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Histopathological changes in *Oreochromis mossambicus* (Peters, 1852) ovaries after a chronic exposure to a mixture of the HIV drug nevirapine and the antibiotics sulfamethoxazole and trimethoprim

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**Highlights**

- Fish reproduction is affected by chronic exposure to non-steroid pharmaceutical mixture.
- Mixture of antiretroviral and antibiotics caused histopathology in fish gonads.
- Female fish gonads were more affected by the mixture of pharmaceuticals.
- Increased incidence of oocyte atresia was observed in exposed female fish ovaries.

**Abstract**

The burden of the human immunodeficiency virus and acquired immunodeficiency syndrome (HIV/AIDS) infection has transformed the African continent into a major consumer of antiretrovirals (ARVs) drugs. In addition to HIV burden, the African continent has also a high incidence of tuberculosis (TB) and has been experiencing recurring outbreaks of several other viral, bacterial, and parasitic epidemic diseases. The novel severe acute respiratory syndrome coronavirus 2 (SARS-COV-2 or Covid-19) pandemic outbreak is adding to the continent’s infectious diseases burden as experts are predicting that it will be here for a long time. One of the consequences of these infectious diseases is that antiviral and antibiotic compounds have become some of the most consumed pharmaceuticals on the continent. Many of these drugs have been frequently detected in surface waters across Africa. There is limited information available on the adverse effects of the mixtures of different types of pharmaceuticals in African aquatic environments on fish reproduction. The present study investigated the effects of the ARV drug nevirapine (NVP - 1.48 and 3.74 mg/L) and its mixture with the antibiotic sulfamethoxazole (3.68 mg/L) and trimethoprim (0.87 mg/L) on *O. mossambicus* gonads using histopathological endpoints as biomarkers.

The fish (n = 52) were exposed for 30 days in a static renewal system. Female *O. mossambicus* exposed to nevirapine (3.74 mg/L) and to NVP – antibiotic mixture recorded higher ovary indices. Statistically significant differences were found in female ovary indices between the fish exposed to NVP (3.74 mg/L) and the control fish (p = 0.002) as well as between the fish exposed to the NVP - antibiotic mixture and the control fish (p = 0.009). The main observed histopathological changes in the ovaries were increased vitellogenic oocyte atresia and vacuolation of the interstitial tissue in the fish exposed to NVP - antibiotic mixture. It is evident that the presence of NVP - antibiotics mixture in water triggered the observed
1. Introduction

The African continent has the highest prevalence of the human immunodeficiency virus and acquired immunodeficiency syndrome (HIV/AIDS) and tuberculosis (TB) co-infection (74% in 2014) as well as the highest number of patients on combined antiretrovirals (ARVs) and TB therapy (World Health Organization - WHO 2015). In 2018, the incidence of TB in South Africa alone was 3% of the global TB incidence (WHO 2019). In 2019, the population living with HIV in South Africa was estimated at 13.5% of the total population (7.97 million) which is the highest incidence worldwide (Statistics South Africa 2019). The recommended treatment of HIV and TB co-infection involves not only a combination of two or three antiretrovirals (ARVs) drugs but also a combination of two or more antimicrobials to treat or prevent TB and other opportunistic microbial infections (National Department of Health 2015; WHO 2015; Panel on Opportunistic Infections in Adults and Adolescents with HIV, 2019).

In addition to TB and HIV epidemics, the African continent has been experiencing recurring outbreaks of viral, bacterial, and parasitic epidemic diseases including the Ebola virus, Vibrio cholera, malaria, and Yellow fever virus to name a few (Fenollar and Mediannikov 2018). As if these were not enough, the newly worldwide severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2 or Covid-19) pandemic outbreak is hitting Africa badly. The WHO public health experts are predicting that the Coronavirus 2019 is here to stay and will add to the list of endemic infectious diseases worldwide (Farge and Shields 2020). One of the major consequences of these infectious diseases is that antiviral and antibiotic compounds have become some of the most consumed pharmaceuticals on the continent (Kairigo et al., 2020; Madikizela et al., 2020). Most African countries do not have proper waste-water treatment plants (WWTPs), and where those facilities exist, the techniques used were not designed to remove pharmaceuticals (Archer et al., 2017; Kairigo et al., 2020; Madikizela et al., 2020). As a result, many of these pharmaceutical compounds and their transformation products end up in the African aquatic environments creating a cocktail of different types of pharmaceuticals (Archer et al., 2017; Kairigo et al., 2020; Madikizela et al., 2020; Horn et al., 2020). Researchers around the world have indicated that these mixtures of pharmaceuticals could have greater negative effects on aquatic animals than observed for each compound alone (Geiger et al., 2016; Schoenfuss et al., 2016; Mezzelani et al., 2018).

