Martínez-Sales, MI.; García Ximenez, F.; Espinos Gutierrez, FJ. (2015). Zebrafish (Danio rerio) as a possible bioindicator of epigenetic factors present in drinking water that may affect reproductive function: is chorion an issue?. Zygote. 23(3):447-452. doi:10.1017/S0967199414000045.

ZEBRAFISH AS A POSSIBLE BIOINDICATOR OF ORGANIC POLLUTANTS IN DRINKING WATERS WITH EFFECTS ON REPRODUCTION: ARE EFFECTS CUMULATIVE OR REVERSIBLE?

M. Martinez-Sales[1], F. García-Ximénez, FJ. Espinós.

Aquaculture and Environmental Research Group (ACUMA), Universidad Politécnica de Valencia, Camino de Vera 14, 46022, Valencia, Spain.

[1] All correspondence to: M. Martinez-Sales. Aquaculture and Environmental Research Group (ACUMA), Universidad Politécnica de Valencia, Camino de Vera 14, 46022, Valencia, Spain. Tel: +34963879433. E-mail: mimarsa@alumni.upv.es

Short title:

ARE THE POLLUTANTS CUMULATIVE OR REVERSIBLE IN ZEBRAFISH?

ABSTRACT

Due to inefficient detection and removal treatments, organic pollutants are present in drinking waters. For this reason, zebrafish is proposed as a complementary control measure in conventional potabilization treatments.

According to the most sensitive parameters (hatching rate, fertility rate and underdeveloped specimens) detected in our previous work, in the current work we attempt to study, in these parameters, the possible cumulative effect of environmental pollutants likely present in drinking waters, between generations, when specimens are cultured in the same water in both generations and/or the possible reversibility of these effects when cultured in control water.

To this end, batches of 20 embryos with the chorion intact were cultured in 3 drinking waters from different sources (A, B and C) and in one control water up to 5 months, in 20 l tanks. Four replicates were performed in all water groups, with a total of 28 aquariums.

Results in water C revealed a non-reversible effect on fertility rate, and also in water C an alteration of sex ratio towards females, although in this case the alteration was reversible. A transgenerational alteration in the germline via epigenetic mechanism from the previous generation is proposed as the most plausible explanation to this effect.

Keywords: organic pollutants; bioindicator; epigenetic; drinking water; zebrafish.

1. INTRODUCTION

Organic pollutants such as pharmaceutical and medical substances and persistent organic pollutants (POPs) have been dispersed worldwide and as a result are emerging in surface, groundwater and even in drinking waters, in this case due to inefficient removal treatments (Ikehata et al., 2008; Benner et al., 2013). The concentrations of these substances are low but increasingly numerous (year by year) and variable over time (Khetan and Collins, 2007; Rodil et al., 2012). These substances can exert toxicological but also epigenetic effects on many functions, operating on somatic cells and in the germ line, in this case promoting transgenerational effects (Rusiecki et al., 2008; Skinner, 2011).

In our previous work (Martínez-Sales et al., 2015), we defined and narrowed the most sensitive developmental and reproductive parameters in zebrafish, with the long-term aim of establishing the zebrafish as a bioindicator of the possible presence of environmental pollutants. Specifically, the assessment was carried out in three drinking waters from different tap water sources. The most sensitive parameters detected were: hatching rate, fertility rate and underdeveloped specimens. So, in the present work we focused on these parameters in order to study the possible cumulative effect and/or possible reversibility of the effects, between generations, of these environmental pollutants in the same three drinking waters (A, B and C) in both generations, despite the fact that there are other sensitive parameters, for example sex ratio.

2. MATERIAL AND METHODS

Zebrafish maintenance

Both F0 obtained from the original wild zebrafish colony and F1 generations were reared in the laboratory following the protocol described in Westerfield (1995). Briefly, adult zebrafish were kept in 20 L tanks at 28.5ºC, in a 3:2 ratio (females: males) (Westerfield, 2007) and fed on granular food supplemented with recently defrosted hen egg yolk and shrimp meat (Simão et al., 2010 a). The light cycle was regulated at 14h light/ 10h dark (Matthews et al., 2002; Brand et al., 2002). The aquariums had water recirculation systems but without active carbon filters. According to the Westerfield (2007) recommendations, a quarter of the total aquarium water was removed weekly and replaced by clean water to avoid ammonium concentrations.

It must be stated that all environmental conditions were identical to all aquariums and the spatial distribution of the aquariums was randomized.

Water sources

The four different drinking waters used in the present study (the same than in our previous work) were classified depending on their source into: three waters from different tap water distribution networks (A, B and C) and one bottled spring water which was established as a control. Type A was tap water from a city located in a region with intensive farming activity, from the hydrological basin of the Túria

river. Type B was from the tap water distribution network of a medium-sized city, supplied from the Túria and Xúquer rivers. Finally, type C was tap water from a city also located in a region with intensive agricultural activity, but from the hydrological basin of the river Xúquer. Type A and C came from groundwater prospecting.

Before filling the aquariums with water, recipients (where the water was stored) were kept open for at least a week, with a large exchange surface to favour chlorine elimination (Westerfield, 1995).

It should be mentioned that all the waters are potable and also that the chemical parameters defined for tap water for human consumption in Royal Decree 140/2003 of 7 February, which establishes the health criteria for the quality of water intended for human consumption, are suitable for zebrafish breeding and maintenance (Westerfield, 2007).

Specimen management

Fertilized embryos were obtained by siphoning. Batches of 20 fertilized embryos at the Mid Blastula Transition (MBT) stage with the chorion intact (Martinez-Sales et al., 2014; Martinez-Sales et al., 2015) were selected under a stereo microscope between those degenerated and those that initiated aberrant parthenogenetic development. These embryos were left in Petri dishes and cultured until 5 dpf (days post fertilization) at 28, 5ºC in dishes with the same water type where their progenitors were reared (same water origin and water destination: A-A; B-B; C-C;

Control-Control) and, on the other hand, in dishes with control water (different water origin and water destination: A-control; B-control; C-control).

Next, from 5 dpf to complete adulthood (5 months post fertilization) larvae were left in aquariums (20 L) in the same type of water as that in which their progenitors were reared and in aquariums with control water, to assess either the possible cumulative effect when specimens are cultured in the same water or the possible reversibility effect when are cultured in control water. From these combinations, four replicates were established with a total of 28 aquariums.

After three months, marbles were placed in each aquarium with the aim of siphoning all aquariums 2 or 3 times a week throughout the 4^{th} and the 5^{th} month, to evaluate the onset of spawning and the fertility rate. Sex ratio of the surviving adults, underdeveloped specimens and survival and abnormality rates at 5 mpf were also evaluated. Moreover, in the F1 offspring (F2 larvae) we evaluated the survival and abnormality rates at 5 dpf and the hatching rate at 72 hpf (hours post fertilization).

The experimental procedures and animal care in this work fully comply with the standards for use of animals established by the Ethical Committee of the Polytechnic University of Valencia, which specifically approved this study.

Experimental design

Two different analyses were carried out on the most sensitive parameters obtained in our previous work: hatching rate, fertility rate and underdeveloped specimens. The

first analysis studied the possible cumulative effect between generations. To this end, fertility rate and underdeveloped specimens (runts) were compared in the F0 and F1 generation. In turn, the hatching rate at 72 hpf was compared in the F1 and F2 generation. The second analysis studied the possible reversibility of the effects in fertility rate and in underdeveloped specimens in the F1 generation, and hatching rate in the F2 generation (see figure 1).

Statistical analysis

The possible cumulative and reversible effects in all parameters were analysed using Chi-square test (Statgraphics Plus 5.1). The Yates correction for continuity was used when a single degree of freedom was involved. Values were considered statistically different at $P<0.05$.

3. RESULTS

As stated in material and methods, four replicates were performed in all water groups with a total of 28 aquariums at the outset. However, 8 aquariums were discarded due to total mortality of the larvae cultured in Petri dishes until 5dpf for reasons unknown and uncontrolled. This mortality cannot be associated to a water type, as the mortality was random between groups. So, the minimum number of replicates per group was two, with a total of 20 aquariums. In the first group (control-control) the final number of replicates was three, in the second group (A-A) the final number of replicates was two, in the third group (A-control) the final number of replicates was also two, in the fourth group (C-C) the final number was

three, in the fifth group (C- control) the final number was four, in the sixth group (B-B) the final number was two and in the seventh group (B-control) the final number was four.

3.1.- Hatching rate

Hatching rate was evaluated at 72 hpf (Martinez-Sales et al., 2015) in the F1 and F2 generations during 4^{th} and 5^{th} mpf.

Cumulative effect

The analysis showed statistically significant differences ($p<0.05$) between the F1 and the F2 generations in all waters studied (see table 1). In all cases, the worst results were obtained in the second generation. These results reveal a cumulative effect in all waters, even in the control water. The negative cumulative effect in the case of water B should be highlighted.

Reversible effect

The analysis showed statistically significant differences ($p<0.05$) between data from the specimens reared in waters with the same origin and destination and data from the specimens reared in control water in all waters studied (see tables 2, 3 and 4). The worst result was obtained in all waters with the same origin and destination. These results reveal that there was a reversible effect in all waters when specimens were cultured in control water.

200

201 *3.2.- Fertility rate*

202

203 Fertility rate was evaluated through 4^{th} and 5^{th} mpf in the F0 and F1 generations.

204

205 ***Cumulative effect***

206

207 The analysis showed statistically significant differences ($p<0.05$) between the F0
208 and the F1 generations in all waters studied (see table 5). The worst results were
209 obtained in the second generation (F1). These results reveal a cumulative effect in
210 all waters, including the control water.

211

212 ***Reversible effect***

213

214 The analysis showed statistically significant differences ($p<0.05$) between data from
215 specimens reared in waters with the same origin and destination and data from
216 specimens reared in control water in all waters studied (see table 6, 7 and 8). In the
217 case of waters A and B, the worst result was obtained in waters with the same origin
218 and destination (A-A and B-B), whereas in water C the result did not improve when
219 specimens were cultured in control water. These results revealed that there was a
220 reversible effect in waters A and B when specimens were cultured in control water,
221 but a non-reversible effect in water C.

222

223 3.3.- *Underdeveloped specimens (runts)*

224

In this second work, specimens evaluated at 5 mpf in the F1 generation were all sexes clearly identifiable, and morphologically were also similar. Hence, there were no underdeveloped specimens.

3.4.- Sex ratio

Even though in the previous work sex ratio was not a sensitive parameter, in the present work, water C displayed a feminization process. Therefore, sex ratio in water C was analysed at 5mpf in the F0 and in the F1 generations.

Cumulative effect

The analysis showed statistically significant differences ($p<0.05$) between water C from F0 and water C from F1. The worst result was obtained in water C from F1, where the sex ratio was skewed towards females (males 25%: females 75%) (see table 9). No significant difference ($p>0.05$) was obtained in the other waters (A and B) whose sex ratio percentages were within the normal range in zebrafish in both generations (60 males: 40 females) (Fenske et al., 1999).

Reversible effect

The feminization detected in specimens cultured in water C, disappeared when were reared in control water (see table 10).

4. DISCUSSION

Based upon results from our previous work (Martínez-Sales et al., 2015), hatching rate, fertility rate and underdeveloped specimens were the most sensitive parameters to detect the possible presence of environmental pollutants in drinking waters from different tap water distribution networks (A, B and C). These parameters were selected considering the full life-cycle (from development to reproduction) of zebrafish specimens.

The same waters were used in the present work, but it should be taken into account that although these waters have the same original source, the physical and chemical conditions of the water may have changed due to seasonal variations in quality at the water source (Ouyang et al., 2006), although in order to be drinkable it should meet legal strict limits. Nonetheless, differences between waters also appeared in the same parameters in this experiment, except in the rate of underdeveloped specimens.

The period around hatching is a critical stage during embryogenesis (Henn, 2011), which is why the hatching rate has been extensively used as a parameter in many toxicological studies (Han et al., 2011; Galus et al., 2013) as well as a parameter for reproductive toxicity assessment (Simon et al., 2011). Our results for hatching rate revealed that although the results were high in all waters in both generations, except in water B (86.47% in F1 and 37.5% in F2), there was a negative cumulative effect in the second generation in all waters tested, even in the control water. Surprisingly, water B reached the worst results in both generations compared to the control water, decreasing to 48.97% (86.47%-37.5%) in the second generation compared to the first. These outcomes may suggest either the possible increasing presence of the same pollutants in waters in both experiments (generations) which affect the

hatching process and/or the possible transmission of these negative effects to the next generation via epigenetic mechanisms (Skinner et al., 2010; Skinner, 2011). However, it should be stated that when specimens were cultured in control water, this cumulative effect disappeared, which rules out a possible transgenerational transmission via epigenetic mechanisms.

Fertility rate has also been used in many toxicological studies as a good parameter (Ankley and Johnson, 2004; Liu et al., 2014). Results from fertility show that there was a negative cumulative effect in the second generation compared to the first in all waters, even in the control water. The most pronounced reduction between generations was obtained in water A, 22.28% (42.60%-20.32%), as this water reached the lowest rate (20.32%), followed by water B (24.5%) in the second generation. These outcomes may suggest either the possible increasing presence of the same pollutants in waters in both experiments (generations), which affected the fertility rate and/or the possible transgenerational transmission of these negative effects to the next generation via epigenetic mechanisms (Skinner et al., 2010; Skinner, 2011). It should be noted that when specimens were cultured in control water, there was a reversible effect in waters A and B, which ruled out a possible transgenerational transmission via epigenetic mechanism in these waters, although the cumulative effect remained in water C, the fertility rate decreasing to 12.03% (43.03% -31%) when specimens were cultured in control water.

So, on the basis of these findings we posit the possible presence of environmental pollutants in water A and B that affect fertility rate in both generations without transgenerational transmission, due to the reversibility process in these waters.

Nevertheless, in water C the non-reversible effect also leads us to consider the possible presence of environmental pollutants in water C that affect fertility rate in both generations, but in this case with a possible transgenerational transmission due to the maintenance of the cumulative effects when specimens were cultured later in control water. This could be explained because early exposure during critical periods of development to environmental pollutants, such as endocrine disruptors (Braw-Tal, 2010), can promote an adult-onset alteration (in this case a reduction in fertility rate) long after the compound is removed, even in subsequent generations if the germ line is affected through epigenetic mechanisms (Skinner et al., 2010; Skinner, 2011).

Regarding the non-reversible effect of the fertility rate in water C, although we are unable to describe the mechanism of action behind this effect, a plausible explanation could be an early exposure to some pollutant in water C during a critical period of embryo development (Braw-Tal, 2010), such as the MBT stage in our case, without a germline alteration via epigenetic mechanism, as the crucial period for epigenetic regulation and modification of the germline is during the period of primordial germ cell migration and gonadal sex determination (Skinner et al., 2010), events that take place after the MBT stage (3 hpf) (Dahm, 2002), at the early gastrulation stage (from 6 hpf) (Yoshizaki et al., 2002). So, taking this argument into account, the most likely explanation could be an alteration in the germline transgenerational transmitted from the previous generation (parents) via epigenetic mechanisms to this generation.

Sex ratio is a relevant parameter used in many toxicological studies (Hill and Janz, 2003; Baumann et al., 2013; Liu et al., 2014). However, in our previous work, it was not classified as a sensitive parameter because in all drinking waters tested sex ratios were within the normal ranges. Thus, all percentages of females were around 40%, which agreed with our current results and with other studies on zebrafish (60 males: 40 females) (Fenske et al., 1999), (68:32) (Örn et al., 2003), (56:44) (Vaughan et al., 2001; Hsioa and Tsai, 2003). However, in this second experiment in water C there was an alteration of sex ratio towards females (75%), although this feminization changed towards normal values in zebrafish when specimens were cultured in control water.

These results suggests the possible presence of some environmental pollutants, only in water C, such as endocrine disrupting chemicals (17-ethinylestradiol, even at ng/l) that can disrupt sexual differentiation in fish (Larsen et al., 2009) and cause feminization and retardation of sexual maturation in zebrafish. These substances may trigger disruption of sex hormones during sexual development and alter female sex, male sex or even both sexes. In fish, the hormonal balance between estrogens and androgens appears to be an important factor in the course of sexual differentiation (Liu et al., 2014).

It must be highlighted that all environmental factors were rigorously controlled to avoid any external alteration of our sex differentiation in zebrafish, as this is known to be a difficult process in fish (Liew et al., 2014) that can be affected by several environmental factors in a very complex way (Baroiller et al., 1999).

Evidence from our results gathered to date corroborates that zebrafish is a suitable model for use as a bioindicator to detect environmental pollutants in drinking water. The complexity of detecting these substances in conventional potabilization treatments, due to their interactions and their variable and random presence even at low levels in drinking water, makes their routine chemical detection and control difficult or even impossible (Khetan and Collins, 2007; Benner et al., 2013). For this reason, bioindicators could be used as backup control measures to conventional potabilization treatments.

Finally, the detection in our previous (Martinez-Sales et al., 2015) and current works of the negative effects on reproductive parameters in zebrafish reared in drinkable water is cause for alarm, as the presence of these substances in drinking water may be one of the reasons behind the decline in human reproduction in metropolitan areas (Toft et al., 2006; Jurewicz et al., 2009; Braw-Tal, 2010; Vested et al., 2014).

5. DECLARATION OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

6. FUNDING

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

7. ACKNOWLEDGMENTS

The authors would like to thank Mr. Javier Rubio Rubio for his valuable technical support and Mr. Neil Macowan for improving the English of this manuscript.

8. REFERENCES

Ankley GT & Johnson RD, 2004. Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. Institute for Laboratory Animal Research**,** 45: 469-483.

Baroiller JF, Guiguen Y & Fostier A, 1999. Endocrine and environmental aspects of sex differentiation in fish. Cellular and Molecular Life Sciences, 55: 910-931.

Baumann L, Holbech H, Keiter S, Kinnberg KL, Knörr S, Nagel T & Braunbeck T, 2013. The maturity index as a tool to facilitate the interpretation of changes in vitellogenin production and sex ratio in the Fish Sexual Development Test. Aquatic Toxicology, 128-129: 34-42.

Benner J, Helbling DE, Kohler HPE, Wittebol J, Kaiser E, Prasse C, et al., 2013. Is biological treatment a viable alternative for micropollutant removal in drinking water treatment processes?.Water research, 47(16): 5955-5976.

Brand M, Granato M & Nüslein-Volhard C, 2002. Keeping and raising zebrafish. In: Zebrafish. A Practical Approach, pp. 16. Eds Nüslein-Volhard C and Dahm R. Oxford, England: Oxford University Press.

Braw-Tal R, 2010. Endocrine disruptors and timing of human exposure. Pediatric Endocrinology Reviews (PER), 8(1): 41-46.

Dahm R, 2002. Atlas of embryonic stages of development in the zebrafish (Appendix 2). In: Zebrafish. A Practical Approach, pp. 219-236. Eds Nüslein-Volhard C and Dahm R. Oxford, England: Oxford University Press.

Fenske M, Maack G, Ensenbach U & Segner H, 1999. Identification of estrogens-sensitive developmental stages of zebrafish, Danio rerio. Proceedings of the Ninth Annual Meeting of Society of Environmental Toxicology and Chemistry, 25-29.

Galus M, Kirischian N, Higgins S, Purdy J, Chow J, Rangaranjan S, Li H, Metcalfe C & Wilson JY, 2013. Chronic, low concentration exposure to pharmaceuticals impacts multiple organ systems in zebrafish. Aquatic Toxicology, 132-133: 200-211.

Han Z, Jiao S, Shan Z & Zhang X, 2011. Effects of β-endosulfan on the growth and reproduction of zebrafish (Danio rerio). Environmental Toxicology and Chemistry, 30(11): 2525–2531.

Henn K, 2011. Limits of the embryo toxicity test with Danio rerio as an alternative to the acute fish toxicity test. Ph Thesis. University of Heidelberg, Germany.

Hill R & Janz D, 2003. Developmental estrogenic exposure in zebrafish (Danio rerio). I. Effects on sex ratio and breeding success. Aquatic Toxicology, 63: 417-429.

Hsioa CD & Tsai HJ, 2003. Transgenic zebrafish with fluorescent germ cell: a useful tool to visualize germ cell proliferation and juvenile hermaphroditism in vivo. Developmental Biology, 262: 313-323.

Ikehata K, Gamal El-Din M & Snyder SA, 2008. Ozonation and advanced oxidation treatment of emerging organic pollutants in water and wastewater. Ozone: Science and Engineering, 30(1): 21-26.

Jurewicz J, Hanke W, Radwan M & Bonde J, 2009. Environmental factors and semen quality. International journal of occupational medicine and environmental health, 22(4): 305-329.

Khetan SK & Collins TJ, 2007. Human Pharmaceuticals in the Aquatic Environment: A Challenge to Green Chemistry. Chemical Reviews, 107: 2319-2364.

Larsen MG, Bilberg K & Baatrup E, 2009. Reversibility of estrogenic sex changes in zebrafish (Danio Rerio).Environmental Toxicology and Chemistry, 28: 1789-1785.

Liew WC & Orbán L, 2014. Zebrafish sex: a complicated affair. Briefings in functional genomics, 13(2): 172-187.

Liu SY, Jin Q, Huang XH & Zhu GN, 2014. Disruption of zebrafish (*Danio rerio*) sexual development after full life-cycle exposure to environmental levels of triadimefon. Environmental toxicology and pharmacology, 37(1): 468-475.

Martinez-Sales M, García- Ximénez F & Espinós FJ, 2014. Zebrafish (*Danio rerio*) as a possible bioindicator of epigenetic factors present in drinking water that may affect reproductive function: Is chorion an issue? Zygote, 1-6.

Martinez-Sales M, García- Ximénez F & Espinós FJ, 2015. Zebrafish as a possible bioindicator of organic pollutants with effects on reproduction in drinking waters. Journal of environmental sciences. In press.

Matthews M, Trevarrow B & Matthews J, 2002. A virtual tour of the Guide for zebrafish users. Laboratory Animal, 31(3): 34-40.

Örn S, Holbech H, Madsen TH, Norrgren L & Petersen GI, 2003. Gonad development and vitellogenin production in zebrafish (Danio rerio) exposed to ethinylestradiol and methyl testosterone. Aquatic Toxicology, 65: 397–411.

Ouyang Y, Nkedi-Kizza P, Wu QT, Shinde D & Huang CH, 2006. Assessment of seasonal variations in surface water quality. Water research, 40(20): 3800-3810.

Rodil R, Quintana JB, Concha-Graña E, López-Mahía P, Muniategui-Lorenzo S & Prada-Rodríguez D, 2012. Emerging pollutants in sewage, surface and drinking water in Galicia (NW Spain). Chemosphere, 86(10): 1040-1049.

Rusiecki JA, Baccarelli A, Bollati V, Tarantini L, Moore LE & Bonefeld-Jorgensen EC, 2008. Global DNA hypomethylation is associated with high serum-persistent organic pollutants in Greenlandic Inuit. Environ Health Perspect, 116(11): 1547-1552.

Simão M, Cardona-Costa J, Pérez Camps M & García-Ximénez F, 2010 a. Ultraviolet Radiation Dose to be Applied in Recipient Zebrafish Embryos for Germ-line Chimaerism is Strain Dependent. Reproduction in Domestic Animals, 45: 1098-1103.

Simon O, Mottin E, Geffroy B & Hinton T, 2011. Effects of dietary uranium on reproductive endpoints—fecundity, survival, reproductive success—of the fish Danio rerio. Environmental Toxicology and Chemistry, 30(1): 220-225.

Skinner MK, Manikkam M & Guerrero-Bosagna C, 2010. Epigenetic transgenerational actions of environmental factors in disease etiology. Trends in Endocrinology & Metabolism, 21(4): 214-222.

Skinner MK, 2011. Role of epigenetics in developmental biology and transgenerational inheritance. Birth Defects Research Part C: Embryo Today: Reviews, 93(1): 51-55.

Toft G, Rignell-Hydbom A, Tyrkiel E, Shvets M, Giwercman A, Lindh CH, et al., 2006. Semen quality and exposure to persistent organochlorine pollutants. Epidemiology, 17(4): 450-458.

Vaughan M, Van Egmond R & Tyler CR, 2001. The effect of temperature on sexual differentiation and development in the zebrafish (Danio rerio). Proceedings of the 11th Annual Meeting of the Society of Environmental Toxicology and Chemistry, 6-10.

Vested A, Giwercman A, Bonde JP, & Toft G, 2014. Persistent organic pollutants and male reproductive health. Asian journal of andrology, 16(1): 71.

Westerfield M, 1995. The zebrafish book. University of Oregon, Eugene, OR.

Westerfield M, 2007. The Zebrafish book: A guide for the laboratory use of zebrafish (Danio rerio). Edn 5. University of Oregon Press, Eugene, US.

Yoshizaki G, Takeuchi Y, Kobayashi T, Ihara S & Takeuchi T, 2002. Primordial germ cells: the blueprint for a piscine life. Fish Physiology and Biochemistry, 26(1): 3-12.