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Chapter 2

Zebrafish embryos and larvae in behavioural assays

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Abstract

Zebrafish embryos and early larvae are becoming increasingly important as models in biomedical research because of their low cost, high throughput and potential as a replacement for adult, higher vertebrate model species (especially rodents). The functional domains of drug targets, and other functionally important proteins, are often highly conserved between the zebrafish and mammals. Furthermore, the zebrafish embryo or larva shows a complex behavioural repertoire only a few days after fertilization. Here, we show how behavioural studies in mammals are being translated to the zebrafish embryo/larva model. We give emphasis to behavioural studies that may be relevant to drug screening or safety toxicology. We show how video tracking hardware and software can be used to provide an automated, high data-content readout for zebrafish behavioural responses. Published behavioural assays using zebrafish developmental stages include responses to dark challenge, acoustic stimuli, novel environments and various pharmacological compounds. Future prospects for zebrafish developmental behavioural studies include the potential to move from 96-well format studies into microfluidic-based embryo cultures. The zebrafish embryo model is already becoming a useful system for identifying molecular-genetic pathways associated with behavioural responses.

Introduction

The need for new model systems in behavioural and neuropharmacological studies

It is well known that the number of major new drugs coming onto the market is declining, while the cost of drug development is increasing [89]. This problem is particularly acute in the field of central nervous system (CNS) drugs. In particular, the discovery of new psychiatric drugs has the lowest success rate compared to other therapeutic arenas [90,91]. Because of the lengthy development timelines (7-9 years per drug), extreme costs (up to €1.5 billion per drug), high attrition rates and patent expiration of main blockbuster drugs (the 'patent cliff'), discovery of drugs for disorders of the nervous system is becoming prohibitively expensive and risky [91,92]. This is well illustrated by the recent announcement that major pharmaceutical companies have pulled out of drug discovery in some domains of neuroscience, including psychiatric disorders and pain [91]. In addition to these problems, there is a desire to reduce the use of adult mammals in biomedical research, on ethical and cost grounds (reviewed by [93]). For this reason, alternatives to the use of adult mammals are sought, and the zebrafish embryo is one model commonly considered (Figure 2.1) .

The zebrafish embryo model

The zebrafish is a freshwater teleost fish that is very easy to keep and has low maintenance costs. The embryos can be used in many research contexts because they develop extremely rapidly and can be obtained year round in large numbers [28,43,44]. External fertilisation and development of the eggs (which reduces maternal behavioural influences) and the transparency of the zebrafish embryo (which allows live cell imaging) are another beneficial aspect when studying multiple developmental processes. Zebrafish embryos kept at 28.5°C hatch between 48-72 hours post fertilization (hpf), when they become free-swimming larvae with a complex behavioural repertoire [32]. Before hatching, the embryo is confined within the chorion, a semi-transparent and relatively impermeable membrane which acts as a barrier to the entry of some compounds [94].

The zebrafish genome is almost completely sequenced and is available online at ensembl.org; it contains orthologues of numerous human disease genes [95]. Analysis shows that many of these genes, including those that are implicated in behaviour, are highly

conserved between the zebrafish and humans, at least in their functional domains. An example is the glucocorticoid receptor isoform GR β which our group has shown [96] to be highly similar to the human GR β (Figure 2.2). Other online resources include the zebrafish user community at zfin.org, which has a wide range of protocols and databases relevant to general zebrafish research; and the Zebrafish Neurophenome Project [97] which provides resources for researchers in the field of zebrafish behavioural biology and neurosciences.

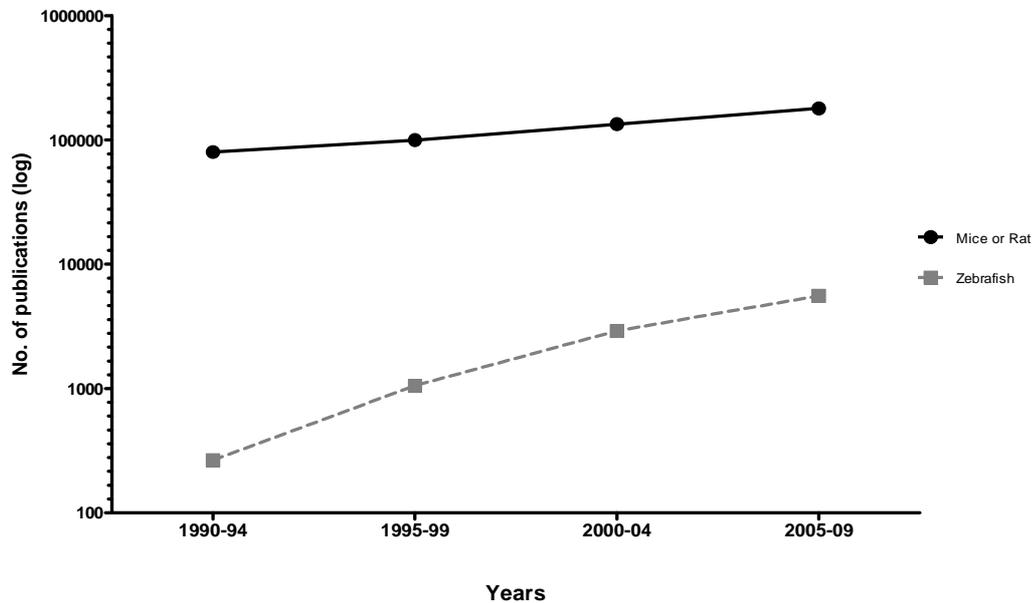


Figure 2.1. The use of zebrafish in biomedical is increasingly rapidly, as can be seen from the number of articles on Pubmed in which “zebrafish” appears in the title/abstract. The search included papers from all disciplines covered by Pubmed at <http://www.ncbi.nlm.nih.gov/sites/entrez/> regardless of whether the zebrafish used were larval or adult. The same figures for a search with “mouse OR mice” is also shown. As can also be seen, the rodent remains by far the more commonly used of the two model species. Indeed, the zebrafish embryo is never likely to replace rodents in academic or pharmaceutical research owing to its lower toxicity predictivity and the demand of regulatory authorities to perform test on mammalian species. Rather, it may represent a complementary model that provides a bridge between cell culture systems and mammalian whole animal models.

A recent study examined behavioural changes in zebrafish embryos after sublethal exposure to a panel of toxic compounds [55]. The majority (57/60) of the compounds induced behavioural changes that were easily detected and analysed by videotracking equipment and software. In another study, the toxicity of compounds to zebrafish embryos was found to be well correlated with the toxicity of those same compounds in rodents [98]. It has also been shown that several drugs which cause QT prolongation (sometimes leading to spontaneous death) and cardiac arrhythmia in humans, also produce cardiac dysfunction (bradycardia and/or dissociation between atrial and ventricular rhythm) in zebrafish embryos [99,100]. This is perhaps not surprising, in view of the fact that the zebrafish has a remarkably similar electrocardiogram (ECG) pattern to humans [101]. It also has an orthologue of the HERG receptor gene that is bound by many drugs with cause cardiac side-effects. Not only is the zebrafish ZERG highly similar in its amino acid sequence to HERG [99], but it also shows similar biophysical properties [102]. Together, these findings indicate that the zebrafish embryo model may be used for identifying drugs that might produce serious cardiac side effects.

The studies cited above suggest that the zebrafish embryo model has promise for safety/toxicity screening. Another area in which the zebrafish embryo model shows great promise, and which will form the focus of this chapter, is as an experimental model in behavioural testing.

Ontogeny of zebrafish locomotor and behavioural responses

Zebrafish embryo development takes place very rapidly. The first behaviour observed during its development is spontaneous movement that begins at 17 hpf, during which embryos repeatedly perform slow, alternating tail coils [32]. Older embryos within the chorion, close to hatching, show rhythmic movement of the pectoral fins, together with sudden changes of position of the embryo, every 20 seconds or so [103]. Forward genetic analysis coupled with behavioural recording has already started to identify genes that may influence behavioural responses [104].

A recent study [105] has found that 30 hpf old larvae which is still in the chorion can produce a startle response to a pulse of light. Hatched larvae (48-72 hpf) occasionally move and remain on the bottom of the tank. At 4-5 dpf, the swim bladder gradually inflates [106], larvae have excellent vision and are able to hunt for food [107]. After quick maturation of

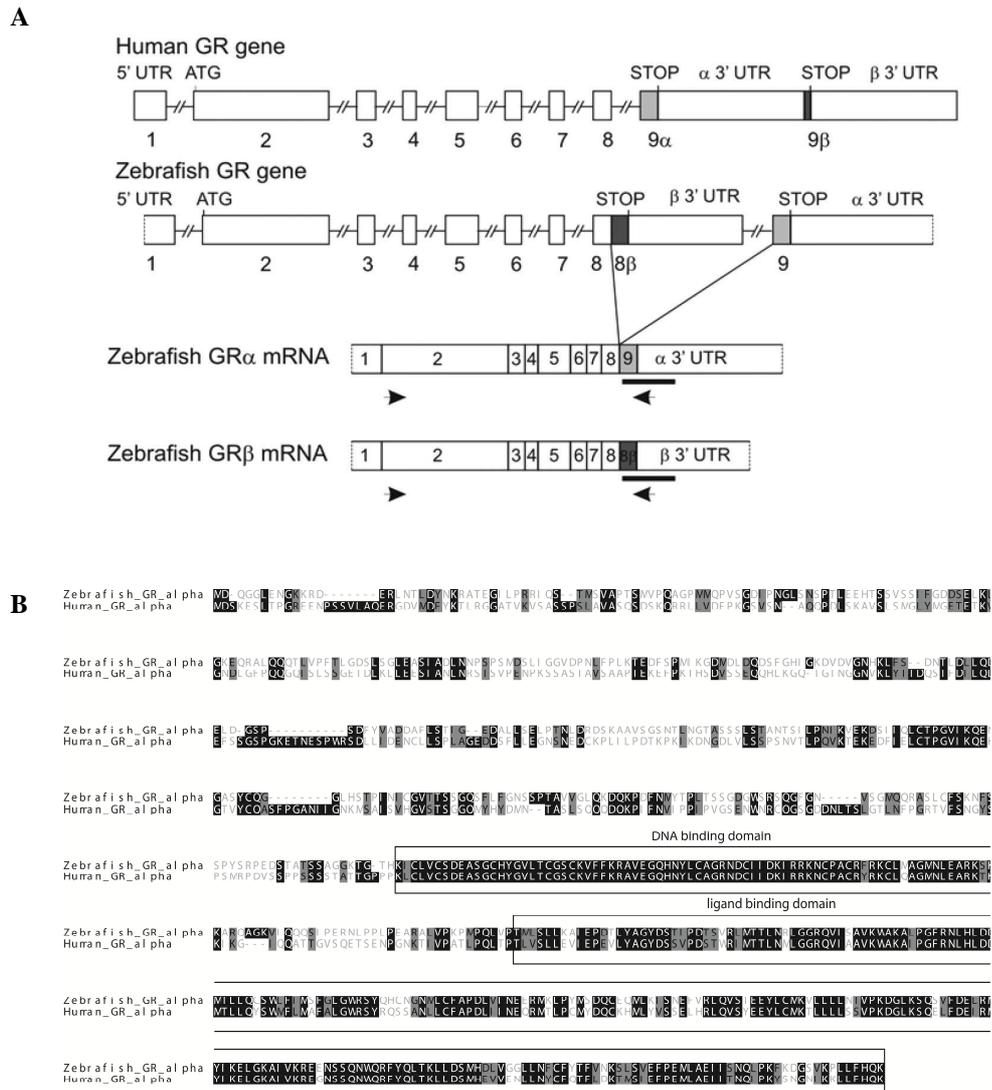


Figure 2.2. **A**. The glucocorticoid receptor (GR) gene provides a good example of the strong conservation between many zebrafish genes and their human orthologues. For example, the exon structure of the GR β isoform is remarkably similar to that of humans. From Schaaf *et al.*, with permission [108]. **B**. An apparently low sequence similarity between zebrafish and human proteins often belies the fact that the functional domains of key genes may be highly conserved. For example, we show here the GR α protein isoform, which may underlie depression-like behaviours in zebrafish. Note the high sequence similarity in the functional (ligand binding and DNA-binding) domains between the human (NP_000167.1) and zebrafish (ABR88075.1) proteins. ClustalW alignment.

the sensory and motor system, zebrafish larvae are able to display more robust and complex behaviours in the first week of development.

Zebrafish larval locomotor assays can be used as screening tools for testing the behavioural effects of many drugs, chemicals and toxins causing hyper- or hypo-activity from a safety pharmacology perspective [109]. This use is driven in part by the desire to reduce the numbers of rodents used in the drug discovery and compound screening process.

Therefore, the various automated systems that have been developed to study rodent behaviour are being adapted to the study of zebrafish behaviour. Using these automated systems, various behaviours can be studied in detail with the help of high-resolution video recordings and analytical software.

Before the zebrafish embryo model can be accepted as a complementary behavioural screen for compounds, it is vital that we understand the predictivity of the zebrafish embryo model (that is, the extent to which it is informative about the effects of compounds in mammalian systems). A range of anxiolytic and stimulant compounds (Table 2.1) were tested for effects on the locomotor activity of zebrafish larvae and were shown to produce responses similar to those seen in mammals [110-113]. Ethanol (which has both stimulant and sedative effects) caused hyperactivity at lower concentrations (1–2%), whereas treatment at higher concentrations (4%) decreased the locomotor activity [114,115]. Thus, these results suggest that zebrafish larvae are sensitive to neuroactive drugs, and that their locomotor response is similar to that of mammals. If further studies confirm these findings, then the zebrafish has promise as a model organism to test the effects of drug treatment on locomotor activity. This could be applied to the early identification of potential neuroactive compounds before regulatory preclinical studies are performed [46,87].

Champagne and colleagues [54,80,116,117] made a series of studies in which they aimed to translate rodent behavioural repertoire to adult zebrafish. They found that zebrafish display behaviours which resemble those of rodents (e.g. in *open field* and *light/dark box* tests). Zebrafish displayed anxiety-like behaviours including dark-avoidance in the *light/dark box*, and thigmotaxis in the *open field* test. These behaviours waned over time and a previous history of stress attenuated the dark-avoidance. These zebrafish behaviours were later translated to larval/juvenile zebrafish [54,118]. While zebrafish can never replace rodent models, the studies cited suggest that they can at least provide a

complement to rodent models, especially in the fields of neurobehavioural research and drug discovery.

Aims of the present chapter

Here, we review the potential value of the zebrafish embryo and larva as a model for behavioural research, neuropharmacology and high throughput drug screening. We concentrate on recent work from our lab and related studies from other groups worldwide. First, we discuss the potential of the zebrafish embryo/larva model system to be adapted to high throughput analysis via automated video-tracking systems, translated from rodent behavioural paradigms. Second, we review the range of behavioural tests that can be applied to the study of zebrafish embryos and larvae. We then consider two illustrative case studies of the recent application of zebrafish embryo/larva-based assays to two applied problems: (i) the study of anxiety-like behaviours and the neuropharmacological screening of anxiolytic compounds and anxiogenics; (ii) the use of behavioural recording in safety/toxicity studies. Finally, we consider some potential future technological developments in this field with special reference to the microfluidic culture of zebrafish embryos. A note on terminology: the zebrafish *embryo* becomes a *larva* at the moment of hatching (around 48 – 72h). We shall include both of these rather arbitrary stages in our discussion here.

Automated video tracking

In this section, we discuss the use of video-tracking software and hardware for studying zebrafish embryo/larva behaviour. Traditionally, a researcher observes the animal and notes behavioural events of interest, either by writing it down on paper or by entering the data into an event-recording program [119]. Manual recording of behaviour can be implemented with a relatively low investment, and, for some behaviours, it may be the only way to detect and record their occurrence. However, automated observation can provide significant advantages.

Behaviours are recorded more precisely because the computer algorithm always works in the same way, and the system does not suffer from observer fatigue or drift. In contrast to manual observation, video tracking software carries out pattern analysis on a video image of the observed animals to extract quantitative measurements of the animals' behaviour. Automated observation using video tracking is particularly suitable for measuring

locomotor behaviour, expressed as spatial measurements (distance, speed, turning, etc.) that the human observer is unable to accurately estimate.

Table 2.1 Effect of different compounds on locomotor activity of larval zebrafish.

Compound	Concentration	Age of zebrafish	Reference
A. Sedative			
4-aminopyridine	0.6mM	5 dpf	[120]
Clozapine	12.5–50 mM	7 dpf	[112]
Cocaine hydrochloride	0.2–50.0 μ M	6 dpf	[121]
d-amphetamine	0.1–20.0 μ M	6 dpf	[121]
Diazepam	10 nM–100 mM	7-14 dpf	[121]
Ethanol	4%	6 dpf	[114,121,122]
Fluoxetine	4.6 mM	3-6 dpf	[111]
Melatonin	10 nM–100 mM	7-14 dpf	[110]
Pentobarbital	10 nM–100 mM	7-14 dpf	[121]
Polybrominated diphenyl	31 μ g/L	5 dpf	[123]
B. Stimulant			
4-aminopyridine	0.8-2.5 mM	5 dpf	[120]
Aconitine	2.5-25 μ M	5 dpf	[120]
Bisphenol A	0.01-1 μ M	5 dpf	[124]
Ethanol	1-2 %	7 dpf	[114,121]
Pentylene-tetrazole	10 mM	5 dpf	[120]

Video tracking systems were introduced in the early 1990s, offering clear advantages of flexibility, spatial precision, and accuracy over manual observation or devices using infrared-beam technology. A major step forward in this technology was achieved by the use of a video digitizer. A modern frame grabber uses a high-speed analog-to-digital converter

to enable real-time conversion of the entire video image to a high-resolution grid of pixels. This enables high-speed data acquisition and, therefore, tracking of animals that are moving relatively fast. However, most of these systems have severe limitations. Some can only track one animal in one arena. Some tend to require highly contrasting backgrounds and can deal with only a limited range of experimental setups. Most of these tracking systems were developed for rodent studies.

With the increasing use of the zebrafish as an alternative for rodent studies there is also a demand for automated video tracking systems for zebrafish and zebrafish larvae. However, because zebrafish are small aquatic animals, there are substantial technological challenges including: (a) water may cause reflections; (b) an aquarium has three dimensions and rodent studies usually are done in one plane, with the animal moving across a surface; (c) many studies require high-throughput; and (d) zebrafish embryos and larvae are transparent at early stages and therefore hard to detect. These are some of the reasons why specialized solutions are required. The companies Noldus Information Technology and Viewpoint have both developed these specialized solutions to automate behavioural studies with zebrafish.

Colwill and Creton [125] recently reviewed zebrafish behavioural assays and highlight how these can help in discovering new drug targets for anxiety. Successful high throughput screens using zebrafish hinge on using automated imaging. Systems like DanioVision and ZebraBox have made it possible to track and analyse behavioural traits. This removes the need for time-consuming manual scoring, and provides high resolution data gathered in real time.

The zebrafish larva is small (approximately 3.0 – 3.5 mm in length during the first few hours after hatching; [33]). This makes it suitable for large-scale analysis, with a single 96 well-plate accommodating one embryo per well. Using automated imaging, the average lab can process hundreds of zebrafish per day. For example, EthoVision XT (Noldus Information Technology, The Netherlands) has been employed in isolating seizure-resistant zebrafish larvae in a large-scale mutagenesis screen [126]. The behaviour of zebrafish is

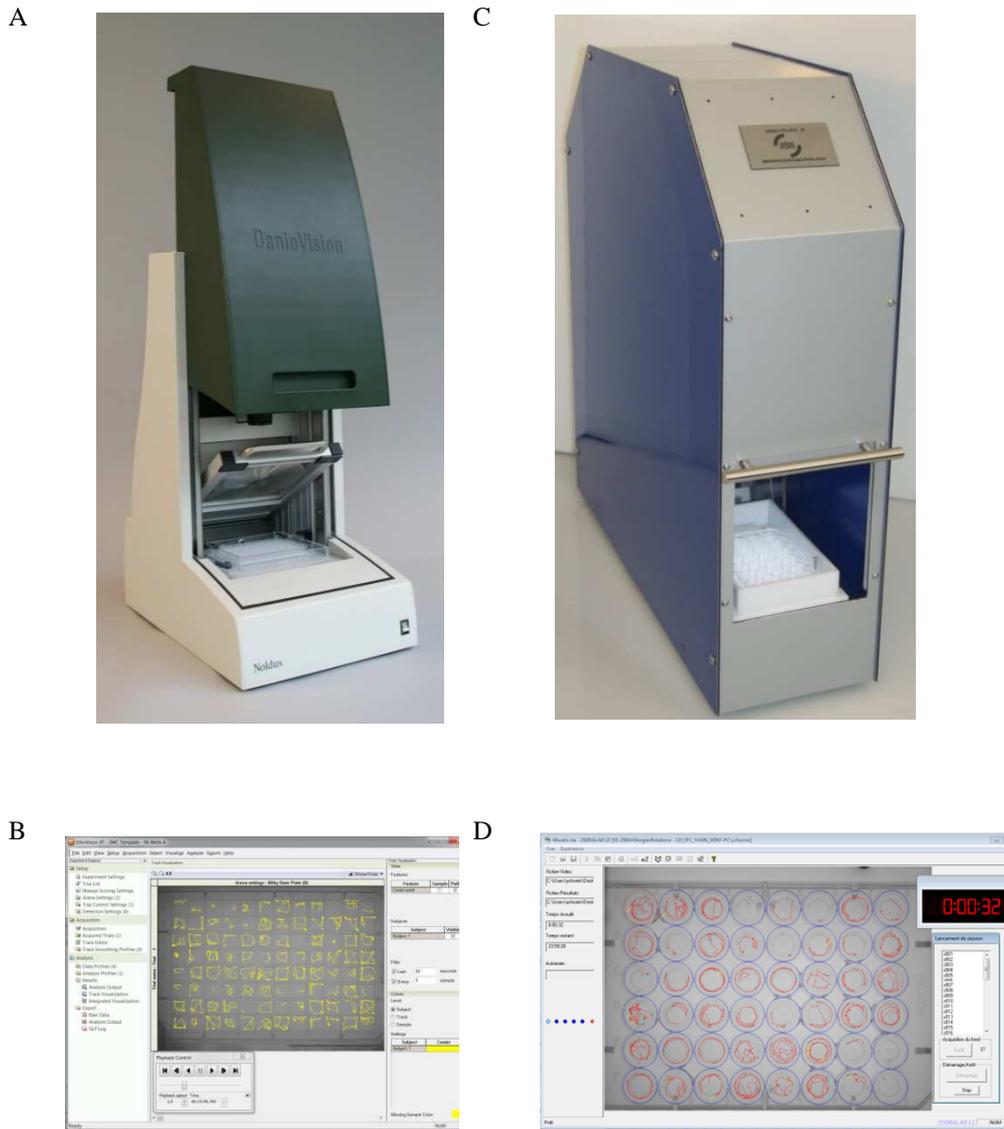


Figure 2.3. Commercial solutions for the video-tracking of zebrafish embryo behaviour. A, B. Noldus Daniovision system. A, Hardware with 96-well plate. B, Screen print showing video tracking of embryo swimming pattern. C, D. Viewpoint ZebraLab videotrack systems. C, Hardware with 24-well plate installed. D, Screen print showing tracked swimming patterns in a 48-well plate.

studied by measuring variables such as swimming speed, distances swum, time spent in the different sections of the tank or maze, time spent immobile, erratic movements, turn angle, etc. For an accurate, objective, and efficient measurement of these parameters, tracking software provides a practical tool for researchers. The two principal commercial video tracking solutions for zebrafish developmental stages are shown in Figure 2.3.

Behavioural testing of embryonic and larval Zebrafish

The development of new behavioural assays for zebrafish is in full swing, and the possibilities are vast. The existence of sophisticated behavioural tracking and analysis tools

are fuelling this growing field of research, and adding to the arsenal of high throughput screens being used in the discovery of new drug targets. The use of behavioural assays is increasing in pre-clinical safety evaluation because they can be carried out at medium/high-throughput.

Optimizing experimental conditions for zebrafish testing

There are several conditions which need to be controlled in order to get consistent results. Due to rapid development of zebrafish larvae, it is essential to perform an experiment at the right developmental stage, i.e. when appropriate organs and functions are developed. Certain behaviours are only observed at a specific stage. MacPhail *et al.* [122] found that time of day has an impact on the locomotor activity of the zebrafish larvae; they are more active in the morning than in the afternoon. Zebrafish larvae at 5 dpf also display diurnal rhythm in locomotor activity; locomotor activity during the day is higher compared to night [127].

The rearing density of zebrafish larvae may also influence their behaviour. Zellner and colleagues [128] found that larvae raised in groups had higher levels of activity in the dark period compared to larvae raised individually. Ambient light conditions in the test apparatus are of vital importance to zebrafish larvae, which can show either decreased or increased locomotor activity depending on the intensity of light [129]. Another factor which can affect larval zebrafish locomotor behaviour is the amount of acclimatization-time before testing. In the visual motor response test (see below) acclimatization time and light conditions before testing can have a big influence on the outcome [122,128].

The temperature and lighting conditions at which embryos are raised and tested is another factor which can alter the locomotor behaviour and development of the zebrafish

larvae [130-132]. Typically, researchers use a water temperature of 28°C. The containing vessel used for experiments varies widely between labs. Formats for behavioural testing include Petri dishes, 12-, 24- 48- and 96-well microtitre plates. A recent study [133] has found that testing zebrafish larvae in bigger space dimensions (24-well plates compared to 48- or 96- well plates) results in elevated locomotor activity. Finally, when performing tests on the locomotor activity in the zebrafish, developmental malformations should be taken in to account which can affect their movements [129]. All these variables should be controlled in order to promote consistent behavioural assessments and reproducible outcomes especially involving locomotor activity of the zebrafish in order to screen for drugs/compounds.

Table 2.2. Different types of behavioural patterns exhibited by zebrafish larvae.

Behaviour	Time	Stimulus	Reference
Coiling	17-21 hpf	None	[47,48,134,135]
Touch induced escape response	22-27 hpf	Touch	[48,136,137]
Evoked Swimming	27 hpf - until hatching	Touch	[134,138]
Photomotor response	30 hpf	Light intensity	[105]
Optokinetic response	73-80 hpf	Moving objects	[50,139-142]
Optomotor response	5 dpf	Moving objects	[52,53,143,144]
Shadow response	8 dpf	Approaching object	[62,145]
Turning behaviour	6-9 dpf	Touch, approaching object, sudden change of light conditions, sound	[137,146]
Prey capture	9 dpf	Prey	[137,146-148]

Behavioural tests

Many assays have been developed in zebrafish larvae by taking advantage of its inherent visual reflexes. Among these are the optomotor response [52,53,143,144], optokinetic

response (OKR) [50,139-142] and light-dark challenge test [98]. A list of behaviours displayed by zebrafish larvae (up to 9 dpf) is given in Table 2.2.

Coiling

Zebrafish embryo development takes place very rapidly. The first behaviour observed during its development is spontaneous movement that begins at 17 hpf, during which embryos repeatedly perform slow, alternating tail coils [47,48]. This spontaneous movement is independent of sensory stimulation and is driven by activity in the spinal cord [134]. Frequency of coiling decreases after 21 hpf [48]. Compounds such as perfluorooctanesulphonic acid and chlorpyrifos have been shown to affect coiling in zebrafish as shown in Table 2.3.

Touch-induced escape response

Beginning at 21 hpf, embryos demonstrate a new motor behaviour in response to mechanical stimuli. Zebrafish larvae react with rapid tail coils in response to touch stimuli with two or three rapid trunk contractions [47,48]. These coils are stronger than the spontaneous coils and can be distinguished. Though embryos first react to touch at 21 hpf, it is suggested that use of embryos at 26 hpf or later makes it easier to distinguish the touch response since much of the spontaneous coiling has stopped [48]. Many compounds have been shown to affect touch-induced escape response (see Table 2.4).

Table 2.3. Effect of different compounds on tail coiling in zebrafish embryos

Compound	Concentration	Exposure time	Effect	Reference
Perfluorooctanesulphonic acid (PFOS)	4 mg/L	Exposed at 6 hpf, tested at 25 hpf	Tail bend frequency increased	[149]
Chlorpyrifos	0.625 mg/L	Exposed at 2 hpf, tested at 24 hpf	Tail coiling duration and frequency increased	[150]

The zebrafish larva reacts to the touch stimuli in different ways. It moves away from the stimulus upon touching the head which may lead to swimming. However, it does not re-orientate itself upon touching the tail, which can lead to a brief forward movement. Both of these responses only occur if the hindbrain and spinal cord are intact [156]. There are two types of mechanosensory neurons acting on touch stimuli: Rohon-Beard neurons are activated on tail stimulation, while trigeminal neurons are activated on head and yolk stimulation [156].

The mechanisms behind the response are still unknown. One interesting observation is that zebrafish show a differential developmental response to strychnine [48]. This is significant because strychnine is a competitive antagonist of glycine at inhibitory postsynaptic spinal glycine receptors in zebrafish [157]. However, more information on the developmental changes in glycine receptor expression is needed in zebrafish development, especially in terms of changing expression detected by *in situ* hybridisation. Pietri, Manalo *et al.* [158] suggested that the touch response is dependent on AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)-type glutamate receptor activation. They concluded that the circuits required for touch-evoked responses are confined to the spinal cord and that

only the most rostral part of the spinal cord is sufficient for triggering the full response. This rostral sensory connection is thought to be established via commissural primary ascending (CoPA) interneurons, as they project to the rostral spinal cord.

Table 2.4 The compounds affecting the touch-induced escape response.