Studies have shown that the combination of two or more drugs may result either in a synergistic, additive or an antagonistic physiological effect (Yin et al., 2014; Yuan and Chen 2019). The multi-drug combination is used in complex diseases such as HIV, TB, cancers and other complicated diseases which do not follow the original one-target one-drug concept (National Department of Health 2015; Panel on Opportunistic Infections in Adults and Adolescents with HIV, 2019). For these complex diseases, the combination of drugs targeting the different sites of the disease network in the body is needed for better results (Luo et al., 2019; Yuan and Chen 2019). The choice of the drugs to combine in a prescription is done very carefully to avoid the risk of toxicity due to drugs interactions (Luo et al., 2019; Yuan and Chen 2019). For aquatic animals which are unintentionally exposed to the mixtures of different types of pharmaceuticals present in surface waters their whole life, there is concerns that those drugs interactions may have greater negative effects on their health than a single drug exposure could cause (Schoenfuss et al., 2016; Mezzelani et al., 2018).

Amongst the pharmaceuticals of emerging concern in African surface waters are antiretrovirals and antibiotics (Madikizela et al., 2020). In South Africa, recent studies have shown that environmental levels of efavirenz (EV) and nevirapine (NVP) may have effects on liver tissue of fish (Robson et al., 2017; Nibamureke et al., 2019). Ro bson et al. (2017) reported significant histopathological changes in the liver of O. mossambicus exposed to the ARV drug EFV (10.3 ng/L 459 and 20.6 ng/L) for 96 h. However, the same study did not find significant histopathology in the fish gonadal tissue. In another study from the same research group, Nibamureke et al. (2019) found significant histopathological changes in the liver of adult O. mossambicus exposed to the ARV drug NVP (1.48 μg/L and 3.74 μg/L) for 30 days. These two studies focused on the liver tissue as the two ARV drugs, EFV and NVP, have previously been associated with liver toxicity in humans (Rivero et al., 2007). Still, information is lacking on the long-term effects of ARVs alone or in mixture with other pharmaceuticals in surface water on fish reproduction. Different studies on human HIV patients have argued that the ARVs treatment may have effects on the reproductive system which may be the cause of infertility cases observed in many HIV/AIDS patients (Pavilli et al., 2010; Kushnir and Lewis 2011; Savasi et al., 2019). Studies on the reproductive system of HIV/AIDS patients have shown abnormalities in male semen and female oocyte mitochondrial DNA as well as histopathological changes in the seminiferous tubules (Da Silva et al., 1990; Azu 2012; Savasi et al., 2019). Studies with laboratory rats treated with ARVs have also reported histological alterations of seminiferous tubules and a decrement in sperm motility (Adaramoye et al., 2012; Azu et al., 2014; Oyeyipo et al., 2018).

The broad range antimicrobial activity antibiotics such as sulfamethoxazole (SMX) and trimethoprim (TMP) are frequently prescribed to HIV patients worldwide for the prevention or treatment of opportunistic infections such as Pneumocystis pneumonia, Toxoplasma gondii, malaria and other infections (National Department of Health South Africa 2015; Panel on Opportunistic Infections in Adults and Adolescents with HIV, 2019). Together with ARVs, those two antibiotics consumption ranks amongst the highest in Africa (aus der Beek 2016; Madikizela et al., 2020); hence, their ubiquitously presence in African surface waters (Archer et al., 2017; Kairigo et al., 2020; Madikizela et al., 2020). Despite their known broad antimicrobial activity, SMX-TMP combination in humans may sometimes cause strong side effects during the first semester of pregnancy; these include a high risk of abortion or physical deformation of the foetus (Straub 2016). In addition to these, SMX is amongst antibiotics suspected to have potential endocrine disrupting properties (Kwon et al., 2016; Archer et al., 2017). Internationally, little attention is given on the possible effects of non-steroid pharmaceuticals mixtures in surface waters on the fish reproductive health (Madureira et al., 2011). Madureira et al. (2011) reported increased oocyte atresia in female zebrafish exposed to a mixture of five non-steroid pharmaceuticals (SMX, TMP, carbamazepine, fenofibrate, propranolol hydrochloride) at their environmental levels. In South Africa, the present...
study is the first one to approach the problem of ARVs and anti-
biotics mixtures effects on fish reproduction.

Since the introduction of ARVs treatment, the HIV related deaths have declined considerably worldwide, and the well-being and socio-economic situation of families and people affected by HIV have improved (Ncube et al., 2018; Mosiekiemang et al., 2019). Thus, ARVs and associated antibiotics for HIV/AIDS and TB treatment are needed more than ever and should be accessible to all people in need. According to the WHO, each country should reach at least 90% of its HIV patients on ARVs treatment by the end of 2020 (Human Sciences Research Council - HSRC 2018). On the other hand, as new and recurring infection diseases outbreaks continue to happen, new powerful drugs and multi-drug combinations are developed. Consequently, antivirals, especially, ARVs and antibiotics upload in African aquatic environments will continue to increase if nothing is done to reduce the pharmaceutical concentration of WWTPs’ effluents. Pharmaceutical mixtures in the aquatic environment are a threat to the ecological integrity of aquatic ecosystems as studies have shown that exposure of fish to mixtures of different types of xenobiotics may affