Assessment of developmental delay in the zebrafish embryo teratogenicity assay.

Authors: Teixidó, E., Piqué, E.*, Gómez-Catalán, J., Llobet, J.M.

GRET-CERETOX and Toxicology Unit, Public Health Department, School of Pharmacy, University of Barcelona. Av. Joan XXIII s/n. 08028 Barcelona, SPAIN.

***Corresponding author:** Ester Piqué

E-mail: esterpique@ub.edu

Phone and FAX: +34.93.403.58.16

Toxicology Unit, School of Pharmacy

University of Barcelona

Av. Joan XXIII s/n

E- 08028 Barcelona

SPAIN

ABSTRACT

In this study we analyzed some aspects of the assessment of developmental delay in the zebrafish embryotoxicity/teratogenicity test and explored the suitability of acetylcholinesterase (AChE) activity as a biochemical marker and as a higher throughput alternative to morphological endpoints such as head-trunk angle, tail length and morphological score. Embryos were exposed from 4 to 52 hours post-fertilization (hpf) to a selection of known embryotoxic/teratogen compounds (valproic acid, retinoic acid, caffeine, sodium salicylate, glucose, hydroxyurea, methoxyacetic acid, boric acid and paraoxon-methyl) over a concentration range. They were evaluated for AChE activity, head-trunk angle, tail length and several qualitative parameters integrated in a morphological score. In general, the different patterns of the concentration-response curves allowed distinguishing between chemicals that produced growth retardation (valproic and methoxyacetic acid) and chemicals that produced non-growth-delay related malformations. An acceptable correlation between the morphological score, AChE activity and head-trunk angle as markers of developmental delay was observed, being AChE activity particularly sensitive to detect delay in the absence of malformations.

Keywords (5): Acetylcholinesterase, zebrafish embryo, developmental delay, teratogenicity assay.

Abbreviations:

AChE: acethylcolinesterase

DMSO: Dimethyl sulfoxide

TMS: Total morphological score

WEC: Whole Embryo Culture

DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid)

hpf: hours post-fertilization

1. Introduction

Animal testing will increase dramatically over next decade as a consequence of implementation of the new EU regulation for the Registration, Evaluation and Authorization of Chemicals, REACH (Pedersen et al., 2003; Van der Jagt et al., 2004). One of the toxic responses that must be evaluated is the effect on development, principally teratogenesis. Reproductive and developmental toxicity studies will use by far the most animals and resources within REACH (particularly 23% of animals and 32% of resources in developmental studies). Thus, the introduction of valid alternatives and research into new alternative methods for developmental toxicity testing is specifically urgent in order to reduce the number of animals used (Piersma, 2006). Furthermore, medium-to-high throughput assays of developmental toxicity would be valuable during the screening phase of new drugs research.

In the last decade assays using embryonic stages of the vertebrate zebrafish (Danio rerio) have attracted the attention of toxicologists due to their several advantages. In particular, fish embryos are considered as non-protected life stages and –similar to in vitro assays- an alternative to animal testing (EU directive 2010/63/EU), although they use whole organisms. The fish embryo test with zebrafish (FET) has been suggested as replacement of the acute tests performed with juveniles or adults (Braunbeck et al., 2005) and a draft for an OECD guideline is currently under review (OECD, 2006). Approval of this OECD guideline would prompt the application of the fish embryo test for the chemical safety evaluation on an international scale. The FET is also employed for effluent testing in different countries replacing adult fish tests (DIN, 2001). Analysis of acute toxicity in embryos can also include the screening for developmental disorders as an indicator of teratogenic effects.

Zebrafish are easy to maintain and produce large numbers of embryos that develop outside the mother. The transparency of their embryos allows the scoring of teratological and embryotoxic effects easily. In addition, the development is fast and has been well characterized, including morphological, biochemical and physiological information at all stages of early development (Hill et al., 2005). The development process is highly conserved across vertebrates and the zebrafish genome is completely characterized. Hence, zebrafish embryos represent an attractive model allowing reduction and refinement of animal use in research (Yang et al., 2009).

Numerous studies have been reported exploring the capacity of zebrafish assays for the assessment of the teratogenic potential of chemicals showing a good concordance with in vivo results in mammals (Brannen et al., 2010; Hermsen et al., 2011; Nagel, 2002; Selderslaghs et al., 2009; Van den Bulck et al., 2011). They focused on three manifestations of deviant development: death, malformation and growth retardation (Wilson and Fraser 1977). However, they applied different experimental protocols and the number and the variety of assayed substances were limited. Currently there is no consensus about the optimal procedure in some basic features as the specific endpoints and scoring systems to use, the time of exposure and the stage of embryonic/larval development to do the observations.

General retardation of development is a phenomenon often observed in teratogen-exposed embryos (Liang et al., 2010; van den Brandhof and Montforts, 2010). Developmental delay is usually considered as a reversible and unspecific effect. However, it might lead to persistent delays or deficits in function (Daston et al., 2010) and permit teratogens to act for a longer time during sensitive stages, and thus intensify the severity of the produced anomalies (Weis and Weis, 1987). Some studies have aimed to distinguish between growth retardation and other developmental effects in the zebrafish assays. Nagel (2002) considered that “growth-retardation” at 24 or 48 hpf and tail length at 120 hpf were “teratogenic endpoints”, whereas defects on development of somites, eyes or blood circulation were “development endpoints”. Brannen et al (2010) proposed an extensive scoring system that takes into account the severity of the effects, but those endpoints more related to growth retardation were finally eliminated because they were non-discriminating or not cost-effective. More recently, Van den Bulck et al (2011) considered “growth retardation” as a teratogenic endpoint. However, Hermsen et al (2011) split the assessment in two endpoint categories and two respective scores, a general morphology score and a teratogenic score, suggesting that the first score gives a “semi-quantitative assessment of (mal)development”.

For the purpose of attain a better characterization of the developmental retardation in the zebrafish embryo assays we delineated a scoring system based on some qualitative morphological features characterizing the zebrafish stages described by Kimmel and co-workers (1995) during the 52 hpf. This score has a similar design as the scoring system developed for mammalian whole embryo culture assays (WEC) by Klug and co-workers (1985). Furthermore, we measured the length of the tail and head-trunk angle as quantitative morphological markers of development (Bachmann, 2002; Kimmel et al., 1995). Finally, we determined the acetylcholinesterase (AChE) activity in whole 52hpf embryos. In zebrafish, AChE is expressed in a variety of

tissues, including non-cholinergic cells (Hanneman and Westerfield 1989). Its expression starts early before synapse formation (Layer, 1990) and increases with age along the embryo development (Behra et al., 2002; Bertrand et al., 2001). Therefore, AChE activity was a reasonable candidate as a sensitive biochemical marker of developmental delay with a medium-high throughput potential. Embryos were exposed to a selection of eight compounds characterized by diverse known embryotoxic/teratogen activities (Table 1). Most of these compounds belong to the set of chemicals tested in the ECVAM international study of validation on the three in vitro embryotoxicity tests namely embryonic stem cells test, limb bud micromass test and post-implantation whole-embryo culture test (Genschow et al., 2004). The concentration-response curves obtained for the different endpoints were analyzed and compared in order to assess their relative performance as markers of developmental delay and in connection with the teratogenic effects. In addition, an irreversible AChE inhibitor was tested so as to compare AChE inhibitors and compounds that decrease AChE activity since they induce developmental delay.

2. Materials and methods

2.1. Chemicals and test media

All the selected chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Danieau's buffer (58 mM NaCl; 0.7 mM KCl; 0.4 mM $MgSO_4 \cdot 7\ H_2O$; 0.6 mM $Ca(NO_3)_2$; 5 mM HEPES; pH 7.4) was used as the medium for all solutions during the experiments to keep the pH stable and constant between assays due to the different pKa of the chemicals tested.

2.2. Zebrafish maintenance and egg production

Adult female and male zebrafish were obtained from a commercial supplier (Pisciber, Barcelona) and housed separately in a closed flow-through system in standardized dilution water as specified in ISO 7346-1 and 7346-2 (ISO, 1996; 2 mM $CaCl_2 \cdot 2\ H_2O$; 0.5 mM $MgSO_4 \cdot 7\ H_2O$; 0.75 mM $NaHCO_3$; 0.07 mM KCl). Fish were maintained at 26±1 ºC on a 14-h light and 10-h dark cycle and were fed with commercial dry flake food and live brine shrimp. The day before eggs were required, males and females were placed in breeding tanks (Aquaneering, San Diego, California) with a 2:1 male:female ratio. On the next morning, the eggs could be collected 30 minutes after the light had been turned on. Eggs were collected and successively cleaned with dilution water corresponding to the reconstituted water according to ISO-standard 7346, which was diluted 1:5 using deionized water.

2.3. Embryo exposure

All stock solutions were prepared with Danieau's buffer except retinoic acid that was initially prepared in 100% dimethylsulfoxide (DMSO) and subsequently diluted in Danieau's buffer with a final DMSO concentration of 0.05% (v/v). For all substances, the tests were carried out using five concentrations with a negative control, test medium only or solvent control with 0.05% of DMSO. The tested concentrations covered the range between those producing non-abnormal development and those producing high indices of teratogenesis as determined in at least one previous range-finding assay and/or lethality assay (data not shown).

After egg collection, fertilization success was checked and only batches of eggs with at least a fertilization rate of 80% were used. Fertilized eggs were exposed at 4 hours post-fertilization (hpf) to test media in a 6-well culture plate (Greiner Bio-one, Germany). Ten embryos were randomly distributed into wells and filled with 5 ml of each solution. Each 6-well plate held five different concentrations of the test compound and the negative or solvent control. Embryos were incubated at 27 ± 1ºC on a 14-h light and 10-h dark cycle for 48 hours. The exposure was semi-static and solutions were renewed at 28 hpf.

At 8, 28 and 52 hpf, mortality of embryos was checked using a stereomicroscope (SMZ-168, Motic). According to Nagel (2002) four lethal endpoints were determined which are coagulation of eggs, non-development of somites, non-detachment of the tail and no presence of heartbeat. At 52 hpf embryos were evaluated for developmental effects and acetylcholinesterase activity.

For each substance and concentration, ten fertilized eggs were exposed. Six independent replications were done, using eggs from different spawning events. AChE activity was measured in pool homogenates of each of the 6 replicates. Morphological parameters were evaluated in at least 4 of the replicates.

2.4. Evaluation of developmental effects

2.4.1. Total morphological score (TMS)

We developed a scoring system based on zebrafish development described by Kimmel and co-workers (1995) in order to compute morphological differentiation of embryos at around 52 hpf. This score has a similar design as the scoring system developed for mammalian whole embryo culture assays (WEC) by Klug and co-workers (1985). We selected nine morphological features based in three developmental stages of zebrafish (segmentation period, pharyngula period and hatching period). These nine features were chosen

because they show a clear change between developmental stages and they are easy to observe (Table 2). Each feature was examined in an individual embryo and assigned the appropriate score. A score of 4 was given for features developed as in hatching period, score 3 was given for features that reach developmental stage of pharyngula period and score 2 to features showing a developmental stage as in segmentation period. Finally, score 1 was attributed when features showed a malformation. If the stage of development of a feature was between two defined stages, a half mark was assigned. The numerical total of scores for all nine features is the morphological score for the embryo (a maximum score of 36).. The frequency of embryos in each concentration and control group presenting scores of 35 or less, 34 or less, 33 or less, 32 or less and 31 or less (namely -1, -2, -3, -4 and -5 or more points less than the total score) were calculated and represented as a set of concentration-response curves. Also, the fraction of abnormal embryos (defined as the embryos with some score 1 in any morphological feature) was determined for each concentration and control group and represented together with the morphological score. Scoring of embryos was supervised by one of the authors (E.T.) and any dubious specimen was photographed and reevaluated.

2.4.2. Tail length and head-trunk angle

Embryos that have not yet hatched were dechorionated and all embryos were anesthetized with buffered tricaine methanesulfonate (0.5 mM, Sigma-Aldrich, St. Louis, MO) and photographed (Moticam 2000, Motic) positioned on their lateral side. The distance between the anus and the posterior end of the notochord was defined as tail length (Bachmann, 2002).

The head-trunk angle was measured between a line drawn through the middle of the ear and the eye and a line parallel to the notochord. The resulting angle was subtracted from 180º to express head-trunk angle as described in developmental stages (Kimmel et al., 1995).

All measurements were performed with ImageJ 1.41(http://rsb.info.nih.gov/ij/).

2.5. Acethylcholinesterase (AChE) activity determination

A total of six samples per concentration were analyzed. Each sample was composed of a pool of 10 embryos. The analysis of AChE activity was adapted from Küster (2005). Briefly, the pool of 10 embryos was homogenized in 0.5 ml ice-cold sodium phosphate buffer (0.1 M, pH 7.4, and 0.1% v/v Triton X-100). Homogenization was done for 3x 10s using a Pellet Pestle® Kontes. The homogenates were centrifuged at 4ºC for 15 min at 12,600 rpm. Supernatants were removed and stored at -20ºC until analysis. The

191 determination was carried out in triplicate per sample at 22ºC according to the spectrophotometric method of
192 Ellman (Ellman et al., 1961), adapted to microtitre plates. Kinetic measurement of the optical density change
193 with time (OD/min) was recorded at 405 nm for 5 min. The final concentration of the chromogenic reagent,
194 DTNB, and the substrate, acetylthiocholine-iodide, in the mixture was 0.3 mM and 0.45 mM respectively. The
195 specific enzyme activity was expressed as nmols of substrate hydrolyzed per minute and per mg of protein.
196 Protein concentration of the samples was determined in triplicate at 695 nm using a commercial kit
197 (QuantiPro BCA assay kit, Sigma-Aldrich, St. Louis, MO).
198 Age-dependent AChE activity characterization between 24 and 52 hpf was performed using embryos
199 exposed to Danieau's buffer alone.
200
201 Additionally, an assay of the in vitro AChE inhibitory activity for each substance was done in order to discard
202 a direct inhibitory action (Results in the supplementary file). Total homogenates of ten unexposed fish
203 embryos (52 hpf) were incubated directly for 30 minutes at 30ºC with the highest concentration of each
204 substance tested in the in vivo AChE activity determination. The assay was carried out in triplicate per
205 substance at 22ºC according to the spectrophotometric method of Ellman (Ellman et al., 1961) as described
206 previously.
207
208 2.6. Statistical analysis
209 Concentration-response curves were fitted to all the data using PROAST software (Slob, 2002). The final
210 model, for the concentration-response curves for head-trunk angle, tail length and AChE activity, was
211 selected based on the goodness of fit and the presence of the lowest number of parameters in the equation.
212 For the dichotomous data (teratogenic and lethal effects) a log-logistic model was fitted. The concentration–
213 response curves generated were required to determine EC_{50} (teratogenic effects) and LC_{50} (lethal effects)
214 values. Based on LC_{50} and EC_{50} (teratogenic effects) values, a teratogenic index (TI) was calculated as the
215 ratio LC_{50}/EC_{50}.
216
217 Statistical analysis was performed with SPSS 15.0. One-way analysis of variance (ANOVA) followed by post
218 hoc multi-comparison with the Bonferroni's test was used to analyze homogeneous data of the continuous
219 variables. Kruskal-Wallis test was used to analyze non-homogeneous data. The frequency of abnormal
220 embryos was evaluated with Fisher's exact test. Significance was accepted when $p < 0.05$.

3. Results

The age-dependent AChE activity of zebrafish embryos from Prim-5 to the Long Pec stage (from 24 to 52 hpf) has been characterized. AChE activity increased from about 2 to 43 nmol/min/mg protein (Fig. 1), showing a negative correlation with head-trunk angle (r2 = -0.96).

Zebrafish embryos exhibited diverse and specific morphological abnormalities after exposure to each substance. The incidence of abnormal embryos (fraction of embryos with some teratogenic effect) and retarded embryos (fraction of embryos with some retardation as measured by the applied morphological score) were concentration dependent in all compounds except glucose. The defects described below for each compound were observed in all or most of the embryos at high concentrations. Table 1 shows the LC_{50} (for lethal effects) and EC_{50} (for teratogenic effects), derived from the concentration response curves, and the TI values. All the concentration data are nominal values.

Valproic acid was tested in the range from 0.05 to 0.6 mM. The main teratogenic effects observed were pericardial edema and tail necrosis. Fig. 2.A shows the concentration-response curves for morphological score performed from the fraction of embryos showing -1, -2, -3, -4 and -5 points less on the total morphological score. For valproic acid, there was a clear shift between the concentration-response curves suggesting that a progressive effect on the development occurs at doses that did not produce malformations. Among all the morphological features, pigmentation was the most affected. A significant decrease of AChE activity was reached at the first concentration (Fig. 2.C). Similarly, head-trunk angle significantly increased from 0.05 mM (Fig. 2.B) whereas for tail length a significant decrease was observed from 0.3 mM (Fig. 2.D). Moreover, all three parameters showed a significant concentration-response relationship.

The concentration range of retinoic acid tested was between 0.5 nM and 10 nM. Embryos exposed to retinoic acid showed a characteristic kink in the end of the tail among other abnormalities like pericardial edema. Concentration-response curves for morphological score shift to the right (higher concentrations) crossing the concentration-response curve for teratogenic effects (Fig. 3.A). Therefore, developmental retardation was observed but at doses that produced a high incidence of malformations. AChE activity was not significantly influenced after retinoic acid exposure (Fig. 3.C), while head-trunk angle showed a statistical

significance from 1 nM (Fig. 3.B). Due to the serious malformations presented by embryos exposed to 10 nM of retinoic acid, tail length and head-trunk angle were not measured in this concentration. Tail length was significantly decreased in embryos exposed to retinoic acid at 5 nM.

For caffeine, tested concentrations were between 0.1 mM and 2.5 mM. The most observed abnormalities were head, heart and tail malformations. Concentration-response curve for morphological score and teratogenic effects showed almost no shift (Fig. 3.A). Tail length and head-trunk angle were not significantly influenced (Fig. 3.B and D), however they could not be measured in embryos exposed to 1.5 mM and 2.5 mM of caffeine due to the gross morphological abnormalities. Only the highest concentrations (1.5 mM and 2.5 mM) showed a significant decrease in AChE activity (Fig. 3.C).

The concentrations of glucose tested were between 1 mM and 100 mM. All embryos exposed to glucose showed a normal development without growth retardation or teratogenic effects (Total morphological score data not show). Glucose also had no significant effect on tail length (Fig. 4.C). Only the highest concentration tested (100 mM) resulted in a significantly decreased AChE activity (Fig. 4.B) in concordance with a significantly increased head-trunk angle (Fig. 4.A).

Sodium salicylate was tested in the range between 3 mM and 16 mM. The characteristic effects of the treated groups were intracranial hemorrhage, the yolk sac and its extension structure changing from transparent to opaque brown and swimming disorders. Concentration-response curve for teratogenic effects (Fig. 3.A) was fitted without considering yolk sac abnormalities as a teratogenic effect. The concentration-response curves for morphological score showed a shift to the right relative to the curve for teratogenic effects. Sodium salicylate had no significant effect on AChE activity (Fig. 3.C) and only the highest concentration (16 mM) showed a significant higher head-trunk angle (Fig. 3.B). Tail length was not influenced after sodium salicylate treatment (Fig. 3.D).

The concentrations tested for hydroxyurea were from 2 mM to 32 mM. Treated embryos showed tail malformations and pericardial edema. The concentration-response curves for morphological score showed some incidence of less pigmented embryos at low concentrations. The slopes were low and the curves were crossed by the steepest curve for teratogenic effects at high concentration (Fig. 2.A). A significant difference

was only observed in AChE activity at the highest concentration tested (Fig. 2.C and D).

For methoxyacetic acid, tested concentrations ranged from 2 mM to 16 mM. Abnormal embryos showed pericardial edema, eye malformation and tail necrosis. As shown in Fig. 2.A the concentration-response curves showed a clear and gradual shift between the morphological assessment and teratogenic effects. The most characteristic developmental effect observed was the non-looping of the heart in the embryos demonstrated by the presence of tube heart. AChE activity showed a significant decrease from the first concentration (Fig. 2.C). Moreover, there was a significant concentration-response relationship between methoxyacetic acid exposure and AChE activity. Comparable concentration-response curves for head-trunk angle and tail length were observed (Fig. 2.B and D).

Boric acid was tested at concentrations between 7 mM and 35.5 mM. Treated embryos showed mostly tail malformations and pericardial and yolk sac edema. Concentration-response curves showed almost no shift between the morphological assessment and teratogenic effects. Head-trunk angle increased significantly from 15.8 mM (Fig. 4.B), but AChE activity only decreased significantly at 35.5 mM (Fig.4.C). Tail length showed a significant decrease from 23.7 mM (Fig. 4.D).

Paraoxon-methyl was tested in the range from 1 µM to 20 µM. Embryos did not show any developmental delay, but had abnormalities on the chorda-structure and spasms. Concentration-response curve for teratogenic effects (Fig. 4.A) was fitted based on these alterations. A significant decrease in head-trunk angle was observed (Fig. 4.B) due to the spinal curvature produced by spasms. Tail length only decreased significantly from 10 µM (Fig. 4.D) and AChE activity was significantly decreased from 5 µM and reached almost zero at the highest concentration (Fig. 4.C).

Correlation analysis was done between AChE activity and the two morphometric endpoints, head-trunk angle and tail length (Fig. 5) using the EC_{10} values for each compound (Table 4, supplementary file). LC_{50} was used in those cases where an EC_{10} value could not be calculated because the endpoint did not change after chemical exposure (AChE activity for retinoic acid and tail length for sodium salicylate). Paraoxon-methyl exposure produced a decrease in the head-trunk angle instead of an increase as a result of the body curvature caused by spasms. Therefore, LC_{50} value was used in the correlation analysis.

Both developmental endpoints showed a moderate correlation with AchE activity with an R^2 of 0.75 for head-trunk angle and 0.79 for tail length. Slope values were about 0.81 for head-trunk angle and 0.88 for tail length.

4. Discussion

Numerous studies have been reported that explored the suitability of zebrafish embryo assays to predict the teratogenic potency of chemical substances in mammals (Van den Bulck et al., 2011). Results are promising but some additional effort is necessary both in the standardization of the methodology and in the extension and diversity of the compound database analyzed. These studies have assessed a wide diversity of endpoints, mainly morphological, at different times of development, from 24 hours to 5 days post-fertilization. These endpoints have been integrated in some semi-quantitative scores in order to characterize the concentration-effect relationship. Data is fitted by a curve and some teratogenic concentration is computed (e.g. the teratogen EC_{50}). In addition, some teratogenicity index has to be calculated as a ratio by comparison with some non-specific embryotoxic effect of the substance as lethality (e.g. LC_{50}, NOAEC). These relative teratogenicity indices allows to normalize the teratogenic potency in relation to unspecific toxicity and also, to some extent, to compensate for the limited compound uptake by the fish (Van den Bulck et al., 2011). Therefore, these teratogenicity indices are the final results that can be compared with the available teratogenicity data in mammals. Evidently, the predictive capacity of these tests depends on how the teratogenicity indices are computed. The main variables are the endpoints considered and how they are integrated in some semi-quantitative score. A purely empirical methodology could be applied to optimize these variables if a wide enough set of substances is assayed and compared with the in vivo data. Brannen et al (2010) applies this approach to simplify an original battery of 23 endpoints and 31 tested substances discarding seven endpoints because low discriminating power. However, these empirical approaches would not produce a single optimal point since it would be dependent on where the desired midpoint of the sensitivity-specificity-throughput triangle were positioned.

While lacking a wide database, improving in the zebrafish embryotoxicity test is possible characterizing the dose relationships of some endpoints in a limited set of compounds. Our work aimed to improve the characterization of the endpoints related to developmental retardation. In the seminal study of Nagel (2002) describing the “Zebrafish Danio rerio Teratogenic Assay” (DarT), “growth retardation” was considered as a

teratogenic endpoint but no details were given about how it was evaluated, whereas a separate list of "development" endpoints were proposed (e.g. formation of somites, development of eyes, heart beat). These "development" endpoints were used by Bachmann (2002) to calculate an EC_{50} for developmental delay at 24h and 48h in some chemicals. Based in these endpoints, Busquet et al (2008) evaluated growth retardation considering different parameters such as the global size of the fish egg, the eye and the sacculi/otoliths position, the degree of pigmentation, the tail not detached and the frequency of spontaneous movements. On the other hand, some of these "development" endpoints were considered as teratogenic endpoints by other group that did not included any explicit marker of growth retardation (Selderslaghs et al., 2009). Brannen et al (2010) included several endpoints that could be considered as indicators of development delay but finally, some of them were discarded because low discriminating power. The way that growth/developmental retardation is evaluated and whether it is considered a teratogenic event or not, could modify the quantitative results of the assay. We have some expertise using WEC (Flick et al., 2009) and applying the scoring system developed by Klug et al (1985) that assigns numerical scores to readily observable developmental endpoints characteristic of different developmental stages in the rat/mouse embryo. This score allows a semi-quantitative assessment of the retardation degree and a neat discrimination of retardation versus malformation. Therefore, we have adapted the design of the WEC scoring system to zebrafish up to 52 hpf. Details are commented in the Materials and Methods section and in the Table 2. Recently, a study has been published that followed the same idea and defined a scoring system very similar to the presented by us but extended until 72 hpf (Hermsen et al., 2011). A concentration-effect curve can be drawn using the mean morphological score as the quantitative response (Hermsen et al., 2011). However, this curve shows a flat shape and a slight retardation is not apparent. This is a drawback of indices based on high scores for normal embryos because some individuals losing 1 or 2 points in a score of 36 points practically do not produce a perceptible deviation of the curve. In order to facilitate the discussion, the results have been represented in an alternative way as a set of curves corresponding to the fraction of embryos that have lost at least 1, 2, 3, 4 or 5 points on the total morphological score. This representation gives a better image of the gradual increment of the effects, in the form of a gradual shift between curves, and allows a direct comparison with the teratogenicity curve (Fig. 2.A and 3.A)

Curves for teratogenic effect (frequency of abnormal embryos) and for lethality (not shown) can be compared in order to compute the teratogenic index (TI) as the ratio LC_{50}/EC_{50} (Table 1). These values can not be

directly compared with those reported previously in other studies because of the multiple heterogeneities among the respective experimental protocols. However, the results for caffeine, valproic acid and retinoic acid present are comparable with those reported by Selderslaghs et al (2009) in the assay at 48 hpf. The LC_{50} and EC_{50} values of some compounds, for instance methoxyacetic acid and valproic acid, are not in accordance with these studies (Bachmann, 2002; Selderslaghs et al., 2009). These differences could be explained by the fact that they did not use buffered medium in the test solutions, so the EC_{50} value would depend on the pH of the solution. Furthermore, in these studies LC_{50} were calculated from the mortality only accounted for coagulated embryos.

In addition to the score, two quantitative morphological endpoints have been evaluated: head-trunk angle, a parameter described by Kimmel and co-workers (1995) to determine the developing stage of zebrafish between segmentation period and hatching period (20-70 hpf), and tail length. Head-trunk angle is a very low throughput morphometric endpoint, but seems to be appropriate for early embryo stages whereas tail length (or body length) could be more suitable to evaluate growth in older stages (after hatching) (Brannen et al., 2010). Head-trunk angle has also been a useful parameter in measuring developmental delay caused by disruption of thyroid hormones (Walpita et al., 2009).

Furthermore, we have explored the suitability of a biochemical endpoint, the activity of AChE, as a marker of developmental delay in zebrafish embryos. A biochemical endpoint could be more liable to automation and higher throughput than the morphological endpoints. The expression of AChE starts early, before synapse formation (Layer, 1990), and increases with age along the embryo development (Behra et al., 2002; Bertrand et al., 2001). Methods for AChE determination are easy, robust and sensitive. Results from non-exposed embryos showed a good correlation between AChE activity and head-trunk angle during development (Fig. 1), so AChE activity is developmental stage dependent. We hypothesized that any chemical-induced delay in development would imply a lower AChE activity compared to normally developed embryos and, conversely, that a low AChE activity would indicate a chemical-induced delay. Evidently, some exceptions can be foreseen to this hypothesis: any substance with some specific action on AChE expression or activity, as AChE inhibitors, could produce results unrelated to the developmental stage.

The analysis of our results provides an insight on the suitability of the studied endpoints as indicators of

developmental delay. In general, an acceptable degree of accordance among the concentration-effect curves for the morphological score, head-trunk angle, tail length and AChE activity was observed. A moderate correlation was demonstrated by the analysis of correlation between AChE activity and the morphometric endpoints for developmental delay (Fig. 5). The correlation coefficients increase from 0.75 to 0.98 for head-trunk angle, and from 0.79 to 0.93 for tail length if paraoxon-methyl, a compound that interferes directly with AChE activity, is removed from the data analysis.

The analysis of the respective dose-morphological score curves confirms that valproic acid and methoxyacetic acid exposure was characterized by a developmental delay at concentrations that did not induce malformations (Fig. 2.A), whereas boric acid caused also a clear developmental delay overlapped with the teratogenic effect.

AChE activity and head-trunk angle curves for hydroxyurea indicate that this compound produced only a small retardation effect as can be confirmed by the slight shift on the concentration-morphological score curves (Fig. 2.A). This developmental delay was overlapped and masked by a strong teratogenic effect. In the case of caffeine, it behaved as a teratogen that did not induce any significant developmental retardation at low non-teratogenic concentrations. The reduced AChE activity found at teratogenic concentration and without developmental delay could be caused by the weak AChE inhibitory activity of caffeine (Table 3, supplementary file).

Glucose exposure showed a slight change in morphometric endpoints and AChE activity, but did not produce any significant morphological alteration on embryos. These results were in accordance with previous studies reported, in which glucose was used as a negative control for the evaluation of the specific embryotoxic and teratogenic potential of chemicals (Hill et al., 2005). However, it has been recently demonstrated that zebrafish embryos exposed to glucose (25 mM) showed severe growth retardation and developmental delay with heart defects (Liang et al., 2010). The difference from our assay data could be attributed to the fact that in their study the medium was renewed every eight hours because a fast depletion of the glucose levels was observed.

In our study the morphological score curves, head-trunk angle and tail length for retinoic acid suggests that

induces developmental delay at teratogenic concentrations, whereas AChE activity did not seem to be a good marker of this developmental retardation. This lack of correlation could be explained by the fact that retinoic acid modulates AChE activity in neuronal maturation in vitro (Sidell et al., 1984). Salicylate produced intracranial bleeding and yolk sac injuries at concentrations that did not produce any significant developmental delay. In concordance, no effects were observed in AChE activity, tail length and head-trunk angle.

Paraoxon-methyl exposure, a known irreversible AChE inhibitor, produced a decrease in head-trunk angle, the contrary effect that is observed in the other compounds. This effect could be explained by the fact that paraoxon-methyl exposed embryos showed muscular spasm resulting in a body curvature. In this case the reduced AChE activity is unrelated to the developmental stage because of the direct inhibitory action of paraoxon-methyl. At high concentrations the inhibition raised nearly 100%. In contrast, in the cases of valproic acid and methoxyacetic acid, AChE activity decreased parallel to developmental retardation and did not reach extremely low levels.

In summary, developmental delay occurs, in a more or less pronounced degree in the zebrafish embryo exposed to teratogen compounds, but only in some cases, as valproic acid and methoxyacetic acid, the delay appears at non-teratogenic concentrations. The performance of AChE activity, head-trunk angle, tail length and the morphological score as markers of developmental delay was comparable. Specifically, for the two compounds producing retardation at non-teratogenic concentrations the four responses gave similar results, being AChE activity the most sensitive endpoint. Therefore, the morphological score system that we have implemented allows an easy and objective assessment of developmental delay and teratogenesis, that can be complemented with the determination of AChE activity, particularly in those cases in which developmental delay occurs at non teratogenic concentrations. Furthermore, it is worth noting that AChE determination is easy, automatable and relatively fast. In the case that an unknown compound is analysed, false positives can be obtained in the case of strong AChE inhibitors, but can be easily discarded by means of an in vitro assay.

Ours results do not allow concluding about the discriminating efficiency of developmental retardation endpoints in the zebrafish assay to predict teratogenic potential in mammals. Probably, for a major part of

substances, developmental delay is an unspecific effect unrelated or secondary to teratogenic activity and then including this effect would produce a loss of specificity. In the other side, inclusion of developmental delay assessment would increase the sensitivity of the assay for some specific compounds inducing developmental delay related to teratogenic effects.

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Tables

Table 1. Test substance – overview. LC_{50}, EC_{50} with confidence intervals. Slopes and TI values of the test substances. EC_{50} for sodium salicylate was calculated taking into account embryos with brain hemorrhages and swimming disorders. – No effect.

Test substance	Cas no.	Characterization	LC_{50} (mM)	Slope LC_{50}	EC_{50} teratogenic (mM)	Slope EC_{50}	TI (LC_{50}/EC_{50})

Valproic acid	1069-66-5	Antiepileptic drug	1.74 (1.68 – 1.75)	5.0×10^{-2}	0.52 (0.48 – 0.57)	0.15	3.3
Caffeine	58-08-2	Xanthine alkaloid, psychoactive substance	5.0 (4.8 – 5.2)	0.36	0.8 (0.7 – 1.0)	0.69	6.2
All-trans retinoic acid	302-79-4	Vitamin A metabolite	5.1×10^{-5} (4.99×10^{-5} – 5.71×10^{-5})	4.15×10^{-6}	1.9×10^{-6} (1.7×10^{-6} – 2.2×10^{-6})	1.96×10^{-6}	26.8
Methoxyacetic acid	625-45-6	Glycol ether alkoxy acid metabolite	32.8 (32.1 – 36.4)	2.68	11.3 (10.0 – 12.0)	3.54	2.9
Salicylic sodium salt	54-21-7	Aspirin metabolite	41.0 (39.2 – 47.1)	4.15	12.5 (11.7 – 13.4)	7.26	3.2
Hydroxyurea	127-07-1	Antineoplasic drug	42.6 (40.2 – 43.3)	3.09	31.6 (28.3 – 32.0)	2.03	1.3
Boric acid	10043-35-3	Antiseptic, insecticide, flame retardant	53.4 (52.7-57.3)	4.07	18.3 (16.7-20.0)	13.24	2.9
Paraoxon-methyl	950-35-6	AChE inhibitor	8.8×10^{-2} (8.4×10^{-2} – 9.2×10^{-2})	1.99×10^{-2}	1.0×10^{-2} (9.4×10^{-3} -1.08×10^{-2})	1.26×10^{-3}	8.8
D-(+)-glucose	50-99-7	Sugar	-	-	-	-	-

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Table 2. Zebrafish embryo morphological scoring system and criteria employed to evaluate development. Movement was evaluated after dechorionation of the embryo. OVL, otic vesicle length (Estimation of the number of additional otic vesicles that could fit between the eye and the otic vesicle).

Endpoints	Score			
	Abnormal 1	**Segmentation period 2**	**Pharyngula period 3**	**Hatching period 4**
Detachment of the tail	No tail, malformation of chorda or spinal cord	Very short tail or attached to yolk sac	Tail completely detached	Yolk extension retains a posterior end begins to taper, to take on a more conical appearance
Optic system	Abnormal pigmentation, asymmetric eyes.	Optic primordium has a prominent horizontal crease	Eye with the retina surrounding the lens	Eyes pigmented
Otic system	Formation of no, one or more than two otoliths per sacculus. Absence or abnormally shaped vesicles.	Placode has hollowed out into the otic vesicle	Embryos possess two sacculi (vesicles) each with two prominent otoliths (OVL>1)	Otic vesicle close to the eye (OVL<1)
Brain	Brain necrosis, hemorrhage	Not morphological subdivisions		Brain prominently sculptured
Heart	Pericardial edema, big heart, hemorrhage, abnormal chambers		Linear heart tube	S-shaped loop
Tail	Hemorrhage, tail necrosis, bent tail, bent or twisted tip tail		No tail blood circulation	Tail blood circulation
Head-body pigmentation	Abnormal pigmentation		Not pigmented	Pigmented
Tail pigmentation	Abnormal pigmentation		Not pigmented	Pigmented
Movement	Spasms, abnormal movements	Not movement at all	Side-to-side flexures, spontaneous myotomal contractions	Dechorionated embryos lie on their sides when at rest. A touch elicits a vigorous and rapid response

Figures

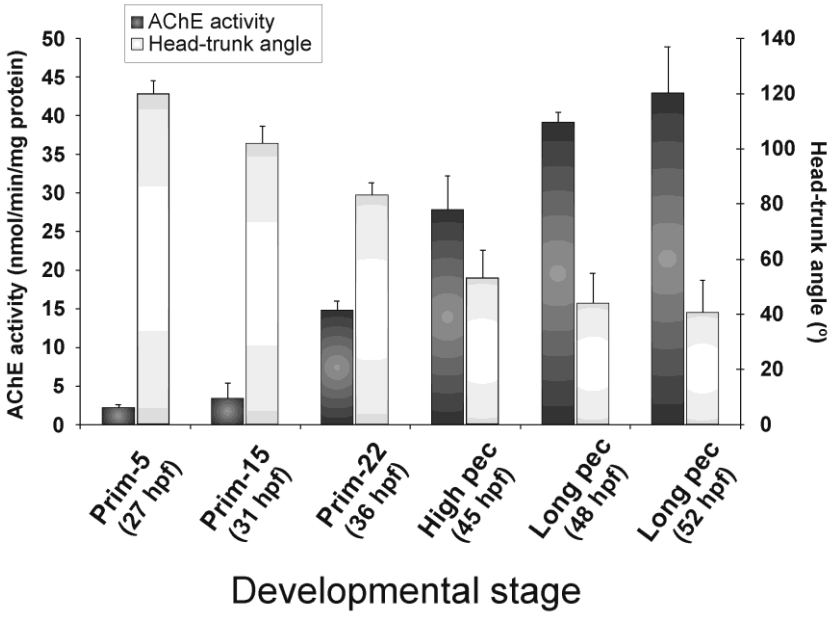


Fig. 1. AChE activity and head-trunk angle in the developing zebrafish embryo from Prim-5 to Long-pec stage. Values are mean ± SD of n= 10 embryos.

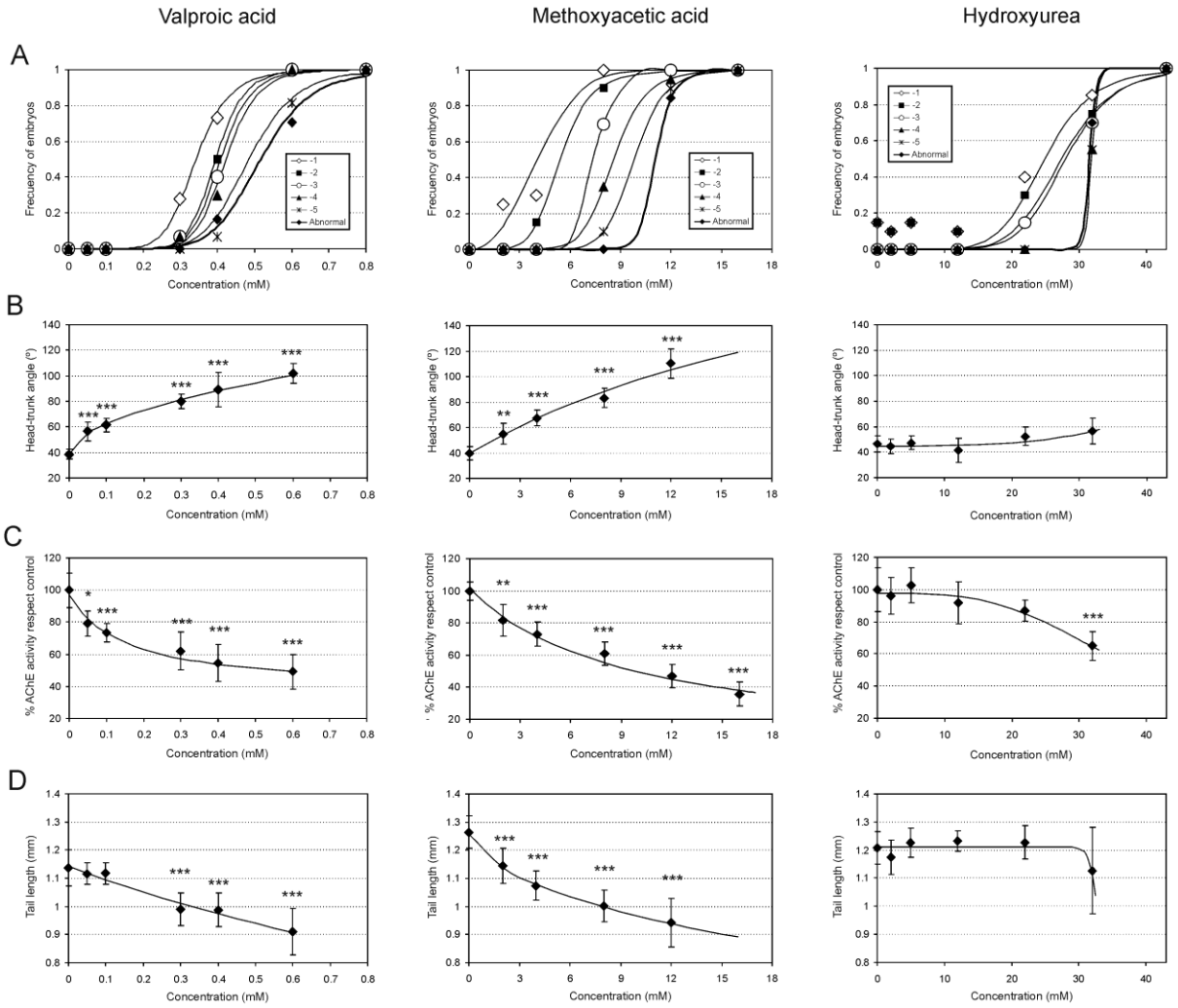


Fig. 2. Concentration-response curves for frequency of embryos with -1, -2, -3, -4 and -5 or more points less on the total morphological score and frequency of abnormal embryos (A), head-trunk angle (B), AChE activity (C) and tail length (D) of valproic acid, methoxyacetic acid and hydroxyurea treated embryos. Embryos were analyzed at 52 hpf after 48 hours of exposure. Error bars indicate SD. *, **, *** indicates significant difference from control values at $P<0.05$, $P<0.01$ and $P<0.001$.

