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Asymmetric patterns in the cranial skeleton of zebrafish (*Danio rerio*) exposed to sodium pentachlorophenate at different embryonic developmental stages

Faviel López-Romero [a], Gerardo Zúñiga [b], Fernando Martínez-Jerónimo [a,*]

[a] *Laboratorio de Hidrobiología Experimental, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Prol. Carpio esq. Plan de Ayala S/N, Col. Santo Tomás, Mexico, D.F., 11340. Mexico*

[b] *Laboratorio de Variación y Evolución Biológica. Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Prol. Carpio esq. Plan de Ayala S/N, Col. Santo Tomás, Mexico, D.F., 11340. Mexico*

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ABSTRACT

Bilaterally symmetric organisms display mirror copies of their structures on both sides of the body, and the development of both sides is regulated by the same set of genes. Environmental variations can directly affect phenotype, and exposure to chemical contaminants at certain stages may modify embryonic development. The pesticide sodium pentachlorophenate (NaPCP) was used at the no-observable-effect concentration (NOEC) to determine the degree of susceptibility of zebrafish (*Danio rerio*) embryos in different developmentally susceptible windows (zygote, blastula, gastrula, segmentation, pharyngula and larva). Shape variation in the zebrafish viscerocranium and fluctuating asymmetry (FA), which increases in direct proportion to environmental stress, induced by exposure to NaPCP were measured with geometric morphometrics. Procrustes ANOVA was performed to estimate the shape variation around a symmetric consensus that accounted for the following factors: shape variation in individuals (I), variation by sides (S), the Individuals × Sides interaction ($I \times S$), and the stages of exposure to the toxicant (Stages). Factors I, S and IxS accounted for most of the morphological variation ($p < 0.0001$). Extensive deformities throughout the viscerocranium occurred during the window of exposure from gastrula to larva. Embryonic mortality occurred and was dependent on the stage of exposure. The NOEC concentration of NaPCP affected embryonic development in *D. rerio* and also induced lethal effects in embryos. FA was determined in both unexposed and NaPCP-exposed embryos and was greater in the control than in some exposure windows; besides, no correlation was found between FA and developmental stages, so our results do not support FA as a bioindicator of chemical stress but confirm its value in the study of morphological effects of toxicants.

1. Introduction

Bilaterally symmetric organisms use the same set of genes to develop structures on both sides of the body, and environmental factors directly influence this process, resulting in the production of asymmetric phenotypes under certain conditions (Dongen, 2006). The absolute value of the difference between the left and right sides ($|L-R|$) of a symmetric character should have a mean tending to zero; small random variations around the mean value are termed fluctuating asymmetry (FA) (Palmer and Strobeck, 2003). FA has been used as a measure of developmental instability in environmentally stressed populations, and it has been determined that FA increases in direct proportion to environmental stress (Møller and Swaddle, 1997; Allenbach et al., 1999; Estes et al., 2006; Palmer et al., 2010). Nevertheless, other studies do not support FA as a measure of environmental stress caused by temperature (Longson et al., 2007) or chemically-induced stress (Floate and Coghlin, 2010); according to Fowler and Whitlock (1994), FA was not related to inbreeding in population. Because the sensitivity and response to environment stress vary depending on the developmental stage at which organisms are exposed, it is also necessary to test for sensitive exposure windows (Gilbert and Epel, 2009).

FA has been evaluated using geometric morphometric techniques and multivariate analysis (Klingenberg and McIntyre, 1998; Allen and Leamy, 2001; Klingenberg et al., 2002; Palmer et al., 2010). Geometric morphometrics are useful tools to study the shape because they eliminate differences in size, location and orientation, unlike traditional morphometrics (Zelditch et al., 2004). Geometric morphometrics use landmarks (biologically homologous

* Corresponding author. Fax: +55 5396 3503.
E-mail address: fjeroni@ipn.mx (F. Martínez-Jerónimo).

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anatomical loci), which permit greater accuracy in the determination of shape variation and minimise measurement error (Leamy and Klingenberg, 2005). FA studies using geometric morphometrics should consider the type of symmetry that was to be evaluated. For example, matching symmetry is present when a mirror image of the structure is found on either side of the body, while in object symmetry the structure is divided into equal halves by a plane running through it (Klingenberg et al., 2002).

The cranium is a symmetric structure, the development of which involves several processes including the induction of the cranial neural crest (CNC) and the specification, migration and differentiation of neural crest cells (NCCs) (Knight and Schilling, 2006). The neurocranium is derived from the CNC and the mesoderm, and the viscerocranium is derived from the CNC, mesoderm and endoderm. NCCs arise from the dorsal and lateral regions of the neural ectoderm, which migrate during segmentation stages (Kimmel et al., 1995) in separate directions to populate the seven pharyngeal arches that are separated by endodermal pouches (Yelick and Schilling, 2002). The specification of NCCs is related to the location of the rhombomeres (hindbrain); the more anterior cells form the mandibular arch and hyoid (Schilling and Kimmel, 1994). Each arch gives rise to different structures: the first arch forms Meckel's cartilage and the dorsal palatoquadrate; the second arch gives rise to the ceratohyal, basihyal, interhyal and hyosimplectic; and the third to seventh arches form the pharyngobranchial, epibranchial, ceratobranchial, hypobranchial and basibranchial (Yelick and Schilling, 2002).

Sodium pentachlorophenate (NaPCP) is a chlorinated hydrocarbon that is used as a herbicide, insecticide, fungicide and bactericide, although it is primarily used as a wood preserver (Wall and Stratton, 1994). NaPCP has toxicological properties: it is probably an endocrine disruptor, mutagen and carcinogen (Chang et al., 2009). In fish exposed to NaPCP, this compound causes negative effects on development, hatching, growth and larval survival (Nguyen and Janssen, 2001, 2002; Nguyen et al., 1999). In aquatic toxicology guidelines, this toxicant is proposed as a reference toxicant (Schirmer et al., 2008).

The zebrafish is a model organism for diverse studies, especially those related to developmental biology (Schilling et al., 1996; Neuhauss et al., 1996; Heisenberg et al., 1996). It has been proposed as a test organism for toxicological studies (Hill et al., 2005) due to its rapid development (somitogenesis is completed at 24 h post-fertilisation), and its transparent embryos make it ideal for toxicological and teratogenic studies (Yang et al., 2009). NaPCP is a potential teratogen and a reference toxicant that can have effects at different developmental stages. The aim of this study was to determine the developmental windows at which zebrafish embryos are more susceptible to NaPCP by evaluating fluctuating asymmetry as well as variation in cranium shape and size using geometric morphometric techniques.

2. Material and methods

2.1. Fish maintenance

Wild-type AB* line zebrafish adults obtained from the Zebrafish International Resource Center in Oregon (USA) were maintained in 40 L aquariums containing reconstituted water (de-ionised water with 60 mg L^{-1} Instant Ocean® marine salts), at pH 7–7.5 and 25 ± 0.5 °C, with a 14 h:10 h (light:dark) photoperiod. The water quality and cleanliness of aquariums were periodically monitored according to Trevarrow (2004). Males and females were maintained separately in different aquariums prior to mating and were fed with nutritionally balanced food pellets twice daily supplementing this diet with live neonates and juveniles of the cladoceran *Daphnia magna*. Zebrafish eggs were collected with a siphon and examined under a stereoscopic microscope to eliminate unfertilised eggs, while the fertilised eggs were maintained up to the moment of exposure to the toxicant in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 10^{-5}% methylene blue) at room temperature (ca. 25 °C).

