Ann. Limnol. - Int. J. Lim. 2020, 56, 10
© EDP Sciences, 2020
https://doi.org/10.1051/limn/2020009

Available online at:
www.limnology-journal.org

RESEARCH ARTICLE

Effects of mancozeb on the testicular histology of the zebrafish (*Danio rerio*)

Merve Abar Gürol, Sezgi Arman* and Nazan Deniz Yön

Sakarya University, Faculty of Arts and Sciences, Department of Biology, Sakarya, Turkey

Received: 3 February 2020; Accepted: 9 April 2020

Abstract – Reproduction is a critical and sensitive process for population continuity of the externally fertilizing aquatic organisms. Environmental pollution may adversely effect the reproductive activities of fish. Pesticides are the mobile chemicals that are known to pollute the aquatic ecosystems. Mancozeb is an ethylene-bis-dithiocarbamate (EBDC) fungicide that is frequently used to protect fruits, vegetables, vineyards and field crops against a wide range of fungal diseases. The aim of the current work was to evaluate the acute toxic effects of mancozeb on the testis tissues of zebrafish (*Danio rerio*). Zebrafish were exposed to 5 ppm and 7.5 ppm of mancozeb concentrations for five days. Testis tissues were removed and fixed in 10% neutral buffered formalin solution. Specimens were embedded in paraffin and 5 μm serial sections were stained with hematoxylin and eosin. The control and the experimental samples were investigated by light microscopy and histopathological changes were evaluated. Mancozeb gave rise to degenerative spermatogenic cells, seminiferous tubule disorganizations, fibrosis, hemorrhage, vacuolization, hypertrophy of spermatocytes, edema, decreased spermatogenic cell clusters and sperms, pyknotic and karyolytic nuclei. These results showed that mancozeb could interrupt the reproductive activity and decrease the fertilization ratio of zebrafish.

Keywords: Mancozeb / testis / *Danio rerio* / zebrafish / histopathology

1 Introduction

Pesticides are an essential part of modern agricultural practices and play a vital role in increasing food production. Pesticide usage is one of the most preferred methods because of its short duration of application and ease of use. However, these chemicals can cause various side effects that they may disrupt the endocrine system and/or induced gonadal abnormalities on non-target organisms (Lone *et al.*, 2014).

Dithiocarbamates, the subclass of carbamate pesticides, are mostly preferred in the agricultural applications and form part of a large group of organic pesticides developed in recent years. Ethylene-bis-dithiocarbamates (EBDCs) are widely used as fungicides in agriculture world-wide due to their low cost, relatively lower acute toxicity and shorter persistence in the environment (Ripley and Cox, 1978; Corsini *et al.*, 2005). Mancozeb is a metal EBDC fungicide and it is a mixture of manganese and zinc EBDC (Mn:Zn, 9:1). It is frequently applied to protect fruits, vegetables, vineyards and field crops against a wide range of fungal diseases (Cycoń *et al.*, 2010; Paro *et al.*, 2012).

Mancozeb-induced toxic effects on some species are available in the literature data. It was reported that mancozeb toxicity on aquatic organisms was moderate to high and 48-h EC_{50} value for *Daphnia magna* was 1000 μg/L (US EPA, 2000; Černohlávková *et al.*, 2009). Chronic exposure to mancozeb caused alterations in gonadal structure and function in female rats (Baligar and Kaliwal, 2001; Santos *et al.*, 2009). By assessing mouse oocytes, it was noted that mancozeb decreased female reproduction performance at the concentrations of ≤1 μg/ml (Cecconi *et al.*, 2007; Paro *et al.*, 2012). It also induced histopathological changes in the liver and adrenal gland of mice (O'Hara and Didonto, 1985; Sakr and Saber, 2007).

