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# Parental exposure to azoxystrobin causes developmental effects and disrupts gene expression in F1 embryonic zebrafish (*Danio rerio*)

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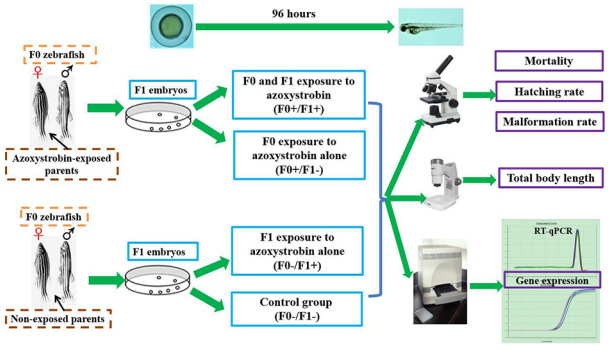
## HIGHLIGHTS

- The effect of parental exposure to azoxystrobin on F1 zebrafish was studied.
- Mortality, development, and transcripts were assessed in F1 zebrafish embryos.
- Mortality and decreased hatching rate were observed in F1 fish exposed to >20 μg/L.
- Transcripts related to oxidative damage in F1 embryos were affected at >20 μg/L.
- Parental exposure alone was sufficient to cause adverse effects in the F1 generation.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The fungicide azoxystrobin induces reproductive toxicity in adult zebrafish. However, data are lacking regarding the impact of azoxystrobin in the F1 generation after parental exposure. To address this knowledge gap, parental zebrafish (F0) were exposed to 2, 20 and 200 μg/L azoxystrobin for 21 days. Following this, fertilized F1 embryos from the exposed parents were either exposed to the same concentration as their corresponding exposed parents (F0+/F1+) or were reared in clean water (F0+/F1−) for 96 h ("+", exposed; "−" unexposed). Likewise, F1 embryos from the non-exposed parents were either reared in clean water (F0−/F0−) as the control group or were exposed to 2, 20 and 200 μg/L azoxystrobin (F0−/F1+) for 96 h. Mortality, deformities, hatching rate, body length, and the expression of transcripts related to the endocrine system, oxidative stress, and apoptosis were measured. Increased mortality, higher malformation rate, decreased hatching rate, and a shorter total body length, as well as up-regulated *cyp19b*, *vtg1*, *vtg2*, *p53*, *casp3*, and *casp9* mRNA and down-regulated *sod1* and *sod2* mRNA were detected in F1 embryos from the F0 and F1 exposure group at 20 and 200 μg/L azoxystrobin (F0+/F1+) when compared with the group from the F0 exposure alone (F0+/F1−). Interestingly, F1 exposure alone (F0−/F1+) did not induce mortality, developmental impairments, nor morphological deformations compared to the control group, but it did increase expression level of *sod1*, *sod2*, *cat*, *p53*, and *casp9* at 200 μg/L azoxystrobin. Taken together, these data suggest that azoxystrobin affects survivability, development, and genes involved in the endocrine system, oxidative stress, and apoptosis in F1 embryos if their parents are initially

*Abbreviations:* hpf, hours post-fertilization; HPG axis, hypothalamic-pituitary-gonad axis; OECD, Organization for Economic Co-operation and Development; PE, pericardial edema; ROS, reactive oxygen species; RT-qPCR, real-time quantitative polymerase chain reaction; SD, spinal deformation; TD, tail deformation; YSD, yolk sac deformation; YSE, yolk sac edema.

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exposed to this fungicide compared to embryos from non-exposed parents. Moreover, the effects are more severe if the offspring are continuously exposed to azoxystrobin similar to their parents.

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## 1. Introduction

Azoxystrobin was one of the top-selling strobilurin fungicides in 2014, with $1.2 billion in global sales according to the data provided by Phillips McDougall in company's reports in 2014 and 2015 (McDougall, 2014, 2015). Due to its ubiquitous use, environmental concentrations of azoxystrobin on a global scale have been reported to reach levels as high as 4.55 μg/L in Salinas estuary, California, USA (Smalling and Orlando, 2011), 29.7 μg/L in runoff-triggered water around Braunschweig, Lower Saxony, Germany (Berenzen et al., 2005), and 34 μg/L in river systems in Shanghai, China (Wang et al., 2009). These levels are concerning as azoxystrobin is highly toxic to aquatic organisms (Bartlett et al., 2001; EFSA, 2010; EPA, 1997). For example, the 96 h-$LC_{50}$ values of azoxystrobin to zebrafish embryos, larvae, and adults are reported to be 0.61, 0.39, and 1.37 mg/L, respectively (Mu et al., 2017). In addition to acute toxicity, studies have demonstrated that azoxystrobin can also induce sub-lethal effects in aquatic organisms at environmentally relevant concentrations. For instance, azoxystrobin caused oxidative damage and genotoxicity in livers of female and male zebrafish after 28 days of 10 μg/L exposure (Han et al., 2016).