Compounds	Concentration	Exposure time	Effect on	Reference
Cadmium	0.25, 0.5 mg/L	Exposed 2-50 hpf,	decreased	[151]
Dichlorodipheny	100 μ M	2 dpf	increased	[152]
DDT	10 μ M	4 dpf	increased	[152]
Diazinon	2 mg/L	0.5 hpf, tested at 5	decreased	[153]
Dieldrin	20 μ M	Exposed 6-48 hpf,	decreased	[152]
Fipronil	1.1 μ M	Exposed 2-48 hpf,	decreased	[154]
Nonylphenol	5 μ M	Exposed 6-48 hpf,	decreased	[152]
Sodium	100 ppm	Exposed 2-3 dpf,	decreased	[155]
2,3,7,8-	0.1 μ M	Exposed 6-48 hpf,	decreased	[152]

Startle response

This is an unconditioned behaviour consisting of rapid and massive acceleration resulting from contraction of the axial muscles. This response is elicited by visual, touch or acoustic

stimuli [46,159-161] and provides a quick measure of sensory and motor integration. It shows a rapid habituation curve with repeated trials which gives information concerning neuroplasticity. The larval visual startle response is less understood than adult visual escape responses. Kimmel and colleagues [145] observed no responses in larvae less than two weeks old to sudden changes in illumination. However, later studies showed that zebrafish larvae performs a startle response at 3 dpf and by 5-6 dpf, they respond to changes in light and dark [55,122] and can follow rotating stripes [162]. Further, a high-throughput analysis [163] was able to show that 6 and 7 dpf larvae show increased frequency of turn swims after flashes of both light and dark. Zebrafish have the ability to detect ultraviolet waves in addition to red, green and blue which is one of the main difference from the mammalian eye [162].

Zebrafish react both to light and dark flashes [49,55] but the latency to respond to light and dark flashes is different [163]. In the case of light flashes, it was 183 ± 93 ms, while with dark flashes, it was even more delayed, with a mean latency of 408 ± 105 ms [163].

Zebrafish also respond behaviourally to an acoustic stimulus after 5 dpf [164]. They are known to exhibit startle response to frequencies of 200 Hz upwards [165]. The acoustic startle response is elicited within 15 ms by sudden, intense acoustic stimuli [166]. This response becomes functional immediately after the zebrafish auditory system has become functional around 5 dpf [167]. The startle response in zebrafish larvae is increased in the case of an aversive event, sensitization, fear/anxiety-potential, or certain drugs (see Table 2.5), while it is decreased by habituation, pre-pulse inhibition, or drugs [160].

Open field test

The open field test has been commonly used to assess animal exploration and is hypothesized to reflect certain emotional states especially in rodents [76,168] and adult zebrafish [80,169]. Due to its several salient features (see chapter 2), the zebrafish has become an excellent model to study locomotion and novel environment exploration [80]. Steenbergen and colleagues [54] used a light/dark box to assess the avoidance behaviour of zebrafish larvae and found aversion to dark environments, which caused signs of anxiety (expressed by thigmotaxis and fewer entries into the dark chamber). The studies using an open field test found that zebrafish larvae display thigmotaxis [32,170] which was attenuated by pre-treatment with anxiolytics or by giving sufficient time for the larvae to habituate in the testing apparatus [32].

Visual motor response test

The zebrafish mainly uses vision to hunt for food and avoid predators [171]. This reliance on vision necessitates the rapid development of the visual system, given that the embryo hatches to a free-swimming larva at around 48 – 72h. Zebrafish initiate an escape response when threatened by a potential predator. This innate behaviour can be used to assess visual performance [172]. It is known that the larval zebrafish has a preference for light and an aversion for dark [122,173], in contrast to adult zebrafish where the preference and aversion are reversed [85].

In the visual motor response test, the larval fish is presented with white light in a closed system (e.g. the commercial set-ups in Figure 2.3) for two minutes to habituate. Video recording is then begun, for the next four minutes of light exposure, to assess basal locomotor behaviour. Then, four minutes of sudden darkness (with infra-red background light to allow video recording to take place) to assess the effect on behaviour. Finally, four minutes of light is given to allow the zebrafish time to recover from the shock of the dark challenge. This test, in principle, relies on the integrity of brain function, nervous system development, locomotor system development and visual pathways and has been used in various assays [55,94,174].

Optokinetic response (OKR)

Stereotyped eye movements in response to movements perceived in the field of vision is known as the optokinetic response. It is aimed at minimizing retinal slip (the displacement of images on the retina due to fast movement of the observer or object [178]). This is one of the more widely studied behaviours due to its reliability. Zebrafish larvae are able to perform it even when immobilized [179]. This behaviour develops between 73 and 80 hpf [180] and persists throughout life. Movements are often provided in the form of moving graphics on liquid crystal display (LCD) monitor or a striped drum, and initiate a series of smooth ocular pursuits followed by a rapid saccade as the eyes flick onto the next stripe. Fish with fewer saccades (fast movements of the eye) are identified as having defective visual function. Different compounds can affect this response (see Table 2.6).

Table 2.6. Effects of selected drugs on the optokinetic response (OKR) in zebrafish larvae.

Drug	Concentration	Exposure time	Effect	Reference
Mixture of (2S)-2-amino-4-phosphonobutanoic acid (L-AP4) and DL threo-β-benzyloxycarboxylate (TBOA)	0.4 mM L-AP4+0.2 mM TBOA	2 hours before experiment at 5 dpf	Abolished the OKR	[175]
Bisoprolol	30-300 μM	5 days of treatment from 3 dpf, tested 8 dpf	No significant effect	[176]
Ethanol	1.5 %	Exposed 2-5 dpf, tested on 5 dpf	Higher visual threshold	[177]

Horizontal and bipolar cell processes in zebrafish larvae invaginate into the photoreceptor terminals which form two types of synapses, which relate to the separation of visual input into ON and OFF channels [181]. The ON and OFF channels are capable of sensing light increments and light decrements respectively [182]. A retinal ganglion cell is a type of neuron located near the inner surface (the ganglion cell layer) of the retina of the eye. A study of OKR [175] suggests that it is mediated by the ON retinal pathway. An electrophysiological characterization of the retinal ganglion cells of no optokinetic response (*nrc*) mutant fish, which display no OKR, revealed these to have no ON retinal ganglion cells [175].

Optomotor response test

This is the locomotor behaviour induced in response to a repetitive pattern and has been used to screen for defects in vision. Zebrafish larvae will swim in the same direction as a pattern of moving stripes, a behaviour that is called the optomotor response (OMR). This

response can be observed at 6 dpf, but is more robust at 7 dpf [144,172,183]. This response can be used to test visual function of the zebrafish larvae and testing a compound at an early stage of discovery using low volume of the compounds [184]. A list of selected compounds and their effects on OMR is given in Table 2.7. This assay can be used to evaluate the effect of compounds on zebrafish vision at a higher throughput; however, compounds producing motility defects can appear positive in OMR. Therefore, the assay may be used as a primary screen and false positive compounds can then be further evaluated for defects in visual systems.

Mutants with OMR defects can appear to overlap to a greater extent with those showing OKR defects [185]. However, both of these assays are used for distinct features of visual function which partially depends on different regions of the brain. For instance, ablation of optical tectum results in abolishing OMR but OKR behaviour remains in place [186].

Colour preference

Environmental features such as intensity of illumination (bright/open zone *vs.* dark/protected zone), colours, and topography (periphery *vs.* centre) serve as cues helping an individual to assess the aversiveness or safety of a given environment [187,188]. The adult zebrafish is reported to have an innate preference for black [85], red, yellow and green [188]. However, studies on preference of larval zebrafish are scarce. Knowledge of colour preference in zebrafish might be applicable for colour-based memory and learning paradigms in the future. The colours which act as aversive cues might be helpful in experiments involving stress/anxiety and fear and non-aversive colours can be used in appetitive experiments.