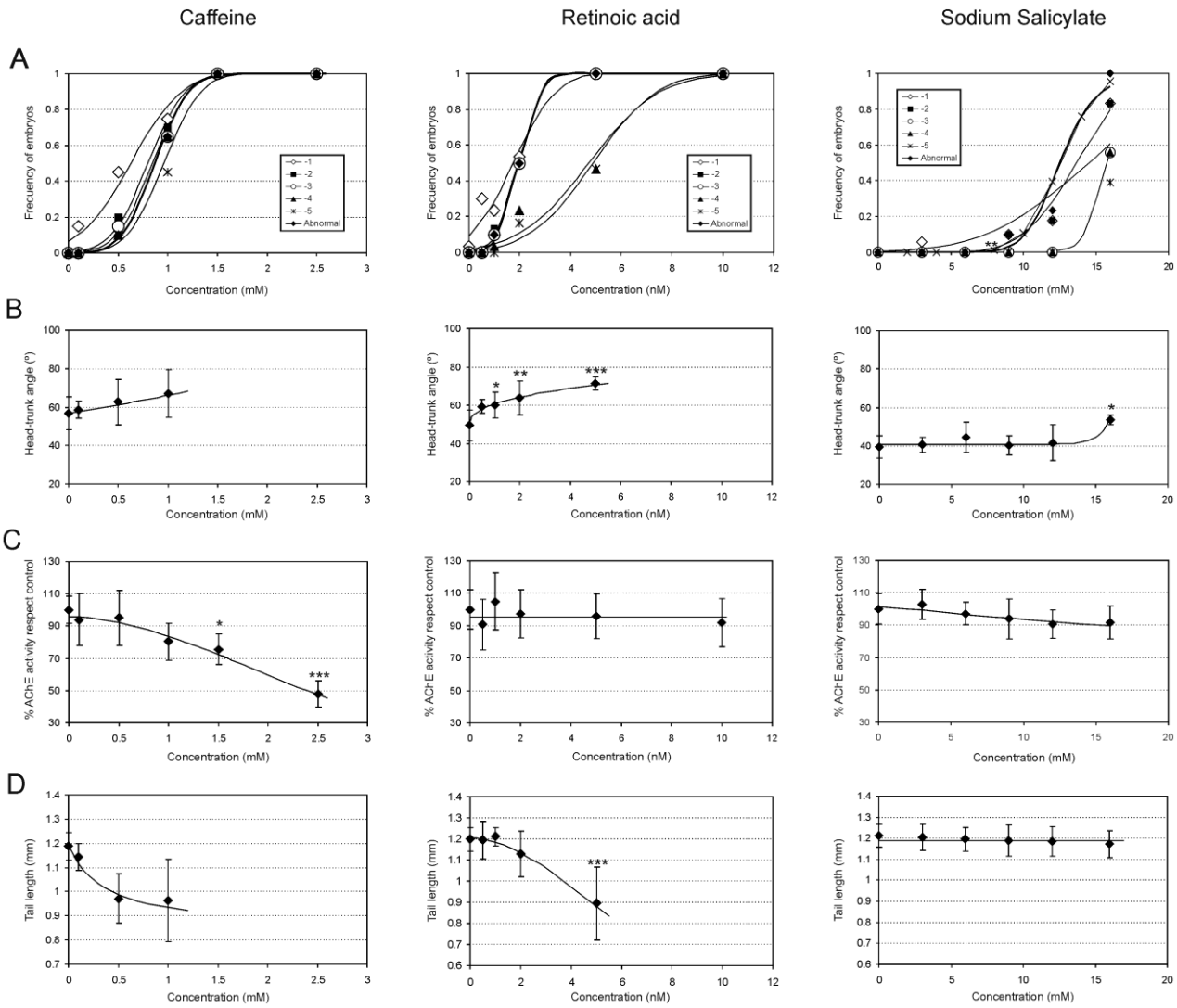


Fig. 3. Concentration-response curves for frequency of embryos with -1, -2, -3, -4 and -5 or more points less on the total morphological score and frequency of abnormal embryos (A), head-trunk angle (B), AChE activity (C) and tail length (D) of caffeine, retinoic acid and sodium salicylate treated embryos Embryos were analyzed at 52 hpf after 48 hours of exposure. Error bars indicate SD. *, **, *** indicates significant difference from control values at $P<0.05$, $P<0.01$ and $P<0.001$.

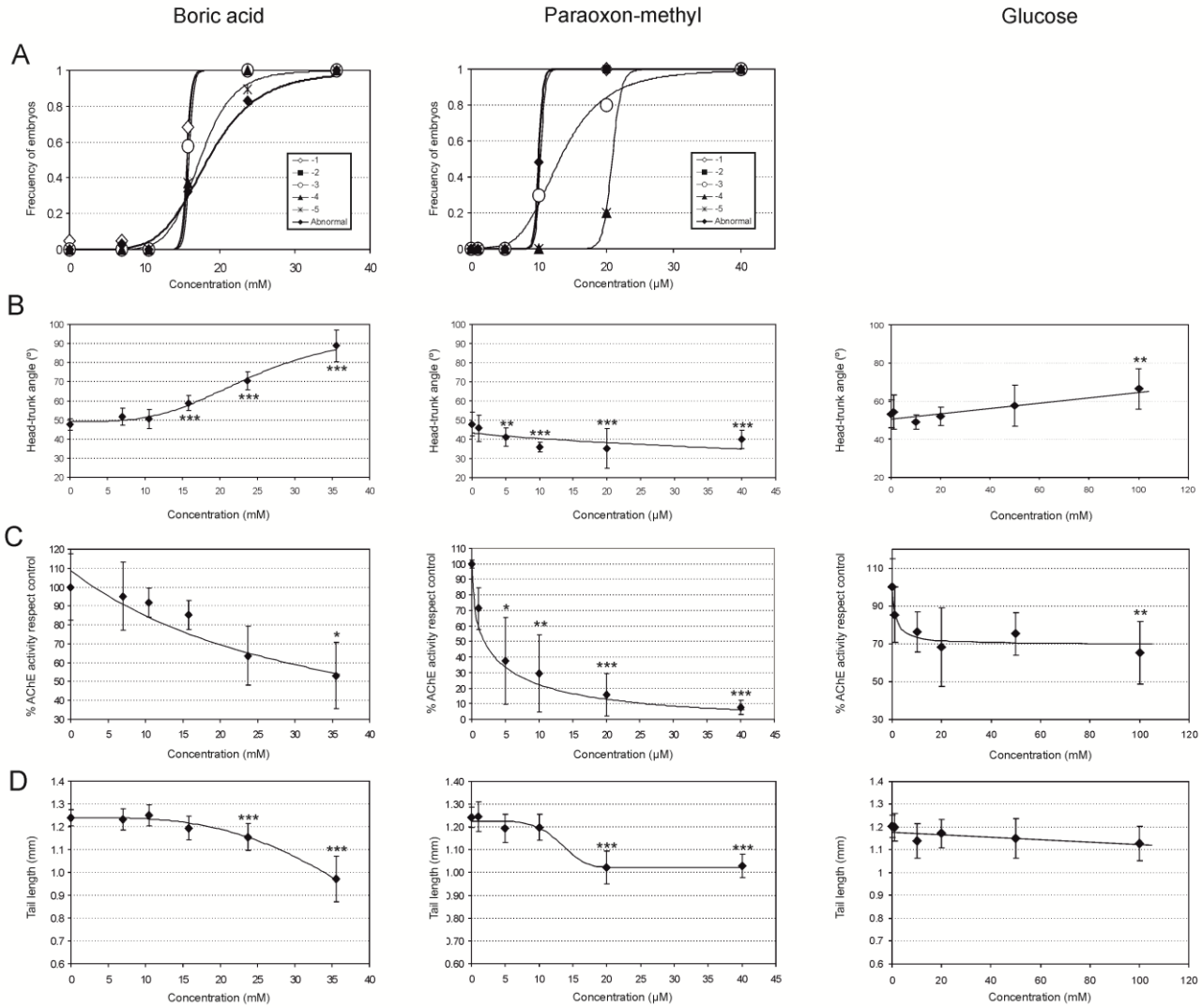


Fig. 4. Concentration-response curves for frequency of embryos with -1, -2, -3, -4 and -5 or more points less on the total morphological score and frequency of abnormal embryos (A), head-trunk angle (B), AChE activity (C) and tail length (D) of boric acid, paraoxon-methyl and glucose treated embryos Embryos were analyzed at 52 hpf after 48 hours of exposure. Error bars indicate SD. *, **, *** indicates significant difference from control values at $P<0.05$, $P<0.01$ and $P<0.001$.

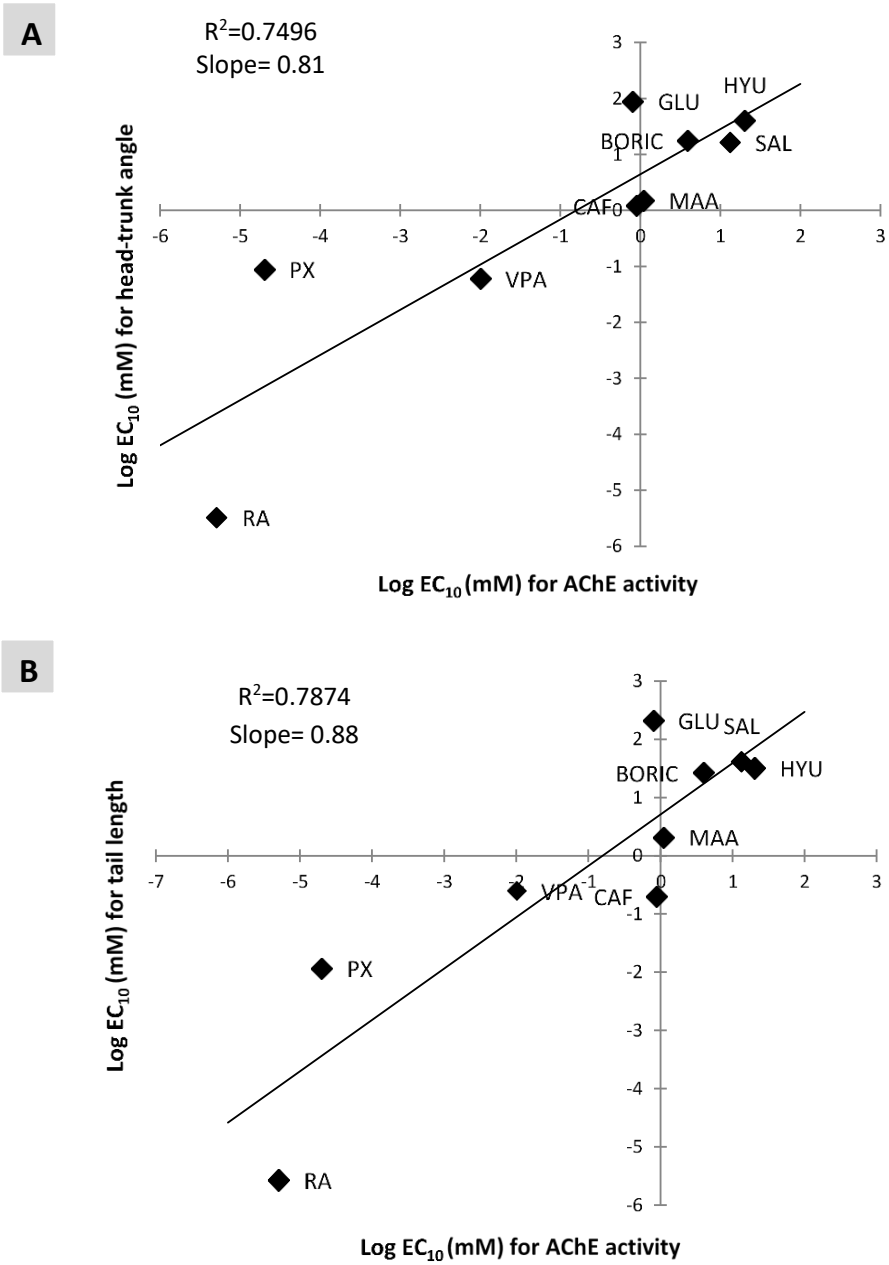
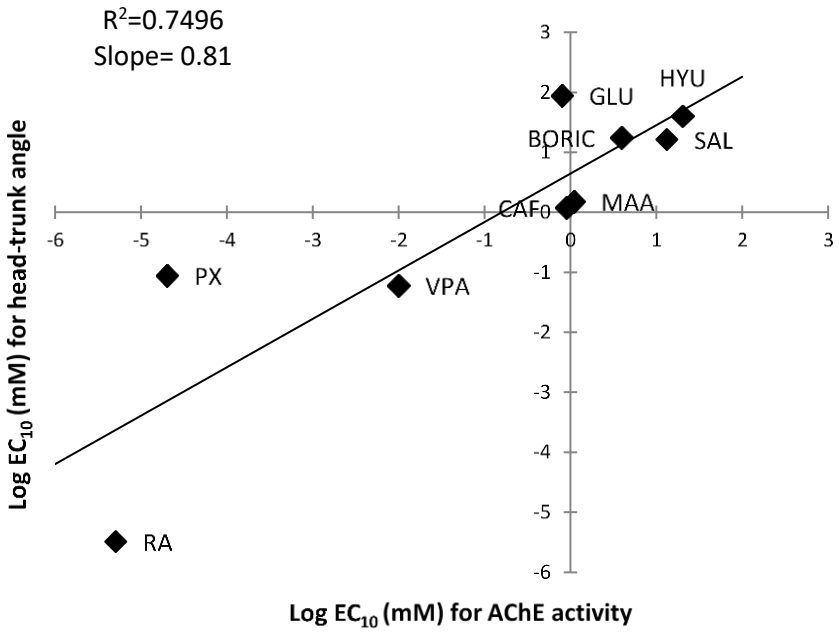


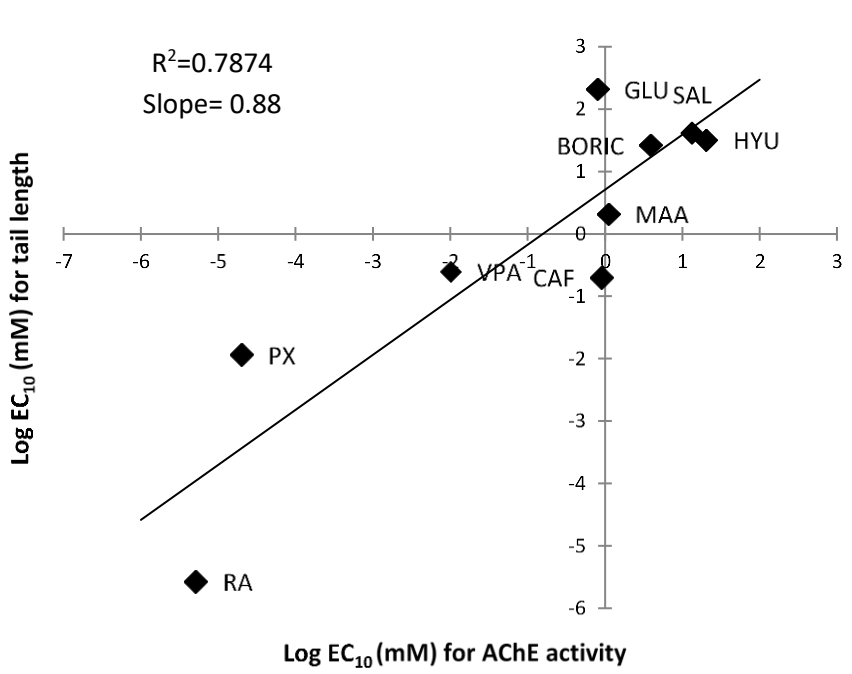
Fig. 5. Correlation between the EC_{10} values for decrease in AChE activity versus EC_{10} for head-trunk angle increase (A) and AChE activity versus tail length decrease (B). Coefficient of correlation of each simple linear regression is shown in the figure.

Each point is labeled with the tested compounds: MAA = methoxyacetic acid; VPA = valproic acid; CAF= caffeine; HYU = hydroxyurea; BORIC = boric acid; PX = paraoxon-methyl; SAL = sodium salicylate; GLU = glucose; RA = retinoic acid

A


R²=0.7496
Slope= 0.81
HYU
GLU
SAL
BORIC
MAA
CAF
PX
VPA
RA
Log EC10 (mM) for head-trunk angle
Log EC10 (mM) for AChE activity

B


R²=0.7874
Slope= 0.88
GLU
SAL
BORIC
HYU
MAA
VPA
CAF
PX
RA
Log EC10 (mM) for tail length
Log EC10 (mM) for AChE activity