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Zebrafish embryos and larvae in behavioural assays

Farooq Ahmad [a], **Lucas P.J.J. Noldus** [b], **Ruud A.J. Tegelenbosch** [b] **and Michael K. Richardson** [a,*]

[a] Institute of Biology, University of Leiden, Sylvius Laboratory, Sylviusweg 72, 2333BE Leiden, The Netherlands

[b] Noldus Information Technology BV, Nieuwe Kanaal 5, P.O. Box 268, 6700 AG Wageningen, The Netherlands

[*]Corresponding author's e-mail address: m.k.richardson@biology.leidenuniv.nl

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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. 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.

Keywords

zebrafish, video tracking, anxiolytics, anxiogenics, microfluidic.

1. Introduction

1.1. 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 (DiMasi et

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al., 2003). 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 (Agid et al., 2007; Craven, 2011). 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 (Kaitin, 2010; Craven, 2011). 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 (Craven, 2011). 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 Lorenzetti et al., 2011). For this reason, alternatives to the use of adult mammals are sought, and the zebrafish embryo is one model commonly considered (Figure 1).

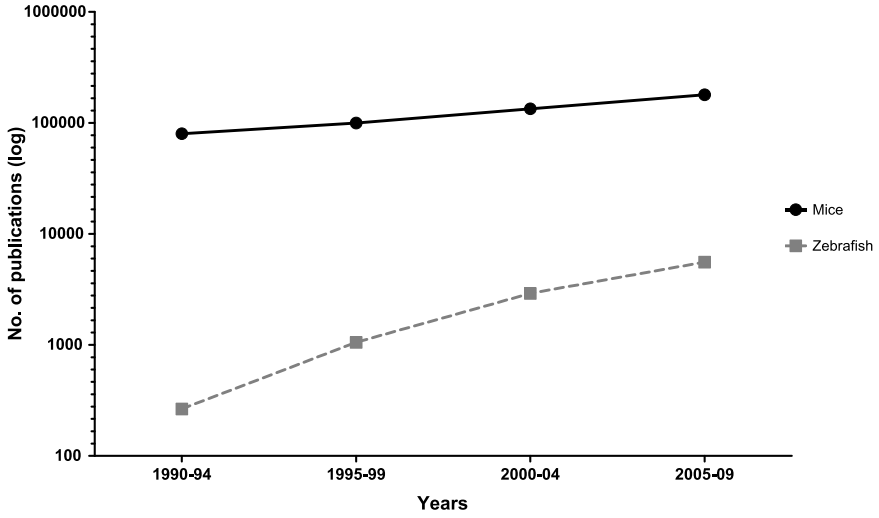


Figure 1. The use of zebrafish in biomedical research is increasing rapidly, as can be seen from the number of articles on Pubmed in which 'zebrafish' appears in the title/abstract. The search included papers from all disciplines covered by Pubmed at http://www.ncbi.nlm.nih.gov/sites/entrez/, regardless 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 mouse remains by far the more commonly used model species. Indeed, the zebrafish embryo is never likely to replace rodents in academic or pharmaceutical research. Rather, it may provide a bridge between cell-culture systems and mammalian whole animal models.

1.2. 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 (Dahm & Geisler, 2006; Spence et al., 2008; Ali et al., 2011b). 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 and 72 h post fertilization (hpf), when they become free-swimming larvae with a complex behavioural repertoire (Colwill & Creton, 2011b). 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 (Ali et al., 2011a).

The zebrafish genome is almost completely sequenced and available online at ensembl.org and contains orthologues of numerous human disease genes (Shin & Fishman, 2002). 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 GRβ which we have shown (Schaaf et al., 2008) to be highly similar to the human GRβ (Figure 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 (Kalueff, 2010–2011) which provides resources for researchers in the field of zebrafish behavioural biology and neurosciences.

A recent study examined behavioural changes in zebrafish embryos after sublethal exposure to a panel of toxic compounds (Ali et al., 2012). 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 (Ali et al., 2012). It has also been shown that several drugs which cause QT prolongation (sometimes leading to spontaneous death) and cardiac arythmia in humans, also produce cardiac dysfunction (bradiacardia and/or dissociation between atrial and ventricular rhythm) in zebrafish embryos (Langheinrich et al., 2003; Lee et al., 2011). This is perhaps not surprising, in view of the fact

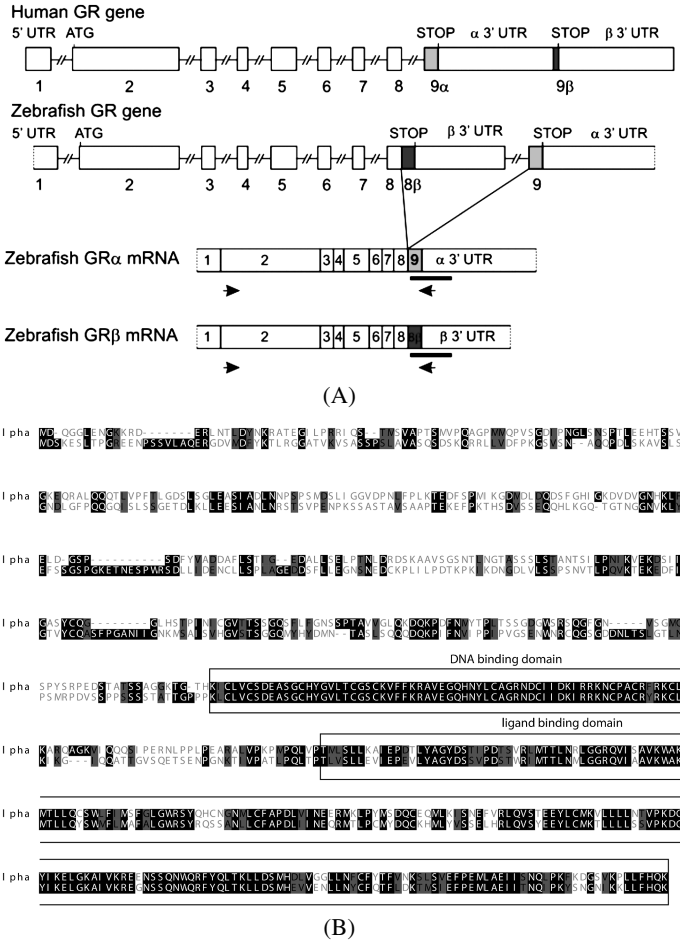


Figure 2. (A) The glucocorticoid receptor (GR) gene provides a good example of the strong conservation between many zebrafish genes and their human orthologues. For example, the exon structure of the GRβ isoform is remarkably similar to that of humans. From Schaaf et al. (2008) with permission. (B) An 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 GRα 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.

that the zebrafish has a remarkably similar electrocardiogram (ECG) pattern to humans (Yu et al., 2012). It also has an orthologue of the HERG receptor gene that is bound by many drugs which cause cardiac side-effects. Not only

is the zebrafish ZERG highly similar in its amino acid sequence to HERG (Langheinrich et al., 2003), but it also shows similar biophysical properties (Scholz et al., 2009). 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 article, is as an experimental model in behavioural testing.

1.3. 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 (Colwill & Creton, 2011b). 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 s or so (Wielhouwer et al., 2011). Forward genetic analysis coupled with behavioural recording has already started to identify genes that may influence behavioural responses (Muto et al., 2005).

A recent study (Kokel et al., 2010) has found that 30-hpf-old larvae which are 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 (Lindsey et al., 2010), larvae have excellent vision and are able to hunt for food (Neuhauss, 2003). After quick maturation of 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 hypoactivity from a safety pharmacology perspective (Porsolt et al., 2002). 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 anxiolytyic and stimulant compounds (Table 1) were tested on locomotor activity of zebrafish larvae and were shown to produce responses similar to those seen in mammals (Zhdanova et al., 2001; Airhart et al., 2007; Boehmler et al., 2007; Irons et al., 2010). 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 (Lockwood et al., 2004; Parng et al., 2007). 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 (Kokel et al., 2010; Rihel et al., 2010).

Champagne and colleagues (2010) 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 (Steenbergen et al., 2011; Schnorr et al., 2012). 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.

1.4. Aims of the present article

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

Table 1.
Effect of different compounds on locomotor activity of larval zebrafish.

Compound	Concentration	Age of zebrafish (dpf)	Reference
Sedative			
4-Aminopyridine	0.6 mM	5	Ellis et al. (2012)
Clozapine	12.5–50 mM	7	Boehmler et al. (2007)
Cocaine hydrochloride	0.2–50.0 μM	6	Irons et al. (2010)
D-Amphetamine	0.1–20.0 μM	6	Irons et al. (2010)
Diazepam	10 nM–100 mM	7–14	Irons et al. (2010)
Ethanol	4%	6	Lockwood et al. (2004); MacPhail et al. (2009); Irons et al. (2010)
Fluoxetine	4.6 mM	3–6	Airhart et al. (2007)
Melatonin	10 nM–100 mM	7–14	Zhdanova et al. (2001)
Pentobarbital	10 nM–100 mM	7–14	Irons et al. (2010)
Polybrominated diphenyl ether (DE-71)	31.0 μg/l	5	Chen et al. (2012)
Stimulant			
4-Aminopyridine	0.8–2.5 mM	5	Ellis et al. (2012)
Aconitine	2.5–25 μM	5	Ellis et al. (2012)
Bisphenol A	0.01–1 μM	5	Saili et al. (2012)
Ethanol	1–2%	7	Lockwood et al. (2004); Irons et al. (2010)
Pentylenetetrazole	10 mM	5	Ellis et al. (2012)

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 48–72 h). We shall include both of these rather arbitrary stages in our discussion here.

2. 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 (Noldus et al., 2000). 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.