Fish may encounter with various pesticides in their natural habitats (Joseph and Raj, 2011). Non-lethal concentrations of these chemicals in the aquatic environments may cause striking structural and functional changes (Sancho *et al.*, 2003; Ullah and Zorriehzahra, 2015). Reproduction is a sensitive process that can be affected by the pesticides contaminating the water sources. Several studies revealed that pesticides reduced the reproductive activity of fish via various ways such as

*Corresponding author: sezgiarman@gmail.com

gonadal histopathology, decreased gonadosomatic indices, endocrine distruption and reduced fertilization capacity (Moore and Waring, 2001; Hanson *et al.*, 2007; Lal, 2007).

Zebrafish (*Danio rerio)* is an important fish for scientific researches and is frequently used as a vertebrate model organism in genetics, developmental, toxicological and other experimental researches (Sprague *et al.*, 2006; Segner, 2009; Meunier, 2012; Dai *et al.*, 2014). Besides, its small size, easy maintenance in lab conditions and lower cost make this fish beneficial for researchers.

In this study, zebrafish was used as an animal model to reveal acute toxic effects of mancozeb on testicular tissue by assessing the histopathological alterations by light microscopy.

2 Materials and methods

2.1 The test chemical and the organism

Mancozeb (Dikozin M-45) was obtained from an agricultural pharmaceutical company (Agrofarm, Istanbul/Turkey). Sexually mature zebrafish (4–5 cm in length, 1–2 g in weight) were used in the experiments. They were maintained in 20 L capacity glass aquaria with dechlorinated tap water (at 26–28 °C, 7.7 pH, 7.9 mg/L dissolved oxygen). They were kept at 14 h light/10 h dark photoperiod and fed with commercial fish feed (Tetramin Pro Energy®) for one week before the treatment.

2.2 Chemical exposure

Experimental samples were divided into three groups ($n = 10$) comprising one control and two experimental groups, each were in triplicates. Mancozeb was dissolved in water. The control group was chemical-free and the experimental groups were exposed to 5 ppm and 7.5 ppm mancozeb for five days. Static test system was conducted. Samples were anaesthetised in iced water and testis tissues were removed for histopathological investigations.

2.3 Histological preparation

Specimens were fixed in 10% neutral buffered formalin and processed for routine histology. The ascending series of ethanol concentrations (70%, 80%, 90% and 100%) was used for dehydration. The tissues were cleared in xylene, embedded in paraffin wax and 5 µm sections were prepared by using Leica RM2125RT microtome (Leica, Germany). The sections were mounted on glass slides, stained with hematoxylin and eosin (H&E) and examined under Leica DM500 compound microscope. Microphotographs were taken with Leica MC170 HD camera.

3 Results

3.1 Control group

In the control group, testis consisted of numerous seminiferous tubules and each tubule was covered by a thin basement membrane. Dark, small and round-shaped Leydig cells were noted in the intersititial tissue among the seminiferous tubules. The germ cells were observed as spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and sperm cells. Spermatogonia were the biggest cells of the spermatogenic cells and they were noticed with their oval-shaped faded nuclei. Primary spermatocytes were smaller than the spermatogonia and they had smaller, dense nuclei covering the majority of the cytoplasm. Secondary spermatocytes had large nuclei and they were smaller than the primary spermatocytes. Spermatids were the smallest cells among the all spermatogenic cells and had dense nuclei. Sperms were observed in the luminal area of the seminiferous tubules. These cells were dark, round-shape nucleated and their cytoplasm could not be noticed (Fig. 1).

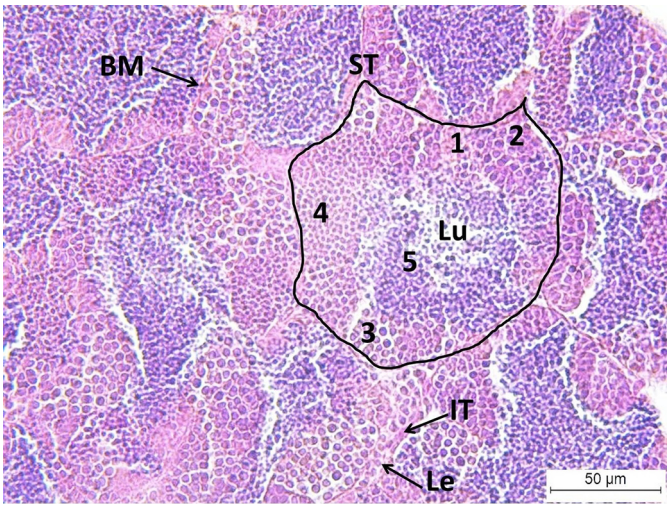

Fig. 1. Testicular histology of the control zebrafish. A seminiferous tubule (ST) (surrounded) covered by a basement membrane (BM) and containing spermatogenic cells peripherally as spermatogonia (1), primary spermatocytes (2), secondary spermatocytes (3), spermatids (4) and sperms (5) in the luminal area (Lu). Intertubular area was full with intersitial tissue (IT) and Leydig cells (Le) were also noticed. (H&E staining).

3.2 Experimental groups

3.2.1 5 ppm mancozeb exposed group

The testis histology of 5 ppm mancozeb treated samples showed thinning of the basement membrane that surrounded some seminiferous tubules, fibrosis (Fig. 2A), fusion of some tubules (Fig. 2B), degenerative appereance of developing spermatogenic cell clusters, separations between the sperms and spermatogenic cells (Fig. 2A,C,D), hemorrhage in the intertubular space (Fig. 2B,C) and vacuole formations (Fig. 2D).

3.2.2 7.5 ppm mancozeb exposed group

The testis of 7.5 ppm mancozeb exposed zebrafish exhibited severe histopathological alterations. Seminiferous tubules were disorganized and their boundaries were not clear (Fig. 3A–B–C). Prominent vacuolization (Fig. 3B,D) and edema (Fig. 3C) were observed. Basement membranes of some tubules were very thin and hypertrophy of spermatocytes were noticed (Fig. 3C). Separations of intertubular area strikingly increased (Fig. 3A–C). Degenerative spermatogenic cell

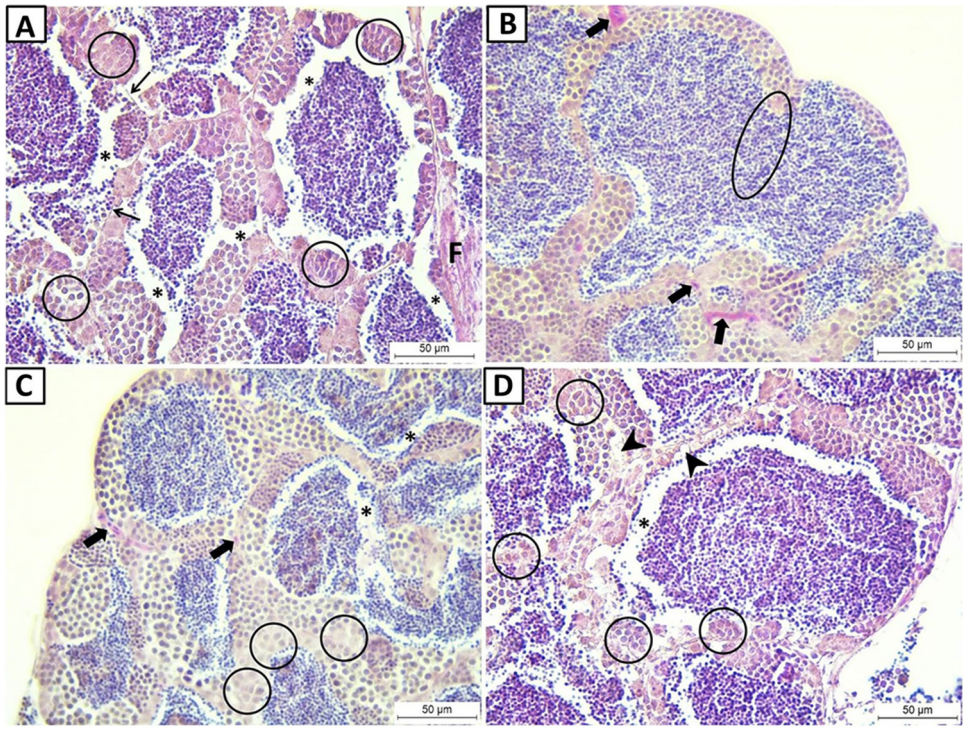

Fig. 2. Testicular histopathology of 5 ppm mancozeb exposed samples. (A) Degenerated spermatogenic cell clusters (encircled), thinned basement mambrane (arrows), separations between the sperms and other developing spermatogenic cell clusters (asterisks) and fibrosis (F). (B) Fusion of the some seminiferous tubules (ellipse) and hemorrhage in the intertubular area (arrows). C) Degenerative appereance of spermatogenic cells (encircled), hemorrhage in the intertubular area (arrows) and separations (asterisks) between the sperms and other developing spermatogenic cell clusters. (D) Degenerated spermatogenic cell clusters (encircled), separation (asterisk) and vacuolization (arrowheads). (H&E staining).

clusters were clearly noticed (Fig. 3A–C). Among these cells, some of them had pyknotic or karyolytic nuclei (Fig. 3D). Even some tubules showed dramatically decreased developing spermatogenic cells and in these tubules only sperm cells were noted in the luminal area (Fig. 3D). Sperms were also severely decreased in some of the seminiferous tubules (Fig. 3B).

4 Discussion

Pesticides are discharged by human activity and contaminate water sources. Fish are exposed to these chemicals directly or indirectly. Pesticides have adverse effects on fish reproduction. Hormonal processes, behavior, gametes and/or gonads can be impacted by pesticide exposure. Several studies have pointed that gonads showed pathological conditions following pesticide treatment (Ewing, 1999; Khan and Law, 2005). Histology is an useful and reliable technique to investigate and evaluate the toxic effects of pesticides on gonads at tissue and cellular level (McHugh *et al.*, 2011).

There are limited data on mancozeb toxicity on testis, and researchers have focused on the effects of this chemical particularly in rodents. Joshi *et al.* (2005) reported that mancozeb treated Wistar albino rats exhibited weight loss of testis, epididymis, seminal vesicle, prostate and significantly decreased sperm cells. In albino mice, mancozeb inhibited the spermatogenesis histologically and spermatogenic cells and sperm counts decreased (Ksheerasagar and Kaliwal, 2010). Another study with albino mice revealed that mancozeb exposure gave rise to decreased sperm cell number and motility; while it increased lipid peroxidation, it reduced antioxidant enzyme activities. The authors also noted that testis was histopathologically altered (Mohammadi-Sardoo *et al.*, 2018). The testis of zebrafish also exhibited altered histology and decreased sperm cell numbers; however in this work enzymatic processes were not investigated.

Previous reports represent histopathological effects of various pesticide types on the testis of different fish species. Diazinon brought about damaged cells and connective tissue, disrupted seminiferous tubules, decreased count of spermatogenic and sperm cells in the testis of *Lepomis macrochirus* (Dutta and Meijer, 2003). Diazinon exposure also caused testicular degeneration in *Clarias gariepinus* (Ola-Davies *et al.*, 2015). *L. macrochirus* testis tissues were affected by endosulfan. Connective tissue and tubule damage, decreased count of spermatids and sperms were noted (Dutta *et al.*, 2006). Mancozeb also caused distinct sperm cell decrease in zebrafish at 7.5 ppm while it was not noted in 5 ppm mancozeb exposed group. It gave rise to seminiferous tubule malformations as fusion and disorganization with uncertain tubule boundaries. Cypermethrin treatment induced cytotoxic damage, condensed spermatogenic cells, vacuole formation and distruped interstitial cells in the testis tissues of *Heteropneustes fossilis* (Singh and Singh, 2008). Vacuolization was also observed in the current study, both experimental specimens showed vacuoles,

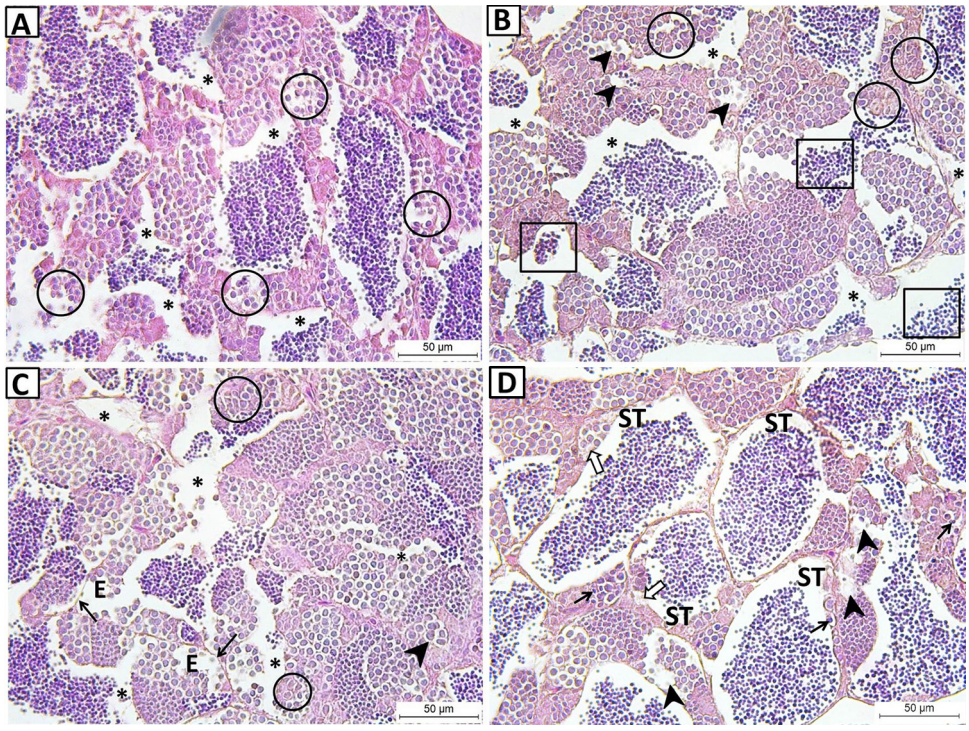

Fig. 3. Testicular histopathology of 7.5 ppm mancozeb exposed samples. (A) Degenerative appereance of spermatogenic cells (encircled) in the disorganized tubules and increased intertubular spaces (asterisks). (B) Vacuole formations (arrowheads), degenerated spermatogenic cells (encirled), decreased sperm cells (squares) and increased separations (asterisks). (C) Edema (E), degenerated spermatogenic cells (encircled), hypertrophy of primary spermatocytes (arrowhead), thinned basement membrane (arrows) and separations (asterisks) (H&E staining). (D) Tubules without developing spermatogenic cells (ST), vacuolization (arrowheads), pyknotic nuclei (arrows) and karyolytic nuclei (white arrows). (H&E staining).

while it was more prominent in 7.5 ppm treated zebrafish testis and edema was also accompanied. Fenitrothion exposed *Oreochromis niloticus* testis showed sterile seminiferous tubules (Benli and Özkul, 2010). In this work, seminiferous tubules without spermatogenic cells were noted in the samples of 7.5 ppm mancozeb treatment. Deltamethrin exposure gave rise to histopathological alterations in the testis of *Xiphophorus helleri* such as not defined seminiferous tubule shape, decreased count of spermatogenic cells and arrested spermatogenic cycle (Koc *et al.*, 2012). Another study revealed that deltametrin treated *Oreochromis niloticus* testis exhibited decreased numbers of spermatogenic cells and sperms, degenerative and hypertrofied spermatogonia, pyknosis and cellular necrosis (Bayar *et al.*, 2014). Degenerative spermatogenic cells were observed in both of the experimental groups; however hypertrohy of spermatocytes, pyknotic and karyolytic cells were noted only in the highest mancozeb concentration treated samples of the current paper.

In the present study, water-borne effects of mancozeb on the testicular structure of zebrafish were noted as degenerative spermatogenic cells, seminiferous tubule disorganizations, fibrosis, tubular fusion, vacuolization, edema, hemorrhage, decreased spermatogenic cell clusters and sperms, hypertrophy of spermatocytes, pyknotic and karyolytic nuclei. In general terms, comparing to previous reports mentioned above, pesticides induced similar hazardous histological changes in male gonads. It is clear that arrested spermatogenic processes and decreased sperm number interrupt and fall through the fertilization potential.

Acknowledgement. This study is based on the MSc thesis of the first author which was supervised by the last author.

References

Baligar PN, Kaliwal BB. 2001. Induction of gonadal toxicity to female rats after chronic exposure to mancozeb. *Ind Health* 39: 235–243.

Bayar AS, Bilici S, Cengiz EI, Satar A, Yanar M. 2014. The effect of vitamin E supplementation on ovary and testis histopathology in *Oreochromis niloticus* exposed to deltamethrin. *Toxicol Environ Chem* 96: 114–135.

Benli AÇK, Özkul A. 2010. Acute toxicity and histopathological effects of sublethal fenitrothion on Nile tilapia, *Oreochromis niloticus*. *Pestic Biochem Phys* 97: 32–35.

Cecconi S, Paro R, Rossi G, Macchiarelli G. 2007. The effects of the endocrine disruptors dithiocarbamates on the mammalian ovary with particular regard to mancozeb. *Curr Pharm Des* 13: 2989–3004.

Černohlávková J, Jarkovský J, Hofman J. 2009. Effects of fungicides mancozeb and dinocap on carbon and nitrogen mineralization in soils. *Ecotoxicol Environ Saf* 72: 80–85.

Corsini E, Birindelli S, Fustinoni S, *et al*. 2005. Immunomodulatory effects of the fungicide Mancozeb in agricultural workers. *Toxicol Appl Pharm* 208: 178–185.

Cycoń M, Piotrowska-Seget Z, Kozdrój J. 2010. Responses of indigenous microorganisms to a fungicidal mixture of mancozeb and dimethomorph added to sandy soils. *Int Biodeterior Biodegrad* 64: 316–323.

Dai YJ, Jia YF, Chen N, *et al*. 2014. Zebrafish as a model system to study toxicology. *Environ Toxicol Chem* 33: 11–17.

Dutta HM, Meijer HJM. 2003. Sublethal effects of diazinon on the structure of the testis of bluegill, *Lepomis macrochirus*: a microscopic analysis. *Environ Pollut* 125: 355–360.

Dutta HM, Misquitta D, Khan S. 2006. The effects of endosulfan on the testes of bluegill fish, *Lepomis macrochirus*: a histopathological study. *Arch Environ Cont Tox* 51: 149–156.

Ewing R. 1999. Diminishing returns: Salmon decline and pesticides. Journal of pesticide reform: a publication of the Northwest Coalition for Alternatives to Pesticides (USA).

Hanson R, Dodoo DK, Essumang DK, Blay J, Yankson K. 2007. The effect of some selected pesticides on the growth and reproduction of fresh water *Oreochromis niloticus, Chrysicthys nigrodigitatus* and *Clarias gariepinus*. *Bull Environ Contam Toxicol* 79: 544–547.

Joseph B, Raj SJ. 2011. Impact of pesticide toxicity on selected biomarkers in fishes. *Int J Zool Res* 7: 212–222.

Joshi SC, Gulati N, Gajraj A. 2005. Evaluation of toxic impacts of mancozeb on testis in rats. *Asian J Exp Sci* 19: 73–83.

Khan MZ, Law FC. 2005. Adverse effects of pesticides and related chemicals on enzyme and hormone systems of fish, amphibians and reptiles: a review. *Proc Pak Acad Sci* 42: 315–323.

Koc ND, Akbulut C, Kayhan FE, Kaymak G. 2012. Histopathological changes in testis of the swordtail fish, Xiphophorus helleri (Pisces: Poecilidae) exposed to deltamethrin. *Fresen Environ Bull* 21: 2866–2870.

Ksheerasagar RL, Kaliwal BB. 2010. Effect of mancozeb on thyroid, testis, accessory reproductive organs and biochemical constituents in albino mice. *Recent Res Sci Technol* 2.

Lal B. 2007. Pesticide-induced reproductive dysfunction in Indian fishes. *Fish Physiol Biochem* 33: 455–462.

Lone S, Shah R, Lone A. 2014. Mancozeb-ımpact on biochemical profile of adult mice (*Mus musculus*). *Am Res J* 1: 1–5.

McHugh KJ, Smit NJ, Van Vuren JHJ, *et al*. 2011. A histology-based fish health assessment of the tigerfish, Hydrocynus vittatus from a DDT-affected area. *Phys Chem Earth Parts A/B/C* 3: 895–904.

Meunier R. 2012. Stages in the development of a model organism as a platform for mechanistic models in developmental biology: Zebrafish, 1970–2000. *Stud Hist Philos Biomed Sci* 43: 522–531.

Mohammadi-Sardoo M, Mandegary A, Nabiuni M, Nematollahi-Mahani SN, Amirheidari B. 2018. Mancozeb induces testicular dysfunction through oxidative stress and apoptosis: protective role of N-acetylcysteine antioxidant. *Toxicol Ind Health* 34: 798–811.

Moore A, Waring CP. 2001. The effects of a synthetic pyrethroid pesticide on some aspects of reproduction in Atlantic salmon (*Salmo salar* L.). *Aquat Toxicol* 52: 1–12.

O'Hara GP, Didonto LJ. 1985. Dithane M-45 and ethylene thiourea: 3-month dietary toxicity study in mice. *J Environ Health* 10: 32–38.

Ola-Davies OE, Fagbohun AF, Emikpe BO, Adeyemo OK. 2015. Diazinon-induced clastogenity and pathological changes in ovaries and testes of *Clarias gariepinus*. *Agric Sci* 6: 146.

Paro R, Tiboni GM, Buccione R, *et al*. 2012. The fungicide mancozeb induces toxic effects on mammalian granulosa cells. *Toxicol Appl Pharm* 260: 155–161.

Ripley BD, Cox DF. 1978. Residues of ethylene bis (dithiocarbamate) and ethylenethiourea in treated tomatoes and commercial tomato products. *J Agric Food Chem* 26: 1137–1143.

Sakr SA, Saber A. 2007. Ameliorative effect of ginger (*Zingiber officinale*) on mancozeb fungicide induced liver injury in albino rats. *Aust J Basic Appl Sci* 1: e656.

Sancho E, Fernandez-Vega C, Ferrando MD, Andreu-Moliner E. 2003. Eel ATPase activity as biomarker of thiobencarb exposure. *Ecotoxicol Environ Saf* 56: 434–441.

Santos PM, Simões T, SáCorreia I. 2009. Insights into yeast adaptive response to the agricultural fungicide mancozeb: a toxicoproteomics approach. *Proteomics* 9: 657–670.

Segner H. 2009. Zebrafish (*Danio rerio*) as a model organism for investigating endocrine disruption. *Comp Biochem Physiol C Toxicol Pharmacol* 49: 187–195.

Singh PB, Singh V. 2008. Cypermethrin induced histological changes in gonadotrophic cells, liver, gonads, plasma levels of estradiol-17β and 11-ketotestosterone, and sperm motility in *Heteropneustes fossilis* (Bloch). *Chemosphere* 72: 422–431.

Sprague J, Bayraktaroglu L, Clements D, *et al*. 2006. The Zebrafish Information Network: the zebrafish model organism database. *Nucleic Acids Res* 34: D581–D585.

Ullah S, Zorriehzahra MJ. 2015. Ecotoxicology: a review of pesticides induced toxicity in fish. *Adv Anim Vet Sci* 3: 40–57.

US EPA. 2000. Pesticide Ecotoxicity Database, Office of Pesticide Programs. Environmental Fate and Effects Division. Washington, DC: US EPA.

Cite this article as: Abar Gürol M, Arman S, Yön ND. 2020. Effects of mancozeb on the testicular histology of the zebrafish (*Danio rerio*). *Ann. Limnol. - Int. J. Lim.* 56: 10