Habituation studies

Habituation, dishabituation and sensitization are non-associative forms of learning. Habituation is defined as decreases in responsiveness to a repeated stimulus involving only a single stimulus instead of association between two stimuli [164,191,192]. It has been observed in a wide range of organisms including protozoa [193] and humans [194]. It serves as a mechanism by which the nervous system filters irrelevant stimuli. Despite its simplicity and ubiquity, our understanding of the neurobiology of habituation is still limited.

Table 2.7. The effects of various compounds on the optomotor response in zebrafish larvae.

Compound	Conc.	Exposure time	Effect	Ref.
Allopurinol	300 µM	5 days of treatment from 3 dpf, tested 8 dpf	No effect	[176]
Amoxicillin	1 mM	5 days of treatment from 3 dpf, tested 8 dpf	No effect	[176]
Aspirin	500 µM	Exposed from 3 dpf until day of testing at 8 dpf	No effect	[189]
Atropine	100 µM	Exposed from 3 dpf until day of testing at 8 dpf	No effect	[189]
Bisoprolol	300 µM	5 days of treatment from 3 dpf, tested 8 dpf	Inhibition	[176]
Chlorpromazine	10 µM	Exposed from 3 dpf until day of testing at 8 dpf	Inhibition	[189]
Chloroquine	100 µM	Exposed from 3 dpf until day of testing at 8 dpf	Inhibition	[189]
Chlorpromazine	0.03-1 µM	5 days of treatment from 3 dpf, tested 8 dpf	Inhibition	[176]
Cisapride	3 µM	5 days of treatment from 3 dpf, tested 8 dpf	Slight inhibition	[176]
Cisplatin	30 µM	5 days of treatment from 3 dpf, tested 8 dpf	Inhibition	[176]
Diazepam	10 µM	Exposed from 3 dpf until day of testing at 8 dpf	Inhibition	[189]
Ethanol	1.5%	6-12, 12-24 hpf	Lower visual acuity	[190]
Ethanol	1.5%	48-60, 60-72 hpf	No effect	[190]
Flecainide	100 µM	5 days of treatment from 3 dpf, tested 8 dpf	Inhibition with motility defects	[176]
Furosemide	1 mM	5 days of treatment from 3 dpf, tested 8 dpf	No inhibition	[176]

Gentamicin	1-30 μM	5 days of treatment from 3 dpf	Inhibition	[176]
Indomethacin	10 μM	5 days of treatment from 3 dpf, tested 8 dpf	No inhibition	[176]
Ketoconazole	3-10 μM	5 days of treatment from 3 dpf, tested 8 dpf	No significant inhibition	[176]
Lansoprazole	30 μM	5 days of treatment from 3 dpf, tested 8 dpf	No effect	[176]
Lithium	100 μM	Exposed from 3 dpf until day of testing at 8 dpf	No effect	[189]
Nicotine	6.2 and 62 μM	Exposed from 3 dpf until day of testing at 8 dpf	Inhibition	[189]
Nicotinic acid	1mM	5 days of treatment from 3 dpf, tested 8 dpf	No inhibition	[176]
Ouabain	50 μM	Exposed from 3 dpf until day of testing at 8 dpf	inhibition	[189]
Phenytoin	100 μM	Exposed from 3 dpf until day of testing at 8 dpf	Inhibition	[189]
Quinine	30-100 μM	5 days of treatment from 3 dpf, tested 8 dpf	Inhibition	[176]
Sodium iodate	10mM	5 days of treatment from 3 dpf, tested 8 dpf	No consistent inhibition	[176]
Spirolactone	30 μM	5 days of treatment from 3 dpf, tested 8 dpf	Inhibition with motility defect	[176]
Thioridazine	0.3-1 μM	5 days of treatment from 3 dpf, tested 8 dpf	Inhibition with motility defects	[176]
Vigabatrin	100 μM	5 days of treatment from 3 dpf, tested 8 dpf	No inhibition	[176]

At a simple level, zebrafish larvae show habituation to tactile or shock stimuli, with the response diminishing after a few repetitions [195]. Another study [164] examined habituation of larval zebrafish displaying an escape-response to auditory stimuli. At 7 dpf, larvae were able to exhibit iterative reduction in a startle response. They also showed that selected drugs with CNS effects (rolipram, donepezil, and memantine) increased the acoustic startle response and decreased habituation in zebrafish larvae. Furthermore, this was similar to previous findings in rodent studies. Zebrafish larvae display two temporally distinguishable forms of habituation and these are mediated by different cellular mechanisms [196]. The first form is elicited by low-frequency of auditory stimulation and last for about 1-15 minutes. This form is dependent on activation of *N*-methyl-D-aspartate receptors (NMDARs) while the other form persists for 25 min–1 h and does not require activation of NMDARs.

One of the problems faced in habituation studies is that it is difficult to distinguish whether reduction in response is due to habituation or fatigue. However, habituation can be distinguished from sensory adaptation or motor fatigue by the demonstration of dishabituation which occurs when an entirely different type of stimulus reinitiates the startle response after habituation has occurred [192]. For example, a light pulse was used to reinitiate larval zebrafish response to sound [164]. The inter-stimulus interval (ISI) [197] also plays a major role in determining the time required for habituation. Longer ISIs lead to delay in habituation while short ISIs can enhance habituation. Other criteria of habituation include spontaneous recovery from iterative reduction and relative stimulus specificity [192]

Turning Behaviour

Turning behaviour is different from swimming behaviour due to its inherent asymmetry. A single bend is often highly distinctive from the subsequent swimming behaviour. Turning behaviour of zebrafish larvae has been studied in detail [198]. Zebrafish larvae display two types of turns. The first type of turn is a routine turn, without any apparent stimulus, resulting in a bend of 30° at the completion of first bend. This turn is distinguished from the normal swimming bouts which initiate with little or no change in head direction. These turns are characterized by a slow angular velocity, and lack a counter-bend after the initial turn. After hatching, these routine turns are displayed and continue throughout ontogeny [145,199]; they are helpful in foraging and predator avoidance [200].

The second type of turn is more complex, and is characterized by high-velocity turns; it associated with escape behaviour. In this case, zebrafish larvae make a very fast C-shaped bend. These turns typically include a large counter-bend in a direction opposite to the stimulus. After this escape response, zebrafish larvae display burst-swimming in which routine turns are not observed [198].

Prey capture

After the zebrafish larva has hatched, it lives on nutrient reserves in its yolk sac [201]. Ultimately, however, it needs to acquire exogenous food. This transition from ‘yolk sac larva’ to ‘free-swimming larva’ which is able to hunt takes place around 5-6 dpf [202]. To acquire food, it develops more complex locomotor behaviour which can be called prey capture. Zebrafish use vision to detect and pursue their prey by involving fine axial motor control [203,204]. It has been shown that prey capture consists of a series of small turns followed by a ‘slow-like’ swim [198]. Zebrafish, as young as 8-9 dpf, can visually track the prey or can use suction feeding when 6 dpf old. They either capture the prey after a brief swimming episode towards the it (the so-called ‘slow-strike approach’); or can catch the prey while remaining stationary [198]. Zebrafish prey capture can be divided into two distinct phases. The first is to reach the prey with the help of J-turns which serve to orient larvae toward their prey with minimal forward movement. In the second stage, larvae culminate in a distinct capture swim which results in biting at the prey [203].

Zebrafish embryo behavioural screening for anxiolytics and anxiogenics

Fear and anxiety are closely-related concepts. Fear is a response to imminent threat (Craske *et al.*, 2009), while anxiety represents a response to future or possible threat [205]. The zebrafish has become an excellent model to study these responses in the context of reacting to predators and alarm pheromone (Pfeiffer, 1977). Anxiety is usually manifested in assays as avoidance behaviour [205]. Various drugs have been used to validate these assays, either by attenuating the response (in the case of anxiolytics) or increasing it (as occurs with anxiogenics).

In this section we review studies in which zebrafish embryo behavioural assays have been used to study anxiety-like responses and their pharmacological modulation.

Parameters used to measure anxiety

As in rodents, exposure to novel environment evokes robust anxiety-like behaviours in zebrafish [206]. The exploratory behaviour of zebrafish is used to measure stress and anxiety-like responses in zebrafish which display wide range of behaviours in a highly aversive novel environment. Zebrafish employ cortisol instead of corticosterone as a primary stress response hormone [207], which makes them a useful animal model relevant to human stress physiology compared to the rodent model where corticosterone is released in response to stress. Commonly used setups used to measure anxiety are the open-field test in which central arena of the field is considered aversive and hence thigmotaxis is observed. Zebrafish avoids top of the tank when using a novel tank showing signs of anxiety and prefers to remain at the bottom of the tank. Adult zebrafish also avoids light area and region nearest to predator when tested using light/dark box. However, larval zebrafish prefer light areas over dark areas [54].

Combining behavioural assays with physiological measures provides an even more powerful avenue for analysis: stress hormones such as cortisol have shown to be elevated in ‘stressed’ zebrafish, essentially providing the basis for validating behavioural data [210]. After observing behaviour, it is fairly easy to measure cortisol levels [211], although this is not possible *in vivo* in zebrafish.

Table 2.8. Effect of selected anxiolytics and anxiogenics on zebrafish anxiety-like behaviours.

Compound	Test	Dose	Effect on zebrafish	Reference
A). Anxiolytics:				
Buspirone	Light/dark box	59.24 μ M	Total time spent in dark zone increased, latency to visit dark zone decreased, number of entries in the dark increased	[54]
Buspirone	-	45 μ M	Altered rest-wake profiles, increased freezing/rest	[87]
Diazepam	12-well plate assay	0.05 and 5 mg/L	Reduced thigmotaxis	[208]

Diazepam (Valium)	Light/dark box	2.5 μ M	Total time spent in dark zone increased, latency to visit dark zone decreased,	[54]
Ethanol	Motor activity	0.5-4 %	Hyperactivity with 0.5-2% ethanol and hypoactivity with 4% ethanol treatment	[209]
Ethanol	Light/dark box	0.027 %	Total time spent in dark zone increased, latency to visit dark zone decreased, number of entries in the dark increased	[54]
Fluoxetine (Prozac)	12-well plate	0.2 mg, 2mg/L	No effect on thigmotaxis, decreased avoidance response	[208]
Fluoxetine	-	15 μ M	Altered rest-wake profile, increased rest	[87,111]
Propranolol	96-well plate	10-100 μ M	Decreased activity in the photomotor response assay	[46]
B.) Anxiogenics:				
Caffeine	Light/dark box	438 μ M	Total time spent in dark zone decreased, latency to visit dark zone increased and number of entries in the dark zone decreased	[54]
Caffeine	12-well plate assay	100 mg/L	Increased thigmotaxis	[208]

New options for measuring physiological responses during behavioural tasks are heart rate [212,213] and skin colour monitoring [214]. Zebrafish larvae are optically transparent, and a large number of them can be monitored in multi-well plates. Image analysis software can be used to extract specific parameters of the zebrafish heartbeat. At this point, imaging heart beat in a freely moving zebrafish is still technically challenging, but once further developed, this paradigm can open interesting new avenues for studying the physiology of fear and anxiety. We discuss some commonly used parameters for the study of anxiety-related behaviour and compounds affecting it (Table 2.8).

Thigmotaxis

Thigmotaxis is one of the most commonly used behavioural endpoints measured in preclinical studies. Animals displaying thigmotactic behaviour strongly avoid the centre of

an arena and stay close to the boundaries of a novel environment [215]. Thigmotaxis is displayed by a wide range of species including rodents [76,215,216] and humans [217,218].

Thigmotaxis validated index of anxiety using anxiolytic and anxiogenic drugs in stressful conditions [76,216]. Thigmotaxis has been studied in detail using adult zebrafish [173,219,220] but has also been displayed by zebrafish larvae [32,170,174]. Recently, a thigmotaxis assay in larval zebrafish has also been validated [174] which shows that thigmotaxis can be modulated after exposure to the anxiogenics and anxiolytics in a medium to high-throughput capacity. Thigmotaxis was attenuated in larvae after 24h exposure to the same test apparatus [32] or in presence of other conspecifics [114] which provide them social protection; thus hereby lessened the anxiety-like behaviour. However, special care should be taken when using the term thigmotaxis. In principle, It could result from the larvae unavoidably coming into contact with the sides of the apparatus, and this should be taken into account when analysing the results of experiments. Furthermore, the arena should be large enough to provide the larvae with a choice of spatial movements. For example, zebrafish larvae move greater distances in microwell plates with larger well sizes [118] [133]. For these and other reasons, thigmotaxis is best studied in an arena at least the size of a 24 well plate.

Scototaxis

A preference for darkness is also an indication of anxiety like behaviours in adult zebrafish [221,222]. The test is very simple and similar to the light/dark box [84]. The test mainly relies on fish exploration in a black and white tank for the establishment of preference. Adult zebrafish exposed to anxiolytics spend significantly more time in the white area compared to controls, whereas anxiogenic drugs had the opposite effect and zebrafish spend less time in the white arena. In contrast to the adult, larval zebrafish display preference for the light environment [54,173]. Therefore, anxiogenics such as caffeine decrease the time spent in dark zone while anxiolytics such as diazepam, buspirone and ethanol caused the fish to spend more time in the dark zone [54].

Freezing

Freezing is a classical index of anxiety [67]. It is defined as partial or total absence of movement except for the gills and eyes. Zebrafish larvae show freezing behaviour when they are exposed to complex environments or anxiogenics (Ahmad and Richardson,

unpublished data) like adult zebrafish [80,223]. Young larvae (i.e. 3-4 dpf) spend more time in freezing or rest state than at older larvae (5-7 dpf) [32,170]. The higher rate of freezing in young larvae have been attributed to inflation of the swim bladder and spontaneous swimming, both of which begin around 5 dpf [106,170]. However, this freezing behaviour is attenuated if the embryos are exposed in the test apparatus 24 h before the testing [32]. Thigmotaxis may be a useful parameter in the experiment involving anxiolytics and anxiogenics which can increase or decrease time spent in mobility respectively [118].

Erratic movements

Sudden sharp changes in direction of movement and/or velocity indicating rapid anxiety-like darting behaviours is known as erratic movement. It has been well studied in the adult zebrafish [223-225]. Larval zebrafish also display erratic movement in response to sudden change in the light [49,54,55,98,226,227] but has not been studied in detail. Erratic movements are usually occurred with high speed [228]. These high speed movements can serve as an indirect measure of erratic movements in stressed zebrafish. Automated quantification system with speed measurement can serve as a useful tool to monitor erratic movements in zebrafish [67].

Other Applications of zebrafish embryo or larval behavioural screening

There is a growing list of efficacy and safety studies of drugs in which the zebrafish embryo/larva has been used. On the efficacy side, the model has proven to be a powerful new system to study the underlying basis of diseases such as epilepsy [229,230]. These studies showed that larval zebrafish pentylenetetrazole (PTZ) seizure assay is relevant to human epilepsy and can be used to screen anticonvulsant drugs at medium/high-throughput level by using zebrafish larvae startle response. They were able to detect suppression of PTZ-induced excessive movements with 13 out of 14 standard anti-epileptic drugs. Foetal alcohol syndrome [94] and tuberculosis [231] are other examples in which zebrafish larvae are promising models. In summary, the use of zebrafish embryos as an *in vivo* bioassay tool can contribute to the identification of drug targets, to drug discovery and to assessing the toxicity of candidate drugs.

A wide range of compounds has been tested for toxicity using zebrafish embryos; for reviews, see [55,232]. For example, the LC₅₀ values of 60 water soluble compounds were

determined [98] and it was found that they correlate well with the LD₅₀ values of the same compounds in rodents (Figure 2.4). This finding was in line with previous studies showing good predictivity of the zebrafish model [73,233]. Furthermore, it was found that nearly all of these pharmacologically active compounds or toxins produced changes in zebrafish embryo behaviour in the light-dark challenge test [55].

Technical challenges and future prospects

We consider here some of the limitations of the zebrafish embryo/larva model in behavioural studies, and how these limitations are being addressed by technical innovations.

Limitations of microtitre plates

Current assays with zebrafish embryos/larvae are typically performed either in small tanks, Petri dishes or microtiter plates (see Table 1 in [98] for example). The problems with these assays include: the combining of several embryos in a single well; the evaporation of buffer, which dramatically affects side and corner wells [98] and may affect the lenses in video-tracking hardware apparatus; and the relatively large volume of buffer, and therefore test compound, consumed [103].

Microfluidic assays

Many of these issues can be addressed by culturing zebrafish embryos in microfluidic systems [103,234,235]. These can provide a constant flow of buffer at low volumes (one system from our lab has a static volume of 8µL per well); and because the chip is a closed system, their use can avoid the problems of evaporation of buffer (Figure 2.5). One drawback of microfluidic systems is that the miniaturization of the environment limits the ability to record distance-based metrics such as thigmotaxis. Therefore, in miniaturized biochips, parameters such as the startle response, which do not rely on a large swimming arena, may be more appropriate for examining anxiety and the effects of anxiolytics.

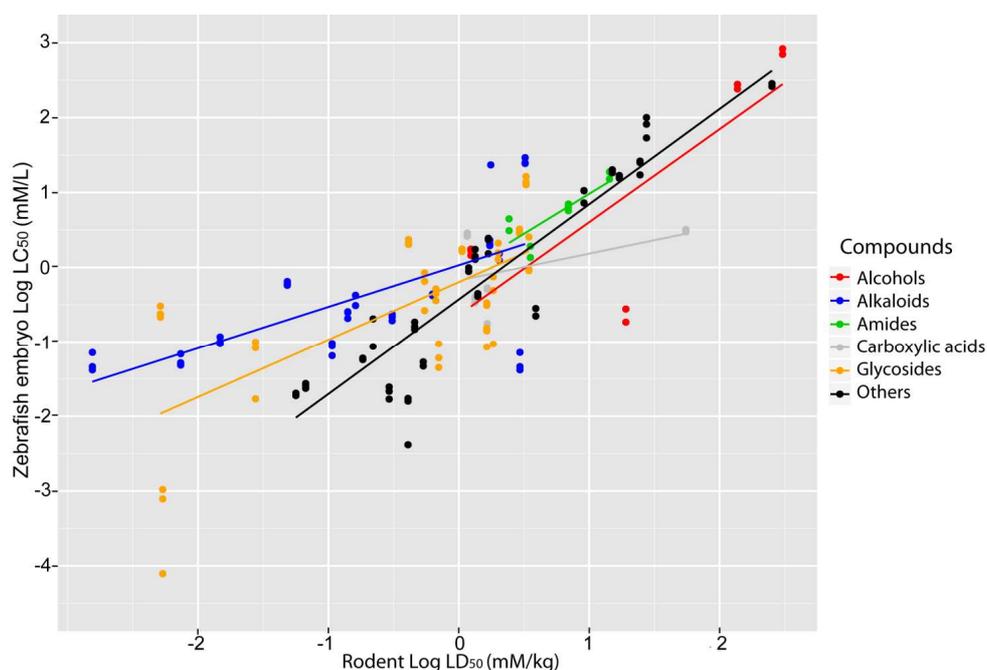


Figure 2.4. Correlation between LC_{50} values of a range of toxins in zebrafish embryo assays, and the LD_{50} values of the same compounds in rodents. From [98]

A recent study [236] presented an automated platform for high-throughput screening using two different assays to detect cardiotoxic compounds and angiogenesis inhibitors. The authors validated these two assays with reference compounds as positive and negative controls, and attempted to screen for novel anti-angiogenic compounds. Rihel and colleagues [87] used larval zebrafish to screen 4000 small molecules for their effects on long-term sleep/wake behaviour using ZebraLab (Viewpoint, France, <http://viewpoint.fr/zerbalab>); and Kokel *et al.* [46] tested approximately 14,000 neuroactive small molecules to look for altered larval responses to high intensity light pulses. Both studies used automated video-tracking systems and microtiter plates to determine behavioural profiles of the zebrafish larvae. Other automated screening platforms are also being developed [237].

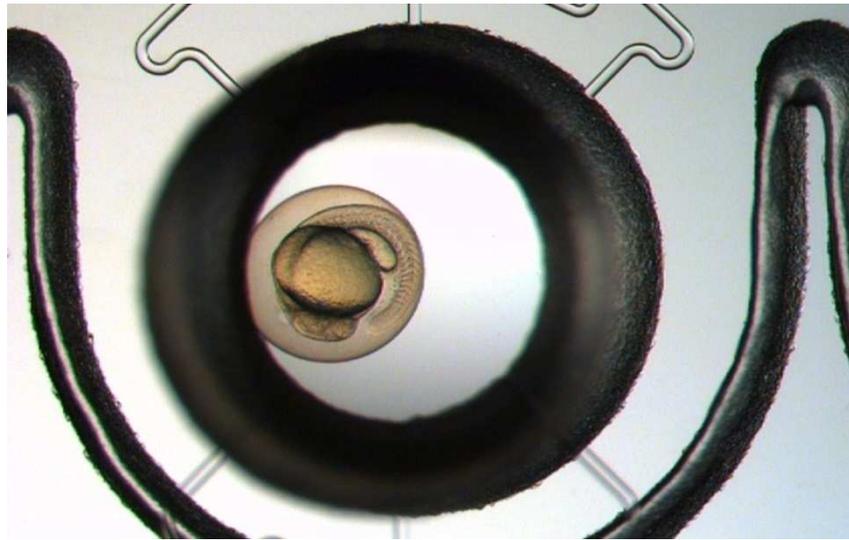


Figure 2.5. A zebrafish embryo cultured in a glass microfluidic biochip (Courtesy Eric Wielhouwer and Michael Richardson, Syntecnos BV). The embryo lies within a well having a volume of approximately $8\mu\text{L}$, and is fed with buffer through the three inlet and three outlet channels at a rate of $2\mu\text{L}/\text{min}/\text{well}$ Automation and high-throughput screening

Conclusions

Young developmental stages of the zebrafish show remarkable similarity to mammals with respect to certain key behaviours. They display responses analagous to anxiety and often respond in a similar way to certain drugs as do rats or humans. There are also many differences between zebrafish and rodent responses, and each zebrafish screen that is developed needs to be fully validated at the pharmacologically, and understood at the molecular and cellular levels. At present, very little is known about how zebrafish embryos/larvae absorb, distribute, metabolise or excrete drugs. Coupled with the fact that it is difficult to screen hydrophobic compounds, because they are poorly soluble in the swimming water, there is considerable industry scepticism about the limitations of the model. However, with the growing number of academic studies in this area, the zebrafish embryo/larva behavioural model is likely to gain in importance.

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