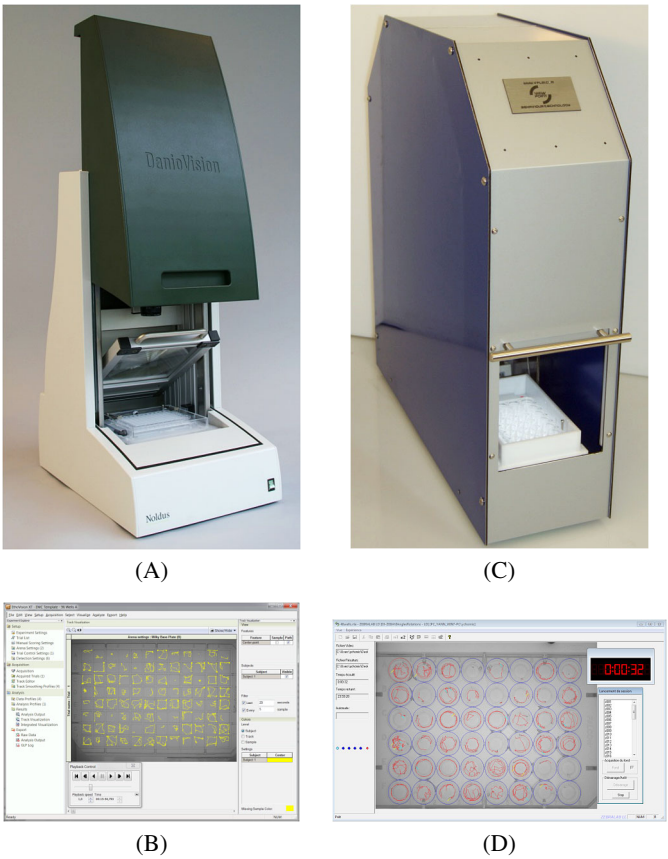
Video tracking systems were introduced in the early 1990s, offering clear advantages of flexibility, spatial precision, and accuracy over manual observation or devices using infrared-beam technology. A major step forward in this technology was achieved by the use of a video digitizer. A modern frame grabber uses a high-speed analogue-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 1 plane (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 & Creton (2011a) 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: Kimmel et al., 1995). 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 a day. For example, EthoVision® XT (Noldus Information Technology, Wageningen, The Netherlands) has been employed in isolating seizure-resistant zebrafish larvae in a large-scale mutagenesis screen. The behaviour of zebrafish is 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 3.



(A) (C)

(B) (D)

Figure 3. Commercial setups for the video-tracking of zebrafish embryo behaviour. (A, B) Noldus DanioVision system. (A) Hardware with a 96-well plate; (B) EthoVision XT screen print showing video tracking of embryo swimming pattern. (C, D) Viewpoint ZebraLab videotrack systems. (C) Hardware with a 24-well plate installed; (D) screen print showing tracked swimming patterns in a 48-well plate. This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/1568539x.

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.

3.1. 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. (2009) 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 (Prober et al., 2006).

The density of rearing zebrafish embryos may also influence their behaviour. Zellner and colleagues (2011) found that larvae that were raised in groups had higher levels of activity in the dark period compared to larvae that had been raised individually. Ambient light conditions in the test apparatus are of vital importance to zebrafish larvae, which can either decrease or increase their locomotor activity depending on the intensity of light (Padilla et al., 2011). 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 (MacPhail et al., 2009; Zellner et al., 2011).

The temperature at which embryos are raised and tested is another factor which can alter the locomotor behaviour and development of the zebrafish larvae (Laale, 1977; Roy et al., 1999; Scheil & Kohler, 2009). 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 (Padilla et al., 2011) 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 into account which can affect their movements (Padilla et al., 2011). 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.

3.2. Behavioural tests

Many assays have been developed in zebrafish larvae by taking advantage of its inherent visual reflexes. Among these are optomotor response (Krauss & Neumeyer, 2003; Maaswinkel & Ti, 2003; Orger & Baier, 2005; Creton, 2009), optokinetic response (OKR) (Qian et al., 2005a,b; Rinner et al., 2005; Brockerhoff, 2006; Huang & Neuhauss, 2008) and light-dark challenge test (Ali et al., 2011c). A list of behaviours displayed by zebrafish larvae (up to 9 dpf) is given in Table 2.

Table 2.
Different types of behavioural patterns exhibited by zebrafish larvae.

Behaviour	Time	Stimulus	Reference
Coiling	17-21 hpf	None	Saint-Amant & Drapeau (1998); Brustein et al. (2003); Downes & Granato (2006); McKeown et al. (2009)
Touch-induced escape response	22-27 hpf	Touch	McKeown et al. (2009); Chen et al. (2012); McClenahan et al. (2012)
Evoked swimming	27 hpf until hatching	Touch	Saint-Amant & Drapeau (1998); Low et al. (2012)
Photomotor response	30 hpf	Light intensity	Kokel et al. (2010)
Optokinetic response (OKR)	73–80 hpf	Moving objects	Qian et al. (2005a,b); Rinner et al. (2005); Brockerhoffn (2006); Huang & Neuhauss (2008)
Optomotor response	5 dpf	Moving objects	Krauss & Neumeyer (2003); Maaswinkel & Ti (2003); Orger & Baier (2005); Creton (2009)
Shadow response	8 dpf	Approaching object	Kimmel et al. (1974); Watkins et al. (2004)
Turning behaviour	6–9 dpf	Touch, approaching object, sudden change of light conditions, sound	Budick & O'Malley (2000); McClenahan et al. (2012)
Prey capture	9 dpf	Prey	Budick & O'Malley (2000); Lorent et al. (2001); McElligott & O'Malley (2005); McClenahan et al. (2012)

Table 3.
Effect of different compounds on tail coiling in zebrafish embryos.

Compound	Concentration (mg/l)	Exposure time	Effect	Reference
Perfluorooctane-sulphonic acid (PFOS)	4	Exposed at 6 hpf, tested at 25 hpf	Tail bend frequency increased	Huang et al. (2010)
Chlorpyrifos	0.625	Exposed at 2 hpf, tested at 24 hpf	Tail coiling duration and frequency increased	Selderslaghs et al. (2010)

3.3. 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 (Downes & Granato, 2006; McKeown et al., 2009). This spontaneous movement is independent of sensory stimulation and is driven by activity in the spinal cord (Saint-Amant & Drapeau, 1998). Frequency of coiling decreases after 21 hpf (McKeown et al., 2009). Compounds such as perfluorooctanesulphonic acid and chlorpyrifos have been shown to affect coiling in zebrafish as shown in Table 3.

3.4. 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 (Downes & Granato, 2006; McKeown et al., 2009). 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 (McKeown et al., 2009). Many compounds have been shown to affect touch-induced escape response (see Table 4).

A zebrafish 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

Table 4.
Effects of selected compounds on the touch response in zebrafish.

Drug	Concentration	Exposure time	Effect	Reference
Cadmium	0.25, 0.5 mg/l	Exposed 2–50 hpf, tested at 3 dpf	Touch response decreased	Hallare et al. (2005)
Dichlorodiphenyl-trichloroethane (DDT)	100 μM	2 dpf	Touch response increased	Ton et al. (2006)
DDT	10 μM	4 dpf	Touch response increased	Ton et al. (2006)
Diazinon	2 mg/l	0.5 hpf, tested at 5 and 8 dpf	Touch response decreased	Scheil et al. (2009)
Dieldrin	20 μM	Exposed 6–48 hpf, tested at 48 hpf	Touch response decreased	Ton et al. (2006)
Fipronil	1.1 μM	Exposed 2–48 hpf, tested at 48 hpf	Touch response decreased	Stehr et al. (2006)
Nonylphenol	5 μM	Exposed 6–48 hpf, tested at 48 hpf	Touch response decreased	Ton et al. (2006)
Sodium benzoate	100 ppm	Exposed 2–3 dpf, tested at 3 dpf	Touch response decreased	Tsay et al. (2007)
2,3,7,8-Tetrachloro-dibenzo-*p*-dioxin (TCDD)	0.1 μM	Exposed 6–48 hpf, tested at 48 hpf	Touch response decreased	Ton et al. (2006)

hindbrain and spinal cord are intact (Drapeau et al., 2002). There are two types of mechanosensory neurons acting on touch stimuli; Rohon–Beard neurons are activated on tail stimulation while trigeminal neurons on head and yolk stimulation (Drapeau et al., 2002).

The mechanisms behind the response are still unknown. It is known that zebrafish show differential developmental response to strychnine (McKeown et al., 2009). This is significant because strychnine is a competitive antagonist of glycine at inhibitory spinal glycine receptors in zebrafish (Brustein et al., 2012). 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 et al. (2009) 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 re-

sponses 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.

3.5. Startle response

This is an unconditioned behaviour displayed by rapid and massive acceleration resulting from contraction of the body axial muscles. This response is elicited by visual, touch or acoustic stimuli (Koch, 1999; Bang et al., 2002; Best et al., 2010; Kokel et al., 2010) 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 (Kimmel et al., 1974) observed no responses in larvae less than two weeks old to sudden changes in illumination. However, later studies showed that zebrafish larvae perform a startle response at 3 dpf and by 5–6 dpf, they respond to changes in light and dark (MacPhail et al., 2009; Ali et al., 2012) and can follow rotating stripes (Fadool & Dowling, 2008). Further, a high-throughput analysis (Burgess & Granato, 2007) 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 differences from the mammalian eye (Fadool & Dowling, 2008).

Zebrafish react both to light and dark flashes (Emran et al., 2008; Ali et al., 2012) but latency to respond to light and dark flashes is different (Burgess & Granato, 2007). 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 (Burgess & Granato, 2007).

Zebrafish also respond behaviourally to an acoustic stimulus after 5 dpf (Best et al., 2008). They are known to exhibit startle response to frequencies of 200 Hz upwards (Zeddies & Fay, 2005). The acoustic startle response is elicited within 15 ms by sudden, intense acoustic stimuli (Liu & Fetcho, 1999). This response becomes functional immediately after the zebrafish auditory system has become functional around 5 dpf (Whitfield et al., 2002). The startle response in zebrafish larvae is increased in the case of an aversive event, sensitization, fear/anxiety-potentiation, or certain drugs (see Table 5), while it is decreased by habituation, pre-pulse inhibition, or drugs (Koch, 1999).

Table 5.
Effects of various drugs on the startle response in zebrafish larvae.

Drug	Concentration (μM)	Exposure time (dpf)	Effect	Reference
Donepezil	3, 10	6–7	Increase acoustic startle response	Best et al. (2008)
Ethanol	30 000	4–6	Vibrational startle response decreased	Carvan et al. (2004)
Memantine	30	6–7	Increase acoustic startle response	Best et al. (2008)
Nicotine	20	1–7	Startle response decreased after mechanical stimulation or tapping the beaker containing zebrafish larvae	Parker & Connaughton (2007)
Rolipram	3	6–7	Increase acoustic startle response	Best et al. (2008)

3.6. Open field test

The open field test has been commonly used to assess animal exploration and this is hypothesized to reflect certain emotional states especially in rodents (Thinusblanc & Foreman, 1993; Prut & Belzung, 2003) and adult zebrafish (Champagne et al., 2010; Stewart et al., 2012). Due to its several salient features (see introduction), the zebrafish has become an excellent model to study locomotion and novel environment exploration (Champagne et al., 2010). Steenbergen and colleagues (2011) used a light/dark box to assess avoidance behaviour of zebrafish larvae and found aversion for a dark environment, 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 (Thirumalai & Cline, 2008; Colwill & Creton, 2011b) which was attenuated using anxiolytics or giving sufficient time for the larvae to habituate in the testing apparatus (Colwill & Creton, 2011b).

3.7. Visual motor response test

The zebrafish mainly uses vision to hunt for food and avoid predators (Fleisch & Neuhauss, 2006a). This reliance on vision necessitates the rapid development of the visual system, given that the embryo hatches to a free-swimming larva at around 48–72 h. Zebrafish initiate an escape response when threatened by a potential predator. This innate behaviour can be used to assess visual performance (Fleisch & Neuhauss, 2006b). It is known that the larval zebrafish has a preference for light and an aversion for dark (MacPhail et al., 2009; Champagne et al., 2010), in contrast to adult zebrafish where the preference and aversion are reversed (Serra et al., 1999).

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 3) for 2 min to habituate. Video recording is then begun, for the next 4 min of light exposure, to assess basal locomotor behaviour. Then, 4 min 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 (Bilotta, 2000; Ali et al., 2011a, 2012).

3.8. Optokinetic response (OKR)

Stereotyped eye movement 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) (Guerraz & Bronstein, 2008). This is one of the more widely studied behaviours due to its reliability. Zebrafish larvae are able to perform it even when immobilized (Valente et al., 2012). This behaviour develops between 73 and 80 hpf (Easter & Nicola, 1997) 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 movement of an eye) are identified as having defective visual function. Different compounds can affect this response (see Table 6).

Horizontal and bipolar cell processes in zebrafish larvae invaginate into the photoreceptor terminals which form two types of synapses, which relate

Table 6.
Effects of selected drugs on the optokinetic response in zebrafish larvae.

Drug	Concentration	Exposure time	Effect	Reference
Mixure of 2S-2-amino-4-phosphonobutanoic acid L-AP4 and DL-threo-β-benzyloxyaspartate (TBOA)	0.4 mM L-AP4 + 0.2 mM TBOA	2 h before experiment at 5 dpf	Abolished the OKR	Emran et al. (2007)
Bisoprolol	30–300 μM	5 days of treatment from 3 dpf, tested 8 dpf	No significant effect	Richards et al. (2008)
Ethanol	1.5%	Exposed 2-5 dpf, tested on 5 dpf	Higher visual threshold	Matsui et al. (2006)

to the separation of visual input into ON and OFF channels (Dowling, 1987). The ON and OFF channels are capable of sensing light increments and light decrements respectively (Schiller et al., 1986). 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 (Emran et al., 2007) 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 (Emran et al., 2007).

3.9. Optomotor response (OMR) 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. This response can be observed at 6 dpf, but is more robust at 7 dpf (Orger & Baier, 2005; Fleisch & Neuhauss, 2006; Portugues & Engert, 2009). 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 (Roeser & Baier, 2003). A list of selected compounds and their effects on OMR is given in Table 7. This assay can be used to evaluate the effect of compounds on zebrafish vision at a higher

Table 7.
The effects of various compounds on the optomotor response in zebrafish larvae.

Compound	Concentration	Exposure time	Effect	Reference
Allopurinol	300 μM	5 days of treatment from 3 dpf, tested 8 dpf	No effect	Richards et al. (2008)
Amoxicillin	1 mM	5 days of treatment from 3 dpf, tested 8 dpf	No effect	Richards et al. (2008)
Aspirin	500 μM	Exposed from 3 dpf until day of testing at 8 dpf	No effect	Berghmans et al. (2008)
Atropine	100 μM	Exposed from 3 dpf until day of testing at 8 dpf	No effect	Berghmans et al. (2008)
Bisoprolol	300 μM	5 days of treatment from 3 dpf, tested 8 dpf	Inhibition	Richards et al. (2008)
Chloropromazine	10 μM	Exposed from 3 dpf until day of testing at 8 dpf	Inhibition	Berghmans et al. (2008)
Chloroquine	100 μM	Exposed from 3 dpf until day of testing at 8 dpf	Inhibition	Berghmans et al. (2008)
Chlorpromazine	0.03–1 μM	5 days of treatment from 3 dpf, tested 8 dpf	Inhibition	Richards et al. (2008)
Cisapride	3 μM	5 days of treatment from 3 dpf, tested 8 dpf	Slight inhibition	Richards et al. (2008)
Cisplatin	30 μM	5 days of treatment from 3 dpf, tested 8 dpf	Inhibition	Richards et al. (2008)

Table 7.
(Continued.)

Compound	Concentration	Exposure time	Effect	Reference
Diazepam	10 μM	Exposed from 3 dpf until day of testing at 8 dpf	Inhibition	Berghmans et al. (2008)
Ethanol	1.5%	6-12, 12-24 hpf	Lower visual acuity	Bilotta et al. (2002)
Ethanol	1.5%	48-60, 60-72 hpf	No effect	Bilotta et al. (2002)
Flecainide	100 μM	5 days of treatment from 3 dpf, tested 8 dpf	Inhibition with motility defects	Richards et al. (2008)
Furosemide	1 mM	5 days of treatment from 3 dpf, tested 8 dpf	No inhibition	Richards et al. (2008)
Gentamicin	1-30 μM	5 days of treatment from 3 dpf, tested 8 dpf	Inhibition	Richards et al. (2008)
Indomethacin	10 μM	5 days of treatment from 3 dpf, tested 8 dpf	No inhibition	Richards et al. (2008)
Ketoconazole	3-10 μM	5 days of treatment from 3 dpf, tested 8 dpf	No significant inhibition	Richards et al. (2008)
Lansoprazole	30 μM	5 days of treatment from 3 dpf, tested 8 dpf	No effect	Richards et al. (2008)
Lithium	100 μM	Exposed from 3 dpf until day of testing at 8 dpf	No effect	Berghmans et al. (2008)
Nicotine	6.2 and 62 μM	Exposed from 3 dpf until day of testing at 8 dpf	Inhibition	Berghmans et al. (2008)

Table 7.
(Continued.)

Compound	Concentration	Exposure time	Effect	Reference
Nicotinic acid	1 mM	5 days of treatment from 3 dpf, tested 8 dpf	No inhibition	Richards et al. (2008)
Ouabain	50 μM	Exposed from 3 dpf until day of testing at 8 dpf	inhibition	Berghmans et al. (2008)
Phenytoin	100 μM	Exposed from 3 dpf until day of testing at 8 dpf	Inhibition	Berghmans et al. (2008)
Quinine	30-100 μM	5 days of treatment from 3 dpf, tested 8 dpf	Inhibition	Richards et al. (2008)
Sodium iodate	10 mM	5 days of treatment from 3 dpf, tested 8 dpf	No consistent inhibition	Richards et al. (2008)
Spironolactone	30 μM	5 days of treatment from 3 dpf, tested 8 dpf	Inhibition with motility defect	Richards et al. (2008)
Thioridazine	0.3–1 μM	5 days of treatment from 3 dpf, tested 8 dpf	Inhibition with motility defects	Richards et al. (2008)
Vigabatrin	100 μM	5 days of treatment from 3 dpf, tested 8 dpf	No inhibition	Richards et al. (2008)

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.

The mutants with OMR defects can appear to overlap to a greater extent with those showing OKR defects (Neuhauss et al., 1999). 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 (Springer et al., 1977).

3.10. 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 (Spence & Smith, 2008; Avdesh et al., 2010). The adult zebrafish is reported to have an innate preference for black (Serra et al., 1999), red, yellow and green (Avdesh et al., 2010). 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.

3.11. 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 (Thompson & Spencer, 1966; Best et al., 2008; Rankin et al., 2009). It has been observed in a wide range of organisms including protozoa (Wood, 1969) and humans (Geer, 1966). 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.

At a simple level, zebrafish larvae show habitation to tactile or shock stimuli, with the response diminishing after a few repetitions (Orger et al., 2004). Another study (Best et al., 2008) 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 (Roberts et al.,

2011). The first form is elicited by low-frequency of auditory stimulation and lasts for about 1–15 min. 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 (Thompson & Spencer, 1966). For example, a light pulse was used to reinitiate larval zebrafish response to sound (Best et al., 2008). The inter-stimulus interval (Levin et al., 2007) 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 (Thompson & Spencer, 1966).

3.12. 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 (Budick & O'Malley, 2000). 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 (Kimmel et al., 1974; Danos & Lauder, 2007); they are helpful in foraging and predator avoidance (Webb & Weihs, 1986).

The second type of turn is more complex, and is characterized by high-velocity turns; it is 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 (Budick & O'Malley, 2000).

3.13. Prey capture

After the zebrafish larva has hatched, it lives on nutrient reserves in its yolk-sac (Liu & Chan, 2002). Ultimately, however, it needs to acquire exogenous food. This transition from 'yolksac larva' to 'free-swimming larva' which is able to hunt takes place around 5–6 dpf (Biehlmaier et al., 2007). 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 (Borla et al., 2002; Gahtan et al., 2005). It has been shown that prey capture consists of a series of small turns followed by a 'slow-like' swim (Budick & O'Malley, 2000). 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 it (the so-called 'slow-strike approach'); or can catch the prey while remaining stationary (Budick & O'Malley, 2000). 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 (Borla et al., 2002).

4. 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 (Craske et al., 2009). 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 (Craske et al., 2009). 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.

4.1. Parameters used to measure anxiety

As in rodents, exposure to novel environment evokes robust anxiety-like behaviours in zebrafish (Blaser & Gerlai, 2006). Exploratory behaviour of

zebrafish is used to measure stress and anxiety-like responses in zebrafish which display a wide range of behaviours in a highly aversive novel environment. Zebrafish employ cortisol instead of corticosterone as a primary stress response hormone (Barcellos et al., 2007), 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 a central arena of the field is considered aversive and, hence, thigmotaxis is observed. A zebrafish avoids the 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 avoid the light area and region nearest to the predator when tested using the light/dark box. However, the larval zebrafish prefers the light area over dark (Steenbergen et al., 2011).

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 (Alsop & Vijayan, 2008). After observing behaviour, it is fairly easy to measure cortisol levels (Alsop & Vijayan, 2008), although this is not possible in vivo in zebrafish.

New options for measuring physiological responses during behavioural tasks are heart rate (Rana et al., 2010; Steele et al., 2011) and skin colour monitoring (Shiraki et al., 2010). 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 heart-beat. 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 8).

4.2. 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 (Treit & Fundytus, 1988). Thigmotaxis is displayed by a wide range of species including rodents (Treit & Fundytus, 1988; Prut & Belzung, 2003; Belzung & Philippot, 2007) and humans (Kallai et al., 2005,

Table 8.
Effect of selected anxiolytics and anxiogenics on zebrafish anxiety-like behaviours.

Compound	Test	Dose	Effect	Reference
Anxiolytics				
Buspirone	Light/dark box	59.24 μM	Total time spent in dark zone increased, latency to visit dark zone decreased, number of entries in the dark increased	Steenbergen et al. (2011)
Buspirone	12-well plate assay	45 μM	Altered rest-wake profiles, increased freezing/rest	Rihel et al. (2010)
Diazepam	12-well plate assay	0.05 mg/l, 5 mg/l	Reduced thigmotaxis	Richendrfer et al. (2012)
Diazepam	Light/dark box	2.5 μM	Total time spent in dark zone increased, latency to visit dark zone decreased,	Steenbergen et al. (2011)
Ethanol	Motor activity	0.5–4%	Hyperactivity with 0.5-2% ethanol and hypoactivity with 4% ethanol treatment	de Esch et al. (2012)
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	Steenbergen et al. (2011)
Fluextine (Prozac)	12-well plate assay	0.2 mg, 2 mg/l	No effect on thigmotaxis, decreased avoidance response	Richendrfer et al. (2012)
Fluextine		15 μM	Altered rest-wake profile, increased rest	Airhart et al. (2007); Rihel et al. (2010)
Propranolol	96-well plate	10–100 μM	Decreased activity in the photomotor response assay	Kokel et al. (2010)
Anxiogenics				
Caffeine	Light/dark box	438 μM	Total time spent in dark zone decreased, latency to visit dark zone increased and number of entries in the dark zone decreased	Steenbergen et al. (2011)
Caffeine	12-well plate assay	100 mg/l	Increased thigmotaxis	Richendrfer et al. (2012)

2007). It is a validated index of anxiety using anxiolytic and anxiogenic drugs in stressful conditions (Prut & Belzung, 2003; Belzung & Philippot, 2007). Thigmotaxis has been studied in detail using adult zebrafish (Blaser et al., 2010; Champagne et al., 2010; Buske & Gerlai, 2012) but has also been displayed by zebrafish larvae (Bilotta, 2000; Thirumalai & Cline, 2008; Colwill & Creton, 2011b). Recently, a thigmotaxis assay in larval zebrafish has also been validated (Bilotta, 2000) 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 24 h exposure to the same test apparatus (Colwill & Creton, 2011b) or in presence of other conspecifics (Lockwood et al., 2004) which provide them social protection, thus lessening 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 (Padilla et al., 2011; Schnorr et al., 2012). For these and other reasons, thigmotaxis is best studied in an arena at least the size of a 24 well plate.

4.3. Scototaxis

A preference for darkness is also an indication of anxiety-like behaviours in adult zebrafish (Maximino et al., 2010, 2011). The test is very simple and similar to the light/dark box (Bourin & Hascoet, 2003). 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 (Champagne et al., 2010; Steenbergen et al., 2011). 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 (Steenbergen et al., 2011).

4.4. Freezing

Freezing is a classical index of anxiety (Champagne et al., 2010). 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 & Richardson, unpublished data) like adult zebrafish (Egan et al., 2009; Champagne et al., 2010). Young larvae, i.e., 3–4 dpf, spend more time in the freezing or the resting state than at 5, 6 or 7 dpf old (Thirumalai & Cline, 2008; Colwill & Creton, 2011b). High rates of freezing in young larvae could be attributed to inflation of the swim bladder and spontaneous swimming which begin around 5 dpf (Thirumalai & Cline, 2008; Lindsey et al., 2010). However, this freezing behaviour is attenuated if the embryos are exposed in the test apparatus 24 h before the testing (Colwill & Creton, 2011b). Thigmotaxis may be a useful parameter in the experiment involving anxiolytics and anxiogenics which can increase or decrease time spent in mobility respectively (Schnorr et al., 2012).

4.5. Erratic movements

Sudden sharp changes in direction of movement and/or velocity, indicating rapid anxiety-like darting behaviours, are known as erratic movement. It has been well studied in the adult zebrafish (Gerlai et al., 2000; Egan et al., 2009; Wong et al., 2010). Larval zebrafish also display erratic movement in response to sudden change in the light (Giacomini et al., 2006; Emran et al., 2008; Champagne et al., 2010; Steenbergen et al., 2011; Ali et al., 2011c, 2012) but this has not been studied in detail. Erratic movements usually occur at high speed (Levin et al., 2007). These high speed movements can serve as an indirect measure of erratic movements in stressed zebrafish. Automated quantification systems with speed measurement can serve as a useful tool to monitor erratic movements in zebrafish (Champagne et al., 2010).

5. Other applications of zebrafish embryo/larval behavioural screening

It is difficult to review the full range of applications in which zebrafish embryos/larvae have been used. However, 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 (Baraban et al., 2005; Berghmans et al., 2007). They showed that the larval zebrafish pentylene tetrazole (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 (Ali et al., 2011a) and tuberculosis (Carvalho et al., 2011) are other examples in which zebrafish larvae are promising models. Taken together, zebrafish embryos as an in vivo bioassay tool can contribute to identify drug targets, discovery and toxicity of the unknown drugs.

A wide range of compounds has been tested for toxicity using zebrafish embryos (for reviews, see Ali et al., 2011c, 2012). For example, the LC_{50} values of 60 water soluble compounds were determined (Ali et al., 2011c) and found that they correlate well with the LD_{50} values of the same compounds in rodents (Figure 4). This finding was in line with previous studies showing a good predictability of the zebrafish model (Parng et al., 2002; Selderslaghs et al., 2009). Furthermore, they found that nearly all of these pharmacologically active compounds or toxins produced changes in zebrafish embryo behaviour in the light-dark challenge test (Ali et al., 2012).

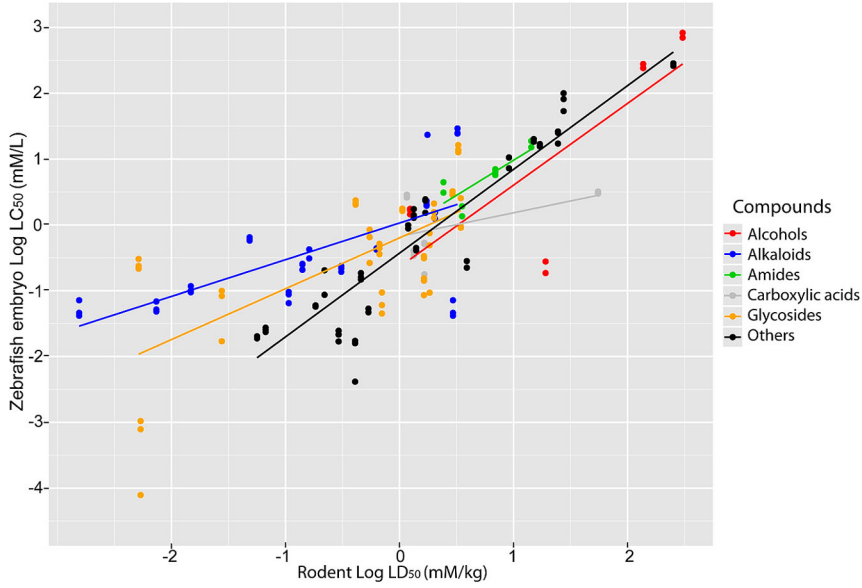


Figure 4. Correlation between LC50 values of a range of toxins in zebrafish embryo assays, and the LD_{50} values of the same compounds in rodents. From Ali et al. (2011c). This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/1568539x.

6. 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.

6.1. 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 Ali et al., 2011c for example). The problem with these assays include: the combining of several embryos in a single well; the evaporation of buffer, which dramatically affects side and corner wells (Ali et al., 2011c) and may affect the lenses in video-tracking hardware apparatus; and the relatively large volume of buffer and, therefore, test compound, consumed (Wielhouwer et al., 2011).

6.2. Microfluidic assays

Many of the issues discussed above can be addressed by culturing zebrafish embryos in microfluidic systems (Wielhouwer et al., 2011; Akagi et al., 2012; Khoshmanesh et al., 2012). These can provide a constant flow of buffer at low volumes (one system from our lab has a static volume of 8 μl per well), while also avoiding the problems of evaporation of buffer because the microfluidic chip can provide a closed system (Figure 5). The obvious drawback of these systems is the miniaturization of the environment, which 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 to examine anxiety and anxiolytics.

6.3. Automation and high-throughput screening

A recent study (Letamendia et al., 2012) presented an automated platform for high-throughput screening using two different assays to detect cardiotoxic compounds and angiogenesis inhibitors. They validated these two assays with known positive and negative compounds, as well as a screening for the detection of unknown anti-anxiogenic compounds. Rihel and colleagues (2010) used larval zebrafish to screen 4000 small molecules for their effects on long-term sleep/wake behaviour using ZebraLab (Viewpoint, France, available online at http://viewpoint.fr/zerbalab) and Kokel et al. (2010) tested

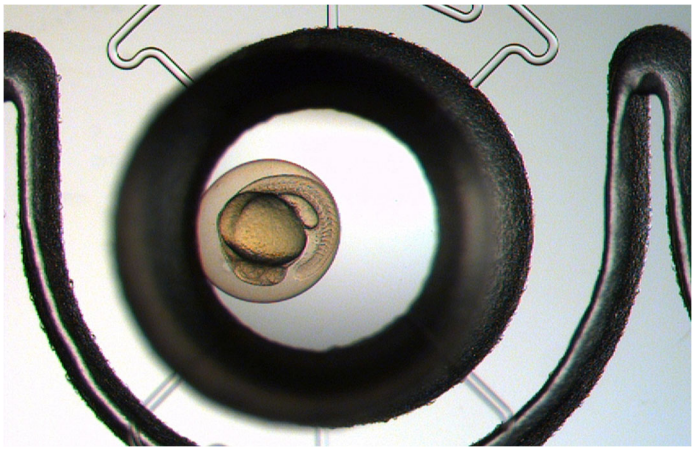


Figure 5. A zebrafish embryo cultured in a glass microfluidic biochip (Courtesy Eric Wielhouwer, Syntecnos, Leiden, The Netherlands). The embryo lies within a well having a volume of approximately 8 μl, and is fed with buffer through the three inlet and three outlet channels at a rate of 2 μl/min per well. This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/1568539x.

approximately 14 000 neuroactive small molecules to look for the altered larval responses to a high intensity light pulses. Both studies used automated video-tracking systems in microtiter plates using behavioural profiles of the zebrafish larvae. Other automated screening platforms are also being developed (Pardo-Martin et al., 2010) which may open new avenues in automated high-throughput screening.

7. Conclusions

In conclusion, young developmental stages of the zebrafish show a remarkable similarity to mammals in certain key behaviours. Larval zebrafish suffer stress and anxiety and often respond in a similar way to drugs as do rats or humans. Of course there are many differences too, and each zebrafish screen that is developed needs to be fully validated and understood at the molecular and cellular level. 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, 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 importance.

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References

Agid, Y., Buzsaki, G., Diamond, D.M., Frackowiak, R., Giedd, J., Girault, J.A., Grace, A., Lambert, J.J., Manji, H., Mayberg, H., Popoli, M., Prochiantz, A., Richter-Levin, G., Somogyi, P., Spedding, M., Svenningsson, P. & Weinberger, D. (2007). Viewpoint — How can drug discovery for psychiatric disorders be improved? — Nature Rev. Drug Disc. 6: 189-201.

Airhart, M.J., Lee, D.H., Wilson, T.D., Miller, B.E., Miller, M.N. & Skalko, R.G. (2007). Movement disorders and neurochemical changes in zebrafish larvae after bath exposure to fluoxetine (PROZAC). — Neurotoxicol. Teratol. 29: 652-664.

Akagi, J., Khoshmanesh, K., Evans, B., Hall, C.J., Crosier, K.E., Cooper, J.M., Crosier, P.S. & Wlodkowic, D. (2012). Miniaturized embryo array for automated trapping, immobilization and microperfusion of zebrafish embryos. — PLoS One 7: e36630.

Ali, S., Champagne, D.L., Alia, A. & Richardson, M.K. (2011a). Large-scale analysis of acute ethanol exposure in zebrafish development: a critical time window and resilience. — PLoS One 6: e20037.

Ali, S., Champagne, D.L., Spaink, H.P. & Richardson, M.K. (2011b). Zebrafish embryos and larvae: a new generation of disease models and drug screens. — Birth Def. Res. C: Embryol. Today Rev. 93: 115-133.

Ali, S., van Mil, H.G. & Richardson, M.K. (2011c). Large-scale assessment of the zebrafish embryo as a possible predictive model in toxicity testing. — PLoS One 6: e21076.

Ali, S., Champagne, D.L. & Richardson, M.K. (2012). Behavioral profiling of zebrafish embryos exposed to a panel of 60 water-soluble compounds. — Behav. Brain Res. 228: 272-283.

Alsop, D. & Vijayan, M.M. (2008). Development of the corticosteroid stress axis and receptor expression in zebrafish. — Am. J. Physiol. Regul. Integr Comp. Physiol. 294: R711-R719.

Avdesh, A., Martin-Iverson, M., Mondal, A., Chen, M., Verdile, G. & Martins, R. (2010). Natural colour preference in the zebrafish (*Danio rerio*). — In: Proceedings of Measuring Behavior 2010, 7th International Conference on Methods and Techniques in Behavioral Research, Eindhoven, The Netherlands, August 24–27, 2010 (Spink, A.J., Grieco, F., Krips, O.E., Loijens, L.W.S., Noldus, L.P.J.J. & Zimmerman, P.H., eds), p. 155-157.

Bang, P.I., Yelick, P.C., Malicki, J.J. & Sewell, W.F. (2002). High-throughput behavioral screening method for detecting auditory response defects in zebrafish. — J. Neurosci. Methods 118: 177-187.

Baraban, S.C., Taylor, M.R., Castro, P.A. & Baier, H. (2005). Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-FoS expression. — Neuroscience 131: 759-768.

Barcellos, L.J.G., Ritter, F., Kreutz, L.C., Quevedo, R.M., da Silva, L.B., Bedin, A.C., Finco, J. & Cericato, L. (2007). Whole-body cortisol increases after direct and visual contact with a predator in zebrafish, *Danio rerio*. — Aquaculture 272: 774-778.

Belzung, C. & Philippot, P. (2007). Anxiety from a phylogenetic perspective: is there a qualitative difference between human and animal anxiety? — Neural Plast.: 59676.

Berghmans, S., Hunt, J., Roach, A. & Goldsmith, P. (2007). Zebrafish offer the potential for a primary screen to identify a wide variety of potential anticonvulsants. — Epilepsy Res. 75: 18-28.

Berghmans, S., Butler, P., Goldsmith, P., Waldron, G., Gardner, I., Golder, Z., Richards, F.M., Kimber, G., Roach, A., Alderton, W. & Fleming, A. (2008). Zebrafish based assays for the assessment of cardiac, visual and gut function - potential safety screens for early drug discovery. — J. Pharmacol. Toxicol. Methods 58: 59-68.

Best, J.D., Berghmans, S., Hunt, J.J., Clarke, S.C., Fleming, A., Goldsmith, P. & Roach, A.G. (2008). Non-associative learning in larval zebrafish. — Neuropsychopharmacology 33: 1206-1215.

Best, J., Adatto, I., Cockington, J., James, A. & Lawrence, C. (2010). A novel method for rearing first-feeding larval zebrafish: polyculture with Type L saltwater rotifers (*Brachionus plicatilis*). — Zebrafish 7: 289-295.

Biehlmaier, O., Makhankov, Y. & Neuhauss, S.C.F. (2007). Impaired retinal differentiation and maintenance in zebrafish laminin mutants. — Invest. Ophthalmol. Vis. Sci. 48: 2887-2894.

Bilotta, J. (2000). Effects of abnormal lighting on the development of zebrafish visual behavior. — Behav. Brain Res. 116: 81-87.

Bilotta, J., Saszik, S., Givin, C.M., Hardesty, H.R. & Sutherland, S.E. (2002). Effects of embryonic exposure to ethanol on zebrafish visual function. — Neurotoxicol. Teratol. 24: 759-766.

Blaser, R. & Gerlai, R. (2006). Behavioral phenotyping in zebrafish: comparison of three behavioral quantification methods. — Behav. Res. Methods 38: 456-469.

Blaser, R.E., Chadwick, L. & McGinnis, G.C. (2010). Behavioral measures of anxiety in zebrafish (*Danio rerio*). — Behav. Brain Res. 208: 56-62.

Boehmler, W., Carr, T., Thisse, C., Thisse, B., Canfield, V.A. & Levenson, R. (2007). D4 Dopamine receptor genes of zebrafish and effects of the antipsychotic clozapine on larval swimming behaviour. — Genes Brain Behav. 6: 155-166.

Borla, M.A., Palecek, B., Budick, S. & O'Malley, D.M. (2002). Prey capture by larval zebrafish: evidence for fine axial motor control. — Brain Behav. Evol. 60: 207-229.

Bourin, M. & Hascoet, M. (2003). The mouse light/dark box test. — Eur. J. Pharmacol. 463: 55-65.

Brockerhoff, S.E. (2006). Measuring the optokinetic response of zebrafish larvae. — Nature Protocols 1: 2448-2451.

Brustein, E., Saint-Amant, L., Buss, R.R., Chong, M., McDearmid, J.R. & Drapeau, P. (2003). Steps during the development of the zebrafish locomotor network. — J. Physiol. 97: 77-86.

Brustein, E., Cote, S., Ghislain, J. & Drapeau, P. (2012). Spontaneous glycine-induced calcium transients in spinal cord progenitors promote neurogenesis. — Dev. Neurobiol., in press, DOI:10.1002/dneu.22050

Budick, S.A. & O'Malley, D.M. (2000). Locomotor repertoire of the larval zebrafish: swimming, turning and prey capture. — J. Exp. Biol. 203: 2565-2579.

Burgess, H.A. & Granato, M. (2007). Modulation of locomotor activity in larval zebrafish during light adaptation. — J. Exp. Biol. 210: 2526-2539.

Buske, C. & Gerlai, R. (2012). Maturation of shoaling behavior is accompanied by changes in the dopaminergic and serotoninergic systems in zebrafish. — Dev. Psychobiol. 54: 28-35.

Carvalho, R., de Sonneville, J., Stockhammer, O.W., Savage, N.D.L., Veneman, W.J., Ottenhoff, T.H.M., Dirks, R.P., Meijer, A.H. & Spaink, H.P. (2011). A high-throughput screen for tuberculosis progression. — PLoS One 6.

Carvan 3rd, M.J., Loucks, E., Weber, D.N. & Williams, F.E. (2004). Ethanol effects on the developing zebrafish: neurobehavior and skeletal morphogenesis. — Neurotoxicol. Teratol. 26: 757-768.

Champagne, D.L., Hoefnagels, C.C., de Kloet, R.E. & Richardson, M.K. (2010). Translating rodent behavioral repertoire to zebrafish (*Danio rerio*): relevance for stress research. — Behav. Brain Res. 214: 332-342.

Chen, L., Huang, C., Hu, C., Yu, K., Yang, L. & Zhou, B. (2012). Acute exposure to DE-71: Effects on locomotor behavior and developmental neurotoxicity in zebrafish larvae. — Environm. Toxicol. Chem. 31: 2338-2344.

Colwill, R.M. & Creton, R. (2011a). Imaging escape and avoidance behavior in zebrafish larvae. — Rev. Neurosci. 22: 63-73.

Colwill, R.M. & Creton, R. (2011b). Locomotor behaviors in zebrafish (*Danio rerio*) larvae. — Behav. Proc. 86: 222-229.

Craske, M.G., Rauch, S.L., Ursano, R., Prenoveau, J., Pine, D.S. & Zinbarg, R.E. (2009). What is an anxiety disorder? — Depr. Anxiety 26: 1066-1085.

Craven, R. (2011). The risky business of drug development in neurology. — Lancet Neurol. 10: 116-117.

Creton, R. (2009). Automated analysis of behavior in zebrafish larvae. — Behav. Brain Res. 203: 127-136.

Dahm, R. & Geisler, R. (2006). Learning from small fry: the zebrafish as a genetic model organism for aquaculture fish species. — Mar. Biotechnol. 8: 329-345.

Danos, N. & Lauder, G.V. (2007). The ontogeny of fin function during routine turns in zebrafish *Danio rerio*. — J. Exp. Biol. 210: 3374-3386.

DiMasi, J.A., Hansen, R.W. & Grabowski, H.G. (2003). The price of innovation: new estimates of drug development costs. — J. Health Econ. 22: 151-185.

Dowling, J.E. (1987). The retina: an approachable part of the brain. — Belknap Press, Cambridge, MA.

Downes, G.B. & Granato, M. (2006). Supraspinal input is dispensable to generate glycine-mediated locomotive behaviors in the zebrafish embryo. — J. Neurobiol. 66: 437-451.

Drapeau, P., Saint-Amant, L., Buss, R.R., Chong, M., McDearmid, J.R. & Brustein, E. (2002). Development of the locomotor network in zebrafish. — Progr. Neurobiol. 68: 85-111.

Easter, S.S. & Nicola, G.N. (1997). The development of eye movements in the zebrafish (*Danio rerio*). — Dev. Psychobiol. 31: 267-276.

Egan, R.J., Bergner, C.L., Hart, P.C., Cachat, J.M., Canavello, P.R., Elegante, M.F., Elkhayat, S.I., Bartels, B.K., Tien, A.K., Tien, D.H., Mohnot, S., Beeson, E., Glasgow, E., Amri, H., Zukowska, Z. & Kalueff, A.V. (2009). Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. — Behav. Brain Res. 205: 38-44.

Ellis, L.D., Seibert, J. & Soanes, K.H. (2012). Distinct models of induced hyperactivity in zebrafish larvae. — Brain Res. 1449: 46-59.

Emran, F., Rihel, J., Adolph, A.R., Wong, K.Y., Kraves, S. & Dowling, J.E. (2007). OFF ganglion cells cannot drive the optokinetic reflex in zebrafish. — Proc. Natl. Acad. Sci. USA 104: 19126-19131.

Emran, F., Rihel, J. & Dowling, J.E. (2008). A behavioral assay to measure responsiveness of zebrafish to changes in light intensities. — J. Vis. Exp. 20: 923, DOI:10.3791/923.

de Esch, C., van der Linde, H., Slieker, R., Willemsen, R., Wolterbeek, A., Woutersen, R. & De Groot, D. (2012). Locomotor activity assay in zebrafish larvae: influence of age, strain and ethanol. — Neurotoxicol. Teratol. 34: 425-433.

Fadool, J.M. & Dowling, J.E. (2008). Zebrafish: a model system for the study of eye genetics. — Progr. Retinal Eye Res. 27: 89-110.

Fleisch, V.C. & Neuhauss, S.C. (2006). Visual behavior in zebrafish. — Zebrafish 3: 191-201.

Gahtan, E., Tanger, P. & Baier, H. (2005). Visual prey capture in larval zebrafish is controlled by identified reticulospinal neurons downstream of the tectum. — J. Neurosci. 25: 9294-9303.

Geer, J.H. (1966). Effect of interstimulus intervals and rest-period length upon habituation of orienting response. — J. Exp. Psychol. 72: 617-619.

Gerlai, R., Lahav, M., Guo, S. & Rosenthal, A. (2000). Drinks like a fish: zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects. — Pharmacol. Biochem. Behav. 67: 773-782.

Giacomini, N.J., Rose, B., Kobayashi, K. & Guo, S. (2006). Antipsychotics produce locomotor impairment in larval zebrafish. — Neurotoxicol. Teratol. 28: 245-250.

Guerraz, M. & Bronstein, A.M. (2008). Ocular versus extraocular control of posture and equilibrium. — Clin. Neurophysiol. 38: 391-398.

Hallare, A.V., Schirling, M., Luckenbach, T., Köhler, H.-R. & Triebskorn, R. (2005). Combined effects of temperature and cadmium on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos. — J. Therm. Biol. 30: 7-17.

Huang, Y.Y. & Neuhauss, S.C.F. (2008). The optokinetic response in zebrafish and its applications. — Front. Biosci.-Landmark 13: 1899-1916.

Huang, H., Huang, C., Wang, L., Ye, X., Bai, C., Simonich, M.T., Tanguay, R.L. & Dong, Q. (2010). Toxicity, uptake kinetics and behavior assessment in zebrafish embryos following exposure to perfluorooctanesulphonicacid (PFOS). — Aquat. Toxicol. 98: 139-147.

Irons, T.D., MacPhail, R.C., Hunter, D.L. & Padilla, S. (2010). Acute neuroactive drug exposures alter locomotor activity in larval zebrafish. — Neurotoxicol. Teratol. 32: 84-90.

Kaitin, K.I. (2010). Deconstructing the drug development process: the new face of innovation. — Clin. Pharmacol. Ther. 87: 356-361.

Kallai, J., Makany, T., Karadi, K. & Jacobs, W.J. (2005). Spatial orientation strategies in Morris-type virtual water task for humans. — Behav. Brain Res. 159: 187-196.

Kallai, J., Makany, T., Csatho, A., Karadi, K., Horvath, D., Kovacs-Labadi, B., Jarai, R., Nadel, L. & Jacobs, J.W. (2007). Cognitive and affective aspects of thigmotaxis strategy in humans. — Behav. Neurosci. 121: 21-30.

Kalueff, A.V., Stewart, A.M., Green, J., Kyzar, E., Roth, A. & Zapolsky, I. (2010–2011). The zebrafish neurophenome project. — Available online at http://www.kaluefflab.com/znp.html (accessed 14 September 2012).

Khoshmanesh, K., Akagi, J., Hall, C.J., Crosier, K.E., Crosier, P.S., Cooper, J.M. & Wlodkowic, D. (2012). New rationale for large metazoan embryo manipulations on chip-based devices. — Biomicrofluidics 6: 24102-2410214.

Kimmel, C.B., Patterson, J. & Kimmel, R.O. (1974). The development and behavioral characteristics of the startle response in the zebra fish. — Dev. Psychobiol. 7: 47-60.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B. & Schilling, T.F. (1995). Stages of embryonic-development of the zebrafish. — Dev. Dynam. 203: 253-310.

Koch, M. (1999). The neurobiology of startle. — Progr. Neurobiol. 59: 107-128.

Kokel, D., Bryan, J., Laggner, C., White, R., Cheung, C.Y., Mateus, R., Healey, D., Kim, S., Werdich, A.A., Haggarty, S.J., Macrae, C.A., Shoichet, B. & Peterson, R.T. (2010). Rapid behavior-based identification of neuroactive small molecules in the zebrafish. — Nature Chem. Biol. 6: 231-237.

Krauss, A. & Neumeyer, C. (2003). Wavelength dependence of the optomotor response in zebrafish (*Danio rerio*). — Vis. Res. 43: 1273-1282.

Laale, H.W. (1977). Biology and use of zebrafish *Brachydanio rerio* in fisheries research — literature-review. — J. Fish Biol. 10: 121-173.

Langheinrich, U., Vacun, G. & Wagner, T. (2003). Zebrafish embryos express an orthologue of HERG and are sensitive toward a range of QT-prolonging drugs inducing severe arrhythmia. — Toxicol. Appl. Pharmacol. 193: 370-382.

Lee, S.H., Kim, H.R., Han, R.X., Oqani, R.K. & Jin, D.I. (2011). Cardiovascular risk assessment of atypical antipsychotic drugs in a zebrafish model. — J. Appl. Toxicol., in press, DOI:10.1002/jat.1768.

Letamendia, A., Quevedo, C., Ibarbia, I., Virto, J.M., Holgado, O., Diez, M., Izpisua Belmonte, J.C. & Callol-Massot, C. (2012). Development and validation of an automated high-throughput system for zebrafish in vivo screenings. — PLoS One 7: e36690.

Levin, E.D., Chrysanthis, E., Yacisin, K. & Linney, E. (2003). Chlorpyrifos exposure of developing zebrafish: effects on survival and long-term effects on response latency and spatial discrimination. — Neurotoxicol. Teratol. 25: 51-57.

Levin, E.D., Bencan, Z. & Cerutti, D.T. (2007). Anxiolytic effects of nicotine in zebrafish. — Physiol. Behav. 90: 54-58.

Lindsey, B.W., Smith, F.M. & Croll, R.P. (2010). From inflation to flotation: contribution of the swimbladder to whole-body density and swimming depth during development of the zebrafish (*Danio rerio*). — Zebrafish 7: 85-96.

Liu, K.S. & Fetcho, J.R. (1999). Laser ablations reveal functional relationships of segmental hindbrain neurons in zebrafish. — Neuron 23: 325-335.

Liu, Y.W. & Chan, W.K. (2002). Thyroid hormones are important for embryonic to larval transitory phase in zebrafish. — Differentiation 70: 36-45.

Lockwood, B., Bjerke, S., Kobayashi, K. & Guo, S. (2004). Acute effects of alcohol on larval zebrafish: a genetic system for large-scale screening. — Pharmacol. Biochem. Behav. 77: 647-654.

Lorent, K., Liu, K.S., Fetcho, J.R. & Granato, M. (2001). The zebrafish space cadet gene controls axonal pathfinding of neurons that modulate fast turning movements. — Development 128: 2131-2142.

Lorenzetti, S., Altieri, I., Arabi, S., Balduzzi, D., Bechi, N., Cordelli, E., Galli, C., Ietta, F., Modina, S.C., Narciso, L., Pacchierotti, F., Villani, P., Galli, A., Lazzari, G., Luciano, A.M., Paulesu, I., Spanoo, M. & Mantovani, A. (2011). Innovative non-animal testing strategies for reproductive toxicology: the contribution of Italian partners within the EU project ReProTect. — Ann. Ist. Superiore Di Sanita 47: 429-444.

Low, S.E., Woods, I.G., Lachance, M., Ryan, J., Schier, A.F. & Saint-Amant, L. (2012). Touch responsiveness in zebrafish requires voltage-gated calcium channel 2.1b. — J. Neurophysiol. 108: 148-159.

Maaswinkel, H. & Ti, L. (2003). Spatio-temporal frequency characteristics of the optomotor response in zebrafish. — Vis. Res. 43: 21-30.

MacPhail, R.C., Brooks, J., Hunter, D.L., Padnos, B., Irons, T.D. & Padilla, S. (2009). Locomotion in larval zebrafish: influence of time of day, lighting and ethanol. — Neurotoxicology 30: 52-58.

Matsui, J.I., Egana, A.L., Sponholtz, T.R., Adolph, A.R. & Dowling, J.E. (2006). Effects of ethanol on photoreceptors and visual function in developing zebrafish. — Est. Ophthalmol. Vis. Sci. 47: 4589-4597.

Maximino, C., de Brito, T.M., Colmanetti, R., Pontes, A.A.A., de Castro, H.M., de Lacerda, R.I.T., Morato, S. & Gouveia, A. (2010). Parametric analyses of anxiety in zebrafish scototaxis. — Behav. Brain Res. 210: 1-7.

Maximino, C., da Silva, A.W.B., Gouveia, A. & Herculano, A.M. (2011). Pharmacological analysis of zebrafish (*Danio rerio*) scototaxis. — Progr. Neuro-Psychopharmacol. Biol. Psychol. 35: 624-631.

McClenahan, P., Troup, M. & Scott, E.K. (2012). Fin-tail coordination during escape and predatory behavior in larval zebrafish. — PLoS One 7: e32295.

McElligott, M.B. & O'Malley, D.M. (2005). Prey tracking by larval zebrafish: axial kinematics and visual control. — Brain Behav. Evol. 66: 177-196.

McKeown, K.A., Downes, G.B. & Hutson, L.D. (2009). Modular laboratory exercises to analyze the development of zebrafish motor behavior. — Zebrafish 6: 179-185.

Muto, A., Orger, M.B., Wehman, A.M., Smear, M.C., Kay, J.N., Page-McCaw, P.S., Gahtan, E., Xiao, T., Nevin, L.M., Gosse, N.J., Staub, W., Finger-Baier, K. & Baier, H. (2005). Forward genetic analysis of visual behavior in zebrafish. — PLoS Genet. 1: e66.

Neuhauss, S.C.F. (2003). Behavioral genetic approaches to visual system development and function in zebrafish. — J. Neurobiol. 54: 148-160.

Neuhauss, S.C.F., Biehlmaier, O., Seeliger, M.W., Das, T., Kohler, K., Harris, W.A. & Baier, H. (1999). Genetic disorders of vision revealed by a behavioral screen of 400 essential loci in zebrafish. — J. Neurosci. 19: 8603-8615.

Noldus, L.P.J.J., Trienes, R.J.H., Hendriksen, A.H.M., Jansen, H. & Jansen, R.G. (2000). The Observer Video-Pro: new software for the collection, management, and presentation of time-structured data from videotapes and digital media files. — Behav. Res. Methods Instrum. Comp. 32: 197-206.

Orger, M.B. & Baier, H. (2005). Channeling of red and green cone inputs to the zebrafish optomotor response. — Vis. Neurosci. 22: 275-281.

Orger, M.B., Gahtan, E., Muto, A., Page-McCaw, P., Smear, M.C. & Baier, H. (2004). Behavioral screening assays in zebrafish. — Methods Cell Biol. 77: 53-68.

Padilla, S., Hunter, D.L., Padnos, B., Frady, S. & MacPhail, R.C. (2011). Assessing locomotor activity in larval zebrafish: influence of extrinsic and intrinsic variables. — Neurotoxicol. Teratol. 33: 624-630.

Pardo-Martin, C., Chang, T.Y., Koo, B.K., Gilleland, C.L., Wasserman, S.C. & Yanik, M.F. (2010). High-throughput in vivo vertebrate screening. — Nature Methods 7: 634-636.

Parker, B. & Connaughton, V.P. (2007). Effects of nicotine on growth and development in larval zebrafish. — Zebrafish 4: 59-68.

Parng, C., Seng, W.L., Semino, C. & McGrath, P. (2002). Zebrafish: a preclinical model for drug screening. — Assay Drug Dev. Technol. 1: 41-48.

Parng, C., Roy, N.M., Ton, C., Lin, Y. & McGrath, P. (2007). Neurotoxicity assessment using zebrafish. — J. Pharmacol. Toxicol. Methods 55: 103-112.

Pfeiffer, W. (1977). The distribution of fright reaction and alarm substance cells in fishes. — Copeia: 653-665.

Pietri, T., Manalo, E., Ryan, J., Saint-Amant, L. & Washbourne, P. (2009). Glutamate drives the touch response through a rostral loop in the spinal cord of zebrafish embryos. — Dev. Neurobiol. 69: 780-795.

Porsolt, R.D., Lemaire, M., Durmuller, N. & Roux, S. (2002). New perspectives in CNS safety pharmacology. — Fund. Clin. Pharmacol. 16: 197-207.

Portugues, R. & Engert, F. (2009). The neural basis of visual behaviors in the larval zebrafish. — Curr. Opin. Neurobiol. 19: 644-647.

Prober, D.A., Rihel, J., Onah, A.A., Sung, R.J. & Schier, A.F. (2006). Hypocretin/orexin overexpression induces an insomnia-like phenotype in zebrafish. — J. Neurosci. 26: 13400-13410.

Prut, L. & Belzung, C. (2003). The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. — Eur. J. Pharmacol. 463: 3-33.

Qian, H., Zhu, Y., Ramsey, D.J., Chappell, R.L., Dowling, J.E. & Ripps, H. (2005a). The optokinetic response of larval zebrafish. — Invest. Ophthalmol. Vis. Sci. 46: e-abstract 5661.

Qian, H.H., Zhu, Y., Ramsey, D.J., Chappell, R.L., Dowling, J.E. & Ripps, H. (2005b). Directional asymmetries in the optokinetic response of larval zebrafish (*Danio rerio*). — Zebrafish 2: 189-196.

Rana, N., Moond, M., Marthi, A., Bapatla, S., Sarvepalli, T., Chatti, K. & Challa, A.K. (2010). Caffeine-induced effects on heart rate in zebrafish embryos and possible mechanisms of action: an effective system for experiments in chemical biology. — Zebrafish 7: 69-81.

Rankin, C.H., Abrams, T., Barry, R.J., Bhatnagar, S., Clayton, D.F., Colombo, J., Coppola, G., Geyer, M.A., Glanzman, D.L., Marsland, S., McSweeney, F.K., Wilson, D.A., Wu, C.F. & Thompson, R.F. (2009). Habituation revisited: an updated and revised description of the behavioral characteristics of habituation. — Neurobiol. Learn. Mem. 92: 135-138.

Richards, F.M., Alderton, W.K., Kimber, G.M., Liu, Z., Strang, I., Redfern, W.S., Valentin, J.P., Winter, M.J. & Hutchinson, T.H. (2008). Validation of the use of zebrafish larvae in visual safety assessment. — J. Pharmacol. Toxicol. Methods 58: 50-58.

Rihel, J., Prober, D.A., Arvanites, A., Lam, K., Zimmerman, S., Jang, S., Haggarty, S.J., Kokel, D., Rubin, L.L., Peterson, R.T. & Schier, A.F. (2010). Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. — Science 327: 348-351.

Rinner, O., Rick, J.M. & Neuhauss, S.C.F. (2005). Contrast sensitivity, spatial and temporal tuning of the larval zebrafish optokinetic response. — Invest. Ophthalmol. Vis. Sci. 46: 137-142.

Roberts, A.C., Reichl, J., Song, M.Y., Dearinger, A.D., Moridzadeh, N., Lu, E.D., Pearce, K., Esdin, J. & Glanzman, D.L. (2011). Habituation of the C-start response in larval zebrafish exhibits several distinct phases and sensitivity to NMDA receptor blockade. — PLoS One 6: e29132.

Roeser, T. & Baier, H. (2003). Visuomotor behaviors in larval zebrafish after GFP-guided laser ablation of the optic tectum. — J. Neurosci. 23: 3726-3734.

Richendrfer, H., Pelkowski, S.D., Colwill, R.M. & Creton, R. (2012). On the edge: pharmacological evidence for anxiety-related behavior in zebrafish larvae. — Behav. Brain Res. 228: 99-106.

Roy, M.N., Prince, V.E. & Ho, R.K. (1999). Heat shock produces periodic somitic disturbances in the zebrafish embryo. — Mech. Dev. 85: 27-34.

Saili, K.S., Corvi, M.M., Weber, D.N., Patel, A.U., Das, S.R., Przybyla, J., Anderson, K.A. & Tanguay, R.L. (2012). Neurodevelopmental low-dose bisphenol A exposure leads to early life-stage hyperactivity and learning deficits in adult zebrafish. — Toxicology 291: 83-92.

Saint-Amant, L. & Drapeau, P. (1998). Time course of the development of motor behaviors in the zebrafish embryo. — J. Neurobiol. 37: 622-632.

Schaaf, M.J.M., Champagne, D., van Laanen, I.H.C., van Wijk, D.C.W.A., Meijer, A.H., Meijer, O.C., Spaink, H.P. & Richardson, M.K. (2008). Discovery of a functional glucocorticoid receptor beta-isoform in zebrafish. — Endocrinology 149: 1591-1599.

Scheil, V. & Kohler, H.R. (2009). Influence of nickel chloride, chlorpyrifos, and imidacloprid in combination with different temperatures on the embryogenesis of the zebrafish *Danio rerio*. — Arch. Envir. Contamin. Toxicol. 56: 238-243.

Scheil, V., Kienle, C., Osterauer, R., Gerhardt, A. & Kohler, H.R. (2009). Effects of 3,4-dichloroaniline and diazinon on different biological organisation levels of zebrafish (*Danio rerio*) embryos and larvae. — Ecotoxicology 18: 355-363.

Schiller, P.H., Sandell, J.H. & Maunsell, J.H. (1986). Functions of the ON and OFF channels of the visual system. — Nature 322: 824-825.

Schnorr, S.J., Steenbergen, P.J., Richardson, M.K. & Champagne, D.L. (2012). Measuring thigmotaxis in larval zebrafish. — Behav. Brain Res. 228: 367-374.

Scholz, E.P., Niemer, N., Hassel, D., Zitron, E., Burgers, H.F., Bloehs, R., Seyler, C., Scherer, D., Thomas, D., Kathofer, S., Katus, H.A., Rottbauer, W.A. & Karle, C.A. (2009). Biophysical properties of zebrafish ether-a-go-go related gene potassium channels. — Biochem. Biophys. Res. Commun. 381: 159-164.

Selderslaghs, I.W., Van Rompay, A.R., De Coen, W. & Witters, H.E. (2009). Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo. — Reproduct. Toxicol. 28: 308-320.

Selderslaghs, I.W., Hooyberghs, J., De Coen, W. & Witters, H.E. (2010). Locomotor activity in zebrafish embryos: a new method to assess developmental neurotoxicity. — Neurotoxicol. Teratol. 32: 460-471.

Serra, E.L., Medalha, C.C. & Mattioli, R. (1999). Natural preference of zebrafish (*Danio rerio*) for a dark environment. — Braz. J. Med. Biol. Res. 32: 1551-1553.

Shin, J.T. & Fishman, M.C. (2002). From zebrafish to human: modular medical models. — Annu. Rev. Genom. Hum. Genet. 3: 311-340.

Shiraki, T., Kojima, D. & Fukada, Y. (2010). Light-induced body color change in developing zebrafish. — Photochem. Photobiol. Sci. 9: 1498-1504.

Spence, R. & Smith, C. (2008). Innate and learned colour preference in the zebrafish *Danio rerio*. — Ethology 114: 582-588.

Spence, R., Gerlach, G., Lawrence, C. & Smith, C. (2008). The behaviour and ecology of the zebrafish *Danio rerio*. — Biol. Rev. 83: 13-34.

Springer, A.D., Easter Jr., S.S. & Agranoff, B.W. (1977). The role of the optic tectum in various visually mediated behaviors of goldfish. — Brain Res. 128: 393-404.

Steele, S.L., Yang, X., Debiais-Thibaud, M., Schwerte, T., Pelster, B., Ekker, M., Tiberi, M. & Perry, S.F. (2011). In vivo and in vitro assessment of cardiac beta-adrenergic receptors in larval zebrafish (*Danio rerio*). — J. Exp. Biol. 214: 1445-1457.

Steenbergen, P.J., Richardson, M.K. & Champagne, D.L. (2011). Patterns of avoidance behaviours in the light/dark preference test in young juvenile zebrafish: a pharmacological study. — Behav. Brain Res. 222: 15-25.

Stehr, C.M., Linbo, T.L., Incardona, J.P. & Scholz, N.L. (2006). The developmental neurotoxicity of fipronil: notochord degeneration and locomotor defects in zebrafish embryos and larvae. — Toxicol. Sci. 92: 270-278.

Stewart, A.M., Gaikwad, S., Kyzar, E. & Kalueff, A.V. (2012). Understanding spatiotemporal strategies of adult zebrafish exploration in the open field test. — Brain Res. 1451: 44-52.

Thinusblanc, C. & Foreman, N. (1993). A rapid test of rodents vision using a modified openfield apparatus. — Phys. Behav. 53: 1003-1006.

Thirumalai, V. & Cline, H.T. (2008). Endogenous dopamine suppresses initiation of swimming in prefeeding zebrafish larvae. — J. Neurophysiol. 100: 1635-1648.

Thompson, R.F. & Spencer, W.A. (1966). Habituation: a model phenomenon for the study of neuronal substrates of behavior. — Psychol. Rev. 73: 16-43.

Ton, C., Lin, Y. & Willett, C. (2006). Zebrafish as a model for developmental neurotoxicity testing. — Birth Defects Res. A: Clin. Mol. Teratol. 76: 553-567.

Treit, D. & Fundytus, M. (1988). Thigmotaxis as a test for anxiolytic activity in rats. — Pharmacol. Biochem. Behav. 31: 959-962.

Tsay, H.J., Wang, Y.H., Chen, W.L., Huang, M.Y. & Chen, Y.H. (2007). Treatment with sodium benzoate leads to malformation of zebrafish larvae. — Neurotoxicol. Teratol. 29: 562-569.

Valente, A., Huang, K.H., Portugues, R. & Engert, F. (2012). Ontogeny of classical and operant learning behaviors in zebrafish. — Learn. Mem. 19: 170-177.

Watkins, J., Miklosi, A. & Andrew, R.J. (2004). Early asymmetries in the behaviour of zebrafish larvae. — Behav. Brain Res. 151: 177-183.

Webb, P.W. & Weihs, D. (1986). Functional locomotor morphology of early life-history stages of fishes. — Trans. Am. Fish. Soc. 115: 115-127.

Whitfield, T.T., Riley, B.B., Chiang, M.Y. & Phillips, B. (2002). Development of the zebrafish inner ear. — Dev. Dyn. 223: 427-458.

Wielhouwer, E.M., Ali, S., Al-Afandi, A., Blom, M.T., Riekerink, M.B.O., Poelma, C., Westerweel, J., Oonk, J., Vrouwe, E.X., Buesink, W., van Mil, H.G.J., Chicken, J., van 't Oever, R. & Richardson, M.K. (2011). Zebrafish embryo development in a microfluidic flow-through system. — Lab. Chip 11: 1815-1824.

Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., Goodspeed, J., Suciu, C., Tan, J., Grimes, C., Chung, A., Rosenberg, M., Gaikwad, S., Denmark, A., Jackson, A., Kadri, F., Chung, K.M., Stewart, A., Gilder, T., Beeson, E., Zapolsky, I., Wu, N., Cachat, J. & Kalueff, A.V. (2010). Analyzing habituation responses to novelty in zebrafish (*Danio rerio*). — Behav. Brain Res. 208: 450-457.

Wood, D.C. (1969). Parametric studies of the response decrement produced by mechanical stimuli in the protozoan, *Stentor coeruleus*. — J. Neurobiol. 1: 345-360.

Yu, F., Zhao, Y., Gu, J., Quigley, K.L., Chi, N.C., Tai, Y.C. & Hsiai, T.K. (2012). Flexible microelectrode arrays to interface epicardial electrical signals with intracardial calcium transients in zebrafish hearts. — Biomed. Microdev. 14: 357-366.

Zeddies, D.G. & Fay, R.R. (2005). Development of the acoustically evoked behavioral response in zebrafish to pure tones. — J. Exp. Biol. 208: 1363-1372.

Zellner, D., Padnos, B., Hunter, D.L., MacPhail, R.C. & Padilla, S. (2011). Rearing conditions differentially affect the locomotor behavior of larval — zebrafish but not their response to valproate-induced developmental neurotoxicity. — Neurotoxicol. Teratol. 33: 674-679.

Zhdanova, I.V., Wang, S.Y., Leclair, O.U. & Danilova, N.P. (2001). Melatonin promotes sleep-like state in zebrafish. — Brain Res. 903: 263-268.