The mechanisms underlying the toxicity of azoxystrobin are primarily related to oxidative stress and apoptosis in aquatic organisms (Cao et al., 2018; Han et al., 2016; Jiang et al., 2018; Liu et al., 2013; Olsvik et al., 2010), but other mechanisms of action can exist for the compound. For example, there is concern regarding the endocrine disrupting effects of azoxystrobin on aquatic organisms. This is due to some reports showing that azoxystrobin can negatively affect reproduction in aquatic organisms. For instance, azoxystrobin exhibited reproductive toxicity in female and male adult zebrafish when exposed to 200 μg/L for 21 days, and transcriptional responses related to the hypothalamic-pituitary-gonad (HPG) axis were dysregulated in male zebrafish at an environmentally relevant concentration of 2 μg/L (Cao et al., 2016). Additionally, a recent study showed that azoxystrobin increased 17β-estradiol (E2) and vitellogenin (Vtg) levels, and upregulated expression levels of gonadal aromatase (*cyp19b*), 17β-hydroxysteroid dehydrogenase (*hsd17b*), and *vtg* in zebrafish larvae at 1 μg/L after 24, 48 and 72 h exposure (Jiang et al., 2018). These studies indicate endocrine disrupting effects of azoxystrobin that are associated with the reproductive axis.

Since the offspring of fish can continue to be exposed to contaminated water after parental exposure in the aquatic environment (Dong et al., 2018), the overall impact of an environmental chemical on developmental toxicity in offspring may be compounded with parental exposure. Additionally, studies support the hypothesis that environmental contaminants exert negative effects on fish offspring after parental exposure, even if the offspring are not exposed to the chemicals (Chen et al., 2017; Hurem et al., 2017; Ji et al., 2013a; Liu et al., 2014). Fish in early life stages are highly susceptible and sensitive to environmental chemicals (Jin et al., 2009; Schulz et al., 2010), not only to a direct waterborne exposure, but also indirectly through their parents.

Exposure to chemicals early in development can have an array of adverse effects in developing organisms. Previous studies report that endocrine disruption, oxidative stress, and higher levels of apoptosis during fish early life stages can lead to developmental defects, teratogenicity, and mortality (Birnbaum, 1995; Colborn et al., 1993; Jin et al., 2013; Mu et al., 2015). To the best of our knowledge, studies are lacking that reveal the specific mechanisms related to the endocrine system, oxidative stress, and apoptosis pathways that can be disrupted following parental exposure to azoxystrobin in the F1 generation. Thus, assessing developmental toxicity of azoxystrobin in the offspring following parental exposure to azoxystrobin, especially at environmentally relevant concentrations, is needed to define the scope of biological impacts exerted by this fungicide.

In our previous study, adult zebrafish (F0) were first exposed to 2, 20 and 200 μg/L azoxystrobin for 21 days (Cao et al., 2016), and fertilized F1 embryos were subsequently collected to determine whether azoxystrobin exerted adverse effects on F1 offspring after parental exposure (present study). We first determined whether parental exposure to azoxystrobin caused changes in different toxicological endpoints in F1 embryos, including mortality, hatching rate, malformation rate, the presence of deformity, and body length of F1 offspring. To determine if there was evidence for endocrine disruption in F1 offspring following exposure to azoxystrobin in the F0 generation, transcripts related to HPG axis in F1 embryos were assessed. In addition, due to reports that azoxystrobin exposures can induce oxidative damage and cell death, transcriptional responses for genes involved in the oxidative stress and apoptosis signaling pathways were measured. This study provides new understanding as to the extent of developmental toxicity of azoxystrobin in F1 embryos after parental exposure.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Azoxystrobin (CAS: 131860-33-8; 98% purity) was obtained from Shenyang Chemical Industry Research Institute, China. A stock solution of 10,000 mg/L azoxystrobin was prepared by dissolving the chemical in acetone (analytical grade) containing 5% Tween 80 and was stored at 4 °C for chemical exposure. All reagents used in this study were of analytical grade. Reconstituted water with a pH value of 7.5 ± 0.5 was prepared according to the formula of ISO-7346-3, which contained 1.27 mM $NaHCO_3$, 0.33 mM $MgSO_4$, 0.33 mM $CaCl_2$, and 0.17 mM KCl (ISO, 1996).

### 2.2. Toxicity assay of azoxystrobin to F1 zebrafish embryos

The present study was a follow-up to the reproductive study of azoxystrobin in parental adult zebrafish (F0) (Cao et al., 2016), and the objective here was to address if parental exposure to azoxystrobin resulted in developmental defects on F1 zebrafish embryos and to investigate the possible mechanisms underlying any azoxystrobin-induced toxicity. The parental exposure assay contained a 14-day pre-exposure and a 21-day exposure. During the 14-day pre-exposure period, adult zebrafish (approximately six months old) were randomly reared in 10-L tanks containing reconstituted water to establish the baseline level of reproduction for each tank. Each breeding tank contained six female and six male zebrafish, and the tanks were equipped with a removable spawning tray of stainless steel mesh at the bottom of the tank to accommodate falling embryos. After a 14-day pre-exposure, adult zebrafish were exposed to blank control, solvent control or 2, 20 and 200 μg/L azoxystrobin (N = 4 biological replicates or tanks/treatment) for 21 days.

At the termination of the parental exposure (F0), all exposed parents (F0+) and non-exposed parents (F0−) were put into reconstituted water without azoxystrobin and the spawning was stimulated in the next morning when the light was turned on. Fertilized F1 embryos at 1-h post-fertilization (hpf) were collected and rinsed with reconstituted water three times for subsequent experiments. F1 embryos originating from the exposed parents were randomly divided into two groups: F1

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