2.2. Exposure to NaPCP

The concentration we used (0.056 mg L^{-1}) was the one reported by Nguyen and Janssen (2001), which, according to these authors, does not have lethal effects (i.e., NOEC); this was dissolved in E3 medium. Our experimental model was designed to establish susceptibility windows. The first exposure treatment (Z–B) was applied between the zygote stage, i.e., 0 h post-fertilisation (hpf), (to ensure that specimens were at this stage; eggs having 2 –4 cells were selected) and the blastula stage (2.25 hpf). Other treatments were applied between the blastula and gastrula stages (2.25–5.5 hpf; B–G), the gastrula and segmentation stages 5.5–10 hpf; G–S), the segmentation and pharyngula stages (10–24 hpf, S–P), and the pharyngula and larva stages (24–72 hpf; P–L); these treatments were staged as described by Kimmel et al. (1995). For each described treatment, 24 embryos were individually exposed to 2 mL of the test solution per well containing the NOEC of NaPCP, in 24-well plates; individual plates were used for each treatment group. In the control series, 24 embryos were also individually distributed in 24-well plates containing 2 mL E3 medium.

After exposure, each set of eggs was washed with E3 medium, placed in a new multi-well plate with fresh medium (E3), and incubated at 28 °C until preservation at 120 hpf in 3.7% PBS-buffered formalin. Although two higher concentrations of NaPCP (0.1 and 0.076 mg L^{-1}) were also assayed, embryos exposed at these concentrations were not used since the toxicant induced the loss of branchial elements, which did not allow for geometric morphometric analysis.

2.3. Staining of larvae with alcian blue

Cranial abnormalities were visualised by staining the cartilage with the alcian blue technique, as described by Javidan and Schilling (2004): 0.5% alcian blue in ethanol:acetic acid solution (80:20) and staining for 6 h. The stained larvae were maintained in a PBS–glycerol solution (30:70) until microscopic examination.

2.4. Image processing

The stained larvae were examined under a Nikon Alphaphot-z YS2-H microscope with a Canon PowerShot SD550 photographic camera attached. The eyes and vitelline sac were removed in most cases to facilitate observation of the cranial cartilage. At least three images were taken of each specimen in the ventral view (viscerocranium) at different depths of the field for subsequent focus stacking with Photoshop CS4; two sets of images were independently obtained for each individual as a way to determine the error in taking images.

2.5. Geometric morphometrics

Symmetrically distributed landmarks in the viscerocranium were established based on the images taken in the ventral view. The initial configuration comprised 41 landmarks, but because of the poor precision of digitisation at the end limits for the hyomandibular and the basibranchials, seven landmarks corresponding to these structures were excluded from the analysis (Fig. 1). Landmarks were digitised with the program ImageJ ver.1.42q (http://rsb. info. nih. gov/ij/); the configuration of each individual was captured twice to estimate measurement error; error in taking images was not considered relevant for the analysis, because

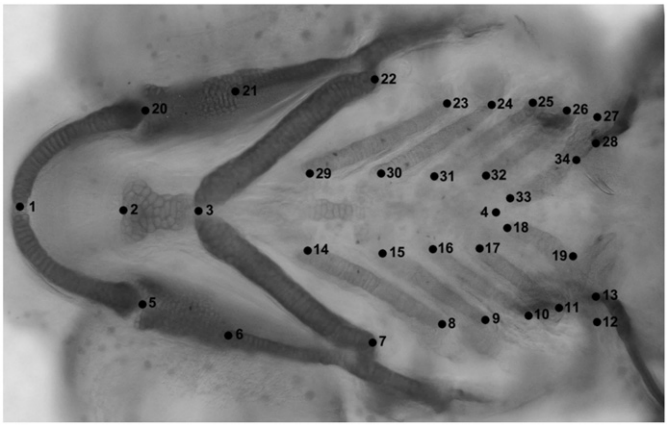

Fig. 1. Landmark configuration in viscerocranium of zebrafish; 4 median and 30 paired landmarks are shown.