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Advantages and disadvantages of the use of Xenopus laevis embryos and Zebra fish as alternative methods to assess teratogens

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Authors'contribution The author contribution was as follows: conceptualization: all authors; writing-original draft: EM; Writing - Review & Editing: FDR, FM, MB; supervision: EM.

Abstract
Traditional teratological protocols in mammals are mandatory for pharmaceutical and chemical product registration, but require hundreds of animals and are complex and money/time-consuming. Among alternative methods, low vertebrate (the teleost zebrafish and the amphibian *Xenopus*) whole embryo developmental toxicological tests appear evolutionary closer to humans than invertebrates and produce more useful data for human health extrapolation.
At least during early stages, in fact, all vertebrate embryos share, from both morphological and molecular point of view, developmental pathways. By consequence similar toxicological responses of embryos to toxicant are expected. Advantages and disadvantages of these two alternative whole organism tests are briefly reviewed.

Introduction

Embryo–fetal developmental toxicity tests (teratology studies) are mandatory for pharmaceutical and chemical product registration, according to relevant guidelines [1]. Traditional approved developmental toxicity tests have been developed using mammal models after the 1960s Thalidomide tragedy and still remain largely unchanged. Protocols show some differences depending on the specific guidelines having in common the use of two different mammalian species, one rodent (rat being the elective model) and one a non-rodent (typically the rabbit). Procedures include the maternal administration of test substance during the whole pregnancy (chemicals, food additives and pesticides) or limited to the organogenetic period (for pharmaceuticals). The sacrifice is programmed one day before the term of pregnancy with necroscopy of the mother and litter evaluation. Viable fetuses are immediately examined for gross morphology and then processed for soft-tissue (visceral) and/or skeletal evaluation. At least three dose-levels (selected on the basis of range-finding tests) and a control group must be included in each main experiment where each group must be composed by 20-25 maternal units. As a consequence, traditional teratological protocols require hundreds of animals and are complex and money/time-consuming.

In spite of minor changes in protocolled developmental toxicity tests, over the last 40 years experimental paradigms have changed for scientists, more interested in elucidating mechanisms and pathogenic pathways involved in developmental toxicity and, therefore, from 1980s a number of *in vitro* alternative tests were protocolled. In the National Research Council 2007 report [2] the use of *in vitro* systems and pathway analysis was suggested to allow the production of high-throughput screening strategies for the detection of developmental toxicants. Finally, new or refined alternative approaches are still on the top of scientific debates, also to fill procedures in compliance with the 3R-principles (reduction, refinement, and replacement of animal use for scientific reason), defined by Russell and Burch in 1959 [3]. For all these reasons, in 1993 the European Union established the European Center for the Validation of Alternative Methods (ECVAM); in 1997, the US government established the Interagency Coordinating Center for the Validation of Alternative Methods (ICCVAM) and, more recently, JaCVAM and KoCVAM were formed in Japan and Korea, respectively. The primary focus of these groups is reduction of animal number and supporting development and validation of alternative tests [Spielmann et al. 2008]. Among them, in regard to developmental toxicity endpoints, the rodent post-implantation whole embryo culture (WEC) is extremely interesting for different reasons, including reduction and refinement [4], while the use of alternative models (fish and amphibian) fulfills also the third R principle (replacement).

Amphibians (as *Xenopus*) and teleosts (as zebrafish) belong both to vertebrate evolutionary tree (Fig. 1), having the same ancestor as mammals. As defined by Haeckel's biogenetic law (1866), embryos appertaining to the same evolutionary group show stages that are a chronological replay of their ancestor evolutionary forms (ontogeny recapitulates evolution). In theory, at least during the early stages, any vertebrate embryo resembles to any other vertebrate both from the morphological and from the molecular point of view. This was morphologically described by Heakel and later well experimentally demonstrated evaluating also the gene expression patterns: based on gene expression, there is a greater similarity among vertebrate embryos than even Haeckel might have imagined, producing the so called "Molecular Haeckel" [5]. Embryonic gene expression concordance evaluation is used to confirm and refine the evolutionary tree of jaw vertebrates: reptiles, birds and mammals represent the monophyletic amniota; amniotes plus amphibians represent the monophyletic tetrapoda, and tetrapods plus bony fishes (including teleosts) represent the monophyletic osteichthyes (Fig. 1). This means a closer evolutionary relationship between amphibians and mammals than between fishes and mammals. Scientists argue that when a gene is expressed in the same organ both in zebrafish and mouse embryos, their last common ancestor had the same organ gene expression, shared with same function in all osteichthyes (including humans). In toxicological frame, therefore, if a toxicant is able to alter in one model developmental patterns known to be common to the evolutionary group, we can assume that the same pattern will be

disrupted in any other embryo (including human) exposed to the same toxicant during the same developmental stages. Molecular Haeckel was drawn till now mainly from embryos of four vertebrate models: mouse, chick, *Xenopus* and zebrafish [5]. Alternative vertebrate for developmental endpoints should, consequently, be selected among these four models.

Advantages to use low vertebrate embryos (*Xenopus* and zebrafish) in compliance to the 3R-principles

Embryologists define the phylotypic period as the embryonic stage window with conserved ontogeny, corresponding in vertebrates to the time of appearance of the pharyngeal arches and somites. During the phylotypic period any vertebrate embryo prominently expresses common developmental genes, essential for embryogenesis [6]. The phylotypic period culminates for mouse with E9.5, for chicken with Hamburger and Hamilton (HH) stage 16, for Xenopus with Nieuwkoop and Faber (NF) stages 28-32, for zebrafish at 24 hours post fertilization (hpf) [7]. In principle, exposing the four model embryos to toxicants during the phylotypic period will produce quite similar effects in spite of the chosen model.

In developmental toxicology, in alternative to mammals, low vertebrate models (zebrafish and *Xenopus*) offer different advantages versus the other Molecular Haekel alternative model (chick): 1. Due to the fact that both zebrafish and *Xenopus* develop from eggs naturally fecundated in water, their embryos can be exposed from fecundation time on, while the chick egg is fecundated in the oviduct and lay at blastodisc stage (cleavage already started and not available for xenobiotic exposure). 2. Additionally, in compliance to the actual EU-directive on the protection of animals used for scientific purposes (Directive 2010/63/EU), not-autonomous feeding larval stages (zebrafish 120 hpf, *Xenopus* stage NF 47) are not included in that directive, allowing the use of zebrafish and *Xenopus* in protocolled developmental toxicity tests outside the rules of this directive. This brings low vertebrate use, at least in Europe, easier at the bureaucratic point of view. Finally, zebrafish and *Xenopus* adults are able to be fertile and reused for long and each mating provides hundreds of fecundated eggs. Consequently, a single couple mating allows different experimental groups formed by a number statistically relevant of embryo (test units). This is an extremely reduction in terms of breed/used animals in comparison to mammalian models. For this point of view chick is a viable alternative as well, even allowing to obtain fertile chicken eggs from widely available reliable sources and on a controlled genetic background.

Zebrafish developmental toxicity tests

Zebrafish (*Danio rerio*) is a teleost fish first used, as a research model, in genetics research in the early 1980s [8]. In the current century, a number of experimental researches on embryo development were produced using the so-called *Danio rerio* embryo test (DarT), a rapid, low-cost and easy method to predict human developmental toxicity [9]. In 2013, OECD validated the zebraFish Embryo Acute Toxicity (zFET) test against a gold standard of adult fish toxicity in view of environmental risk assessment [10,11]. Actually, zFET was not validated for predicting mammalian developmental toxicity; notwithstanding this, with the introduction of some deviations in the zFET protocol, a recent study strongly supports the use of zebrafish developmental toxicity assay as valid alternative method also for screening and assessing the teratogenicity of candidate drugs for regulatory acceptance [12]. A refinement of zFET protocols described as Zebrafish Embryo Developmental Toxicity Assay (ZEDTA) allows test sensitivity increasing by the extension of the protocol with a metabolic activation system (human liver microsomes) and/or skeletal staining of larvae [13].

The most important details of the refined protocol ZEDTA described by Hoyberghs and colleagues in 2020 [13] and some personal notes are briefly described.

- Adult zebrafish (*Danio rerio*) maintained in water recirculating aquaria and kept in adequate breeding conditions are allowed to spawn eggs and fertilize them for about 45 min. Each female normally releases about 100 eggs.

- Embryos checked for normal cell division within 2 hours post fertilization (hpf) are randomly transferred in groups and maintained at 28.5°C. Classically 3-5 concentrations of the test compound are used and one/two control groups (medium and/or solvent) included.
- Samples are evaluated during the whole test period for the morphological parameters.
The last evaluation (at 120 hpf) considers different endpoints and a scoring system is suggested.
- Data are statistically analyzed considering the embryo as the experimental unit.
Special procedures are also described by Hoyberghs and colleagues: **metabolic (m)ZEDTA**, allows metabolic activation of the test substances by 1 h preincubation of test substances with human liver microsomes; **skeletal (s)ZEDTA** allows the bone examination incubating 120 hpf larvae for 1 h in Alizarin Red solution.
Behavioral tests are also reported in literature [14].

Xenopus developmental toxicity tests
Xenopus laevis is a south african anuran amphibian used, in embryological researches, from 1930s [15].
The Frog Embryo Teratogenesis Assay-Xenopus (FETAX), in particular, is a 96-hour whole embryo test developed by Dumont and colleagues [16] and later validated by the American Society of Testing and Materials [17] as an alternative screening test for hazard characterization of chemicals and mixtures. Classical FETAX is conducted using embryos obtained from hormonally-stimulated adults, with the exposure of samples to toxicants covering the whole test period (from mid-blastula to NF stage 46). The teratogenic potential of the tested compounds is determined after analysis of the day-by-day mortality and the morphological examination of live larvae at the end of the test.
Recently, in compliance to animal welfare 3Rs principle and in order to limit the exposures to selected sensitive windows, a refined method (R-FETAX) has been proposed [18*].
The most important details of the R-FETAX and some additional notes are here briefly listed.
- Adults are maintained in automated aquaria under controlled water and room conditions. In compliance with the refinement of the 3R principle and in contrast to the classic FETAX methodology, embryos are obtained without hormonal injection of adults: overnight natural mating in a mating system with controlled humidity and air/water temperature. Each female normally releases up to1000 eggs.
- Normally cleaved embryos at the mid-blastula stage are randomly distributed in groups for testing and maintained at 23°C. Classically 3-5 concentrations of the test compound are used and one/two control groups (medium and/or solvent) included. Exposures can cover the whole length of the procedure (from NF8-mid-blastula to NF 46, test timing 96-120 h depending on protocols) or can be limited to windows covering some developmental phases considered of interest (limiting exposure, for example, to the phylotypic period).
- Samples are evaluated once a day for mortality. At the end of the test, morphological and functional endpoints are recorded.
- Data are statistically analyzed considering the embryos as the experimental unit.
Special procedures have also been described by Battistoni and colleagues [18*]: **skeletal evaluation** allows the cartilage examination processing fixed samples with Alcyan Blue; **swimming test** allows neuro-behavioural evaluation. An extra functional test (**deglutition test**) has been described and allows the indirect evaluation of craniofacial defects [19].

Final remarks
The reason and main advantages for using zebrafish and *Xenopus* developmental alternative tests are described in the paragraphs above. The enormous increase of literature using these developmental toxicity models to test different toxicant agents from different silos (pesticides, pharmaceuticals, air pollutants, food-contact materials, nano-materials, etc.) (as example of recent publications: Dickinson et al., 2022 [20]; Babalola et al., 2021 [21]; Flach et al., 2022 [22]; Islas-

Flores et al., 2018 [23]; Bonfanti et al, 2020;2021 [24,25]; Xu et al., 2022 [26]; Battistoni et al. 2022a [18*]; Battistoni et al. 2022 [19]; Costabile et al 2022 [27]; Gao e Shen, 2022 [28*]; Ismail et al. 2022 [29]; Ge et al, 2021 [30]; Fogliano et al., 2022 [31]; Carotenuto et al., 2022 [32*]; Ames et al., 2022 [33]; Ochenkowska et al 2022 [34*]; Chabchoubi et al 2022 [35*]; Lin et al 2022 [36*]; Coppola et al, 2021 [37]) supports their scientific versatility. Classical teratological endpoints, mechanisms and molecular pathways, functional impairments can all be easily tested in the same experimental sample. Note that functional evaluation and all molecular endpoints (easy to be included in zebrafish and *Xenopus* developmental tests) are not considered in traditional teratological studies in mammals. Finally, in low vertebrate tests it is possible to evaluate different developmental stages in the same sample, with also the possibility to apply vital stainings without stopping development (i.e. vital dye to detect apoptosis). This is of particular interest in comparison to mammal classical teratological studies, where only fetal morphology is routinely screened. The scientific robustness of vertebrate non-mammal models is demonstrated by the "Molecular Heackel" theory (Fig. 1) [5]. The unique possibility of exposure methods typical of *in vitro* approaches (i.e. pulse-exposure, exposure to mixtures of chemicals simultaneously or in sequence) is another advantageous point for low vertebrate developmental toxicity tests. The possibility of using mutants mimicking human diseases (nowadays largely possible in zebrafish developmental model) is an extra tool. Finally, an enormous advantage is the low cost to maintain adults and embryos, the rapidity of the tests, the need of a limited number of adults not involved in treatments and the reduced bureaucracy, are all points in favor of the choice of these alternative models. Some criticisms, however, need to be listed: 1. Low vertebrate developmental tests imply the direct embryo exposure, excluding any maternal and placental pharmacokinetic and pharmacodynamic involvement. This criticism can be at least in part managed by the use of innovative approaches (i.e. applying physiologically based pharmacokinetic (PBPK) models for a reliable extrapolations of pregnancy pharmacokinetic profiles [37] before the selection of test molecules and concentrations 2. The phylogenetic conservation of developmentally relevant genes in vertebrate embryogenesis is almost limited to genes in signaling pathways but does not necessarily extend to the gene products upstream of these conserved pathways (receptors, enzymes and ion channels, that are the initial targets of the small molecules being evaluated for potential human toxicity). A modern strategy to include detailed information also on molecular targets (i.e. data obtained by *in silico* evaluation) in a unique framework could solve this gap [38,39]; 3. Metabolic activation systems using human microsomes are proposed but the performance of these has not been optimized; 4. The exposure of not-phylotypic stages (more relevant in zebrafish, due to the closer evolutionary distance between mammals and *Xenopus*, Fig. 1) can produce species-specific effects; 5. Some mammalian fetal effects are not reproducible in these alternative developmental models; 6. The presence of large quantity of yolk (mainly in zebrafish, when is accumulated at the yolk sac level) can interfere to the adsorption/distribution of the test molecules to the embryonic districts. This is minimized in *Xenopus*, due to the compartmentalization of yolk in each embryonic cell.

In conclusion, due to the described numerous advantages, low vertebrate developmental test usage can be complementary with mammal models to contribute both the basic science research and human safety and health evaluation at least for a general screening of developmental toxicants.

Figure legends

Fig. 1. Phylogenetic tree showing the evolutionary relationships among vertebrate models used in developmental toxicology. Numbers indicate the divergence times in millions of years ago as described by Molecular Heackel studies (from Wheeler and Brandli, 2009, modified). Branch lengths are not proportional to time.

Among low vertebrates, Xenopus is evolutionarily closer to humans than zebrafish, having a common evolutionary history with mammals that is an estimated 90 –100 million years longer than between zebrafish and mammals. By consequence, Xenopus and mammals have many similarities in genomes and organ development, anatomy, and physiology. This is particularly true for the brain, heart, immune system, and the kidney that, in Xenopus tadpoles, develop in a more similar way to their human counterpart than in zebrafish.

In principle, the closer a model organism is to humans in evolutionary terms, the more reliably results can be translated. Consequently, among low vertebrates, Xenopus should represent the elective model.

By contrast, zebrafish offers some unique advantages: several zebrafish mutants mimicking human diseases have been produced; screens using transgenic mutants expressing fluorescent reporter genes allow the in vivo monitoring of organ development.

For review on advantages/ disadvantages of zebrafish/ Xenopus models see Wheeler and Brandli, 2009 [40].

References

[1] P.C. Barrow, ed., Teratogenicity Testing: Methods and Protocols, Humana Press, Totowa, NJ, 2013. https://doi.org/10.1007/978-1-62703-131-8.

[2] National Research Council (U.S.), ed., Toxicity testing in the 21st century: a vision and a strategy, National Academies Press, Washington, DC, 2007.

[3] W.M.S. Russell, R.L. Burch, The principles of humane experimental technique, Special ed, UFAW, Potters Bar, 1959.

[4] C. Harris, Rodent Whole Embryo Culture, in: C. Harris, J.M. Hansen (Eds.), Developmental Toxicology, Humana Press, Totowa, NJ, 2012: pp. 215–237. https://doi.org/10.1007/978-1-61779-867-2_13.

[5] R.P. Elinson, L. Kezmoh, Molecular Haeckel, Dev. Dyn. 239 (2010) 1905–1918. https://doi.org/10.1002/dvdy.22337.

[6] H.-G. Drost, P. Janitza, I. Grosse, M. Quint, Cross-kingdom comparison of the developmental hourglass, Current Opinion in Genetics & Development. 45 (2017) 69–75. https://doi.org/10.1016/j.gde.2017.03.003.

[7] N. Irie, S. Kuratani, Comparative transcriptome analysis reveals vertebrate phylotypic period during organogenesis, Nat Commun. 2 (2011) 248. https://doi.org/10.1038/ncomms1248.

[8] G. Streisinger, C. Walker, N. Dower, D. Knauber, F. Singer, Production of clones of homozygous diploid zebra fish (Brachydanio rerio), Nature. 291 (1981) 293–296. https://doi.org/10.1038/291293a0.

[9] R. Nagel, DarT: The embryo test with the Zebrafish Danio rerio--a general model in ecotoxicology and toxicology, ALTEX. 19 Suppl 1 (2002) 38–48.

[10] OECD, Test No. 236: Fish Embryo Acute Toxicity (FET) Test, OECD, 2013. https://doi.org/10.1787/9789264203709-en.

[11] F. Busquet, R. Strecker, J.M. Rawlings, S.E. Belanger, T. Braunbeck, G.J. Carr, P. Cenijn, P. Fochtman, A. Gourmelon, N. Hübler, A. Kleensang, M. Knöbel, C. Kussatz, J. Legler, A. Lillicrap, F. Martínez-Jerónimo, C. Polleichtner, H. Rzodeczko, E. Salinas, K.E. Schneider, S. Scholz, E.-J. van den Brandhof, L.T.M. van der Ven, S. Walter-Rohde, S. Weigt, H. Witters, M. Halder, OECD validation study to assess intra- and inter-laboratory reproducibility of the zebrafish embryo toxicity test for acute aquatic toxicity testing, Regulatory Toxicology and Pharmacology. 69 (2014) 496–511. https://doi.org/10.1016/j.yrtph.2014.05.018.

[12] Y.-S. Song, M.-Z. Dai, C.-X. Zhu, Y.-F. Huang, J. Liu, C.-D. Zhang, F. Xie, Y. Peng, Y. Zhang, C.-Q. Li, L.-J. Zhang, Validation, Optimization, and Application of the Zebrafish Developmental Toxicity Assay for Pharmaceuticals Under the ICH S5(R3) Guideline, Front Cell Dev Biol. 9 (2021) 721130. https://doi.org/10.3389/fcell.2021.721130.

[13] J. Hoyberghs, C. Bars, C. Pype, K. Foubert, M. Ayuso Hernando, C. Van Ginneken, J. Ball, S. Van Cruchten, Refinement of the zebrafish embryo developmental toxicity assay, MethodsX. 7 (2020) 101087. https://doi.org/10.1016/j.mex.2020.101087.

[14] C. Rao, X. Cao, L. Li, J. Zhou, D. Sun, B. Li, S. Guo, R. Yuan, H. Cui, J. Chen, Bisphenol AF induces multiple behavioral and biochemical changes in zebrafish (Danio rerio) at different life stages, Aquatic Toxicology. 253 (2022) 106345. https://doi.org/10.1016/j.aquatox.2022.106345.

[15] H.A. Shapiro, Experimental Induction of Coupling in Xenopus laevis, with the Production of Fertilised Eggs, Nature. 135 (1935) 510–510. https://doi.org/10.1038/135510a0.

[16] J.N. Dumont, T.W. Schultz, M.V. Buchanan, G.L. Kao, Frog Embryo Teratogenesis Assay: Xenopus (FETAX) — A Short-Term Assay Applicable to Complex Environmental Mixtures, in: M.D. Waters, S.S. Sandhu, J. Lewtas, L. Claxton, N. Chernoff, S. Nesnow (Eds.), Short-Term Bioassays in the Analysis of Complex Environmental Mixtures III, Springer US, Boston, MA, 1983: pp. 393–405. https://doi.org/10.1007/978-1-4613-3611-2_27.

[17] ASTM, Standard guide for conducting the frog embryo teratogenesis assay-Xenopus (FETAX)., Annual Book of ASTM Standards. (1998) 826–36.

[18*]   M. Battistoni, R. Bacchetta, F. Di Renzo, F. Metruccio, A. Moretto, E. Menegola, Modified Xenopus laevis approach (R-FETAX) as an alternative test for the evaluation of foetal valproate spectrum disorder, Reproductive Toxicology. 107 (2022) 140–149. https://doi.org/10.1016/j.reprotox.2021.12.005.

In this paper, authors propose a refined alternative amphibian method (R-FETAX) to evaluate chemical induced embryotoxicity, in compliance to animal welfare 3Rs principle for which there is a great demand for refined tests alternative to classical mammal teratogenicity tests.
They focused on the human foetal valproate spectrum disorder (FVSD) which characteristics are morphological defects (including cranio-facial, neural tube defects) and behavioural alterations due to valproate (VPA) exposure in pregnancy. Different VPA effects were observed depending on the exposure window: concentration-related embryo-lethal and teratogenic effects (neural tube, facial, tail defects) were observed in groups exposed at the organogenetic phylotypic stages, neurobehavioral deficits were described using a functional swimming test in larvae exposed during neurocognitive competent stages. Malformations were compared to those obtained in a mammalian assay (the rat post-implantation whole embryo culture method, WEC).

[19] M. Battistoni, F. Metruccio, F. Di Renzo, R. Bacchetta, E. Menegola, Predictive assays for craniofacial malformations: evaluation in Xenopus laevis embryos exposed to triadimefon, Arch Toxicol. (2022). https://doi.org/10.1007/s00204-022-03327-w.

[20] A.J.G. Dickinson, S.D. Turner, S. Wahl, A.E. Kennedy, B.H. Wyatt, D.A. Howton, E-liquids and vanillin flavoring disrupts retinoic acid signaling and causes craniofacial defects in Xenopus embryos, Developmental Biology. 481 (2022) 14–29. https://doi.org/10.1016/j.ydbio.2021.09.004.

[21] O.O. Babalola, J.C. Truter, J.H. Van Wyk, Lethal and Teratogenic Impacts of Imazapyr, Diquat Dibromide, and Glufosinate Ammonium Herbicide Formulations Using Frog Embryo Teratogenesis Assay-Xenopus (FETAX), Arch Environ Contam Toxicol. 80 (2021) 708–716. https://doi.org/10.1007/s00244-020-00756-5.

[22] H. Flach, A. Lenz, S. Pfeffer, M. Kühl, S.J. Kühl, Impact of glyphosate-based herbicide on early embryonic development of the amphibian Xenopus laevis, Aquatic Toxicology. 244 (2022) 106081. https://doi.org/10.1016/j.aquatox.2022.106081.

[23] H. Islas-Flores, I. Pérez-Alvaréz, L.M. Gómez-Oliván, Evaluation of Teratogenicity of Pharmaceuticals Using FETAX, in: L. Félix (Ed.), Teratogenicity Testing, Springer New York, New York, NY, 2018: pp. 299–307. https://doi.org/10.1007/978-1-4939-7883-0_15.

[24] P. Bonfanti, A. Colombo, M. Saibene, L. Fiandra, I. Armenia, F. Gamberoni, R. Gornati, G. Bernardini, P. Mantecca, Iron nanoparticle bio-interactions evaluated in *Xenopus laevis* embryos, a model for studying the safety of ingested nanoparticles, Nanotoxicology. 14 (2020) 196–213. https://doi.org/10.1080/17435390.2019.1685695.

[25] P. Bonfanti, A. Colombo, M. Saibene, G. Motta, F. Saliu, T. Catelani, D. Mehn, R. La Spina, J. Ponti, C. Cella, P. Floris, P. Mantecca, Microplastics from miscellaneous plastic wastes: Physico-chemical characterization and impact on fish and amphibian development, Ecotoxicology and Environmental Safety. 225 (2021) 112775. https://doi.org/10.1016/j.ecoenv.2021.112775.

[26] Y. Xu, J. Jang, M.C. Gye, The Xenopus laevis teratogenesis assay for developmental toxicity of phthalate plasticizers and alternatives, Environmental Pollution. 300 (2022) 118985. https://doi.org/10.1016/j.envpol.2022.118985.

[27] F. Costabile, S. Decesari, R. Vecchi, F. Lucarelli, G. Curci, D. Massabò, M. Rinaldi, M. Gualtieri, E. Corsini, E. Menegola, S. Canepari, L. Massimi, S. Argentini, M. Busetto, G. Di Iulio, L. Di Liberto, M. Paglione, I. Petenko, M. Russo, A. Marinoni, G. Casasanta, S. Valentini, V. Bernardoni, F. Crova, G. Valli, A.C. Forello, F. Giardi, S. Nava, G. Pazzi, P. Prati, V. Vernocchi, T. La Torretta, E. Petralia, M. Stracquadanio, G. Zanini, G. Melzi, E.

Nozza, M. Iulini, D. Caruso, L. Cioffi, G. Imperato, F. Giavarini, M. Battistoni, F. Di Renzo, M.A. Frezzini, C. Perrino, M.C. Facchini, On the Redox-Activity and Health-Effects of Atmospheric Primary and Secondary Aerosol: Phenomenology, Atmosphere. 13 (2022) 704. https://doi.org/10.3390/atmos13050704.

[28*]   J. Gao, W. Shen, Xenopus in revealing developmental toxicity and modeling human diseases, Environmental Pollution. 268 (2021) 115809. https://doi.org/10.1016/j.envpol.2020.115809.

This review is relevant because summarizes recent advances in using *Xenopus* to study distinct types of tissue/organ development following exposure to environmental toxicants, chemical reagents, and pharmaceutical drugs. Then, the successful use of *Xenopus* as a model for diseases, including fetal alcohol spectrum disorders, autism, epilepsy, and cardiovascular disease, is reviewed. The potential application of *Xenopus* in genetic and chemical screening to protect against embryo deficits induced by chemical toxicants and related diseases is also discussed.

[29]   T. Ismail, H. Lee, Y. Kim, H. Ryu, D. Cho, Z.Y. Ryoo, D. Lee, T.K. Kwon, T.J. Park, T. Kwon, H. Lee, PCNB exposure during early embryogenic development induces developmental delay and teratogenicity by altering the gene expression in *Xenopus laevis*, Environmental Toxicology. 38 (2023) 216–224. https://doi.org/10.1002/tox.23679.

[30]   Y. Ge, F. Ren, L. Chen, D. Hu, X. Wang, Y. Cui, Y. Suo, H. Zhang, J. He, Z. Yin, H. Ning, Bisphenol A exposure induces apoptosis and impairs early embryonic development in Xenopus laevis, Environmental Pollution. 280 (2021) 116901. https://doi.org/10.1016/j.envpol.2021.116901.

[31]   C. Fogliano, C.M. Motta, P. Venditti, G. Fasciolo, G. Napolitano, B. Avallone, R. Carotenuto, Environmental concentrations of a delorazepam-based drug impact on embryonic development of non-target Xenopus laevis, Aquatic Toxicology. 250 (2022) 106244. https://doi.org/10.1016/j.aquatox.2022.106244.

[32*]   R. Carotenuto, M. Tussellino, R. Ronca, G. Benvenuto, C. Fogliano, S. Fusco, P.A. Netti, Toxic effects of SiO2NPs in early embryogenesis of Xenopus laevis, Chemosphere. 289 (2022) 133233. https://doi.org/10.1016/j.chemosphere.2021.133233.

In this paper a double approach to evaluate toxicological effects in early *Xenopus* development was applied, using both morphological endpoints and gene expression evaluation. Genes involved in the early pathways of embryo development were evaluated and related to malformation patterns. suggesting the need of close attention and further studies to better clarify the teratogenic potential of the tested molecules in humans.

[33]   J. Ames, A.A. Miragem, M.F. Cordeiro, F.O. Cerezer, V.L. Loro, Effects of glyphosate on zebrafish: a systematic review and meta-analysis, Ecotoxicology. 31 (2022) 1189–1204. https://doi.org/10.1007/s10646-022-02581-z.

[34*]   K. Ochenkowska, A. Herold, É. Samarut, Zebrafish Is a Powerful Tool for Precision Medicine Approaches to Neurological Disorders, Front. Mol. Neurosci. 15 (2022) 944693. https://doi.org/10.3389/fnmol.2022.944693.

