking applications may provide sufficient sophistication and are crucial in later, high-throughput phases of fear research. Last, it is also recommended that once an interesting effect (or a mutation or a compound) is identified as having fear altering aspects, one can (and should) go back and observe the behavior of the subject to make sure the automated method is correct and did not miss some important changes.

**Analysis of Fear Responses in Larval or Developing Zebrafish**

In this review I deliberately focused on studies conducted in my own laboratory. These studies have been developed for the analysis of the behavior of adult zebrafish. Focusing on the adults, I believe, is important because at this developmental stage one does not expect robust age-dependent (i.e., ontogenetic) changes for several months of the subjects’ lives. That is, after reaching sexual maturity at the age of 3 months, zebrafish remain healthy and reproductively active until about 3 years of age without showing clear signs of aging. This stable period allows one to study time-dependent effects of many manipulations, including repeated environmental stimulus exposure-induced effects or drug or chemical exposure episodes. Such repeated exposure/stimulation effects would be
difficult to disentangle in the rapidly developing embryo or larval fish. Nevertheless, analysis of embryonic development or the larval stage of zebrafish has been the focus of the majority of studies conducted with this species. The behavioral characterization of the larval zebrafish is also important. Specifically, fear responses in the larval zebrafish have been analyzed. Here I only mention a couple of examples for such analyses. Bianco, Kampff, and Engert (2011) analyzed the effect of moving objects (a circular spot presented on a screen using an LCD projector) on 6-7 post-fertilization day old zebrafish (freely swimming stage starts at 5 days post-fertilization). These authors found that when the spot was small, it elicited a prey capture-like response, orientation with the body, and fixation (eye convergence) by the eyes, but when the stimulus was large it induced a significant avoidance reaction. Richendrfer, Pelkowski, Colwill, and Creton (2012) recently published a paper in which a simple and cheap larval zebrafish apparatus and assay for the analysis of fear responses is described. In this paradigm the zebrafish (again, only a few day old freely moving larval zebrafish) are presented with large moving circular objects on a computer screen placed underneath the arenas (wells) in which the subject(s) is/are swimming. The authors showed a significant avoidance reaction induced by the "bouncing ball" and argued that their paradigm allows high throughput screening of anxiolytic and anxiogenic compounds at this early developmental stage, an important development in the field given the short time required for the generation of hundreds of larval zebrafish.

Concluding Remarks

From a translational perspective, zebrafish may be important in the analysis of fear for two main reasons. First, as a practically more feasible study species it may allow one to discover important genetic and other biological mechanisms associated with fear faster and with better efficiency than one could achieve with other laboratory vertebrates or with our own species. Given the evolutionary conservation found at several levels of the biological organization between this fish and humans (from nucleotide sequence, homologies between genes of the two species, physiological and anatomical similarities of these species, to functional similarities of their brains, and the behavioral manifestation of these functions), one can be hopeful that the zebrafish will offer good translational relevance. Second, even if the zebrafish may not always be superior from a research perspective as compared to other model organisms, its analysis adds important knowledge as to how the vertebrate brain functions. Information about mechanisms underlying complex behaviors, such as fear responses, obtained from multiple species allows one to compare the results. Such a comparative approach may allow one to find common features among the studied organisms. It is these commonalities that should truly allow good translation to humans, as it is likely that they represent perhaps the most important evolutionarily conserved features. Finding such features, I believe, may be best achieved if one keeps the species-specific characteristics of the study organism in mind; i.e., if one considers information about the natural history and ecology of the study species in the design of experiments and in the interpretation of their results.

In this review I mainly focused on our own efforts and discussed some points about the behavioral analysis of fear responses in zebrafish. Clearly, our studies demonstrate that
much needs to be done before one can really understand how to induce and measure fear reactions in this novel study species. Nevertheless, we, together with many other researchers (e.g., Blaser, Chadwick, and McGinnis, 2010; Colwill and Creton, 2011; Jesuthasan, 2012; Jesuthasan and Mathuru, 2008; Maximino et al., 2010; Okamoto, Agetsuma, and Aizawa, 2012; Stensmyr and Maderspacher, 2012), have already collected enough evidence that demonstrates that zebrafish will be an important tool with which the mechanisms of vertebrate fear is studied.

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