The authors review the evidences of zebrafish modeling of human diseases for modeling and investigating various neurological disorders. The translatability of findings obtained from zebrafish studies and the broad prospect of human disease modeling paves the way for developing tailored therapeutic strategies. In this review, the predictive power of zebrafish in the discovery of novel, precise therapeutic approaches in neurosciences are discussed. The authors also investigate the newest accomplishments and current challenges in the field and future perspectives.
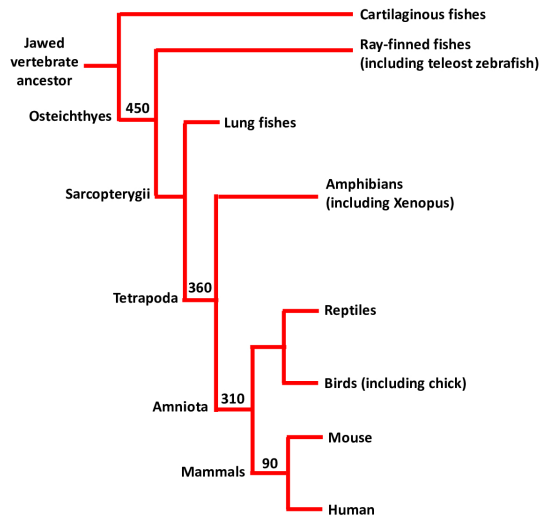
[35*]   I. Ben Chabchoubi, S.S. Lam, S.E. Pane, M. Ksibi, G. Guerriero, O. Hentati, Hazard and health risk assessment of exposure to pharmaceutical active compounds via toxicological evaluation by zebrafish, Environmental Pollution. (2022) 120698. https://doi.org/10.1016/j.envpol.2022.120698.

In this review, efforts were made to reveal correlation between the occurrence in the environment, ecotoxicological and health risks of different pharmaceutical active compounds via toxicological evaluation using zebrafish. This animal model served as a bioindicator for any health impacts after the exposure to these contaminants and to better understand the responses in relation to human diseases. The recent new advances in the morphological assessment of toxicant-exposed zebrafish larvae for the determination of test compounds effects on the developmental endpoints were also discussed.

[36*]   W. Lin, Z. Huang, W. Zhang, Y. Ren, Investigating the neurotoxicity of environmental pollutants using zebrafish as a model organism: A review and recommendations for future work, NeuroToxicology. 94 (2023) 235–244. https://doi.org/10.1016/j.neuro.2022.12.009.

This paper reviews the research on the toxic effects of pollutants in the environment (heavy metals and organic compounds) on the nervous system of zebrafish, summarizing and commenting the main research findings. The discussion of the problems, hot spots in the current research, and the prospects of the contents to be further studied are also included.

[37]   P. Coppola, E. Kerwash, S. Cole, Physiologically Based Pharmacokinetics Model in Pregnancy: A Regulatory Perspective on Model Evaluation, Front. Pediatr. 9 (2021) 687978. https://doi.org/10.3389/fped.2021.687978.

[38]   F. Metruccio, L. Palazzolo, F. Di Renzo, M. Battistoni, E. Menegola, I. Eberini, A. Moretto, Development of an adverse outcome pathway for cranio-facial malformations: A contribution from in silico simulations and in vitro data, Food and Chemical Toxicology. (2020) 111303. https://doi.org/10.1016/j.fct.2020.111303.

[39]   E. Menegola, C.H.J. Veltman, M. Battistoni, F. Di Renzo, A. Moretto, F. Metruccio, A. Beronius, J. Zilliacus, K. Kyriakopoulou, A. Spyropoulou, K. Machera, L.T.M. van der Ven, M. Luijten, An adverse outcome pathway on the disruption of retinoic acid metabolism leading to developmental craniofacial defects, Toxicology. 458 (2021) 152843. https://doi.org/10.1016/j.tox.2021.152843.

[40]   G.N. Wheeler, A.W. Brändli, Simple vertebrate models for chemical genetics and drug discovery screens: Lessons from zebrafish and *Xenopus*, Dev. Dyn. 238 (2009) 1287–1308. https://doi.org/10.1002/dvdy.21967.

Jawed vertebrate ancestor ─── Cartilaginous fishes

Ray-finned fishes
(including teleost zebrafish)

Osteichthyes 450

Lung fishes

Sarcopterygii

Amphibians
(including Xenopus)

Tetrapoda 360

Reptiles

Amniota 310

Birds (including chick)

Mouse

Mammals 90

Human

Highlights

- Developmental toxicology needs refined tools to replace mammals and evaluate pathogenic pathways
- Among alternative models, vertebrate whole embryos are to prefer for human health extrapolations
- Zebrafish/*Xenopus* share different advantages but some differences have to be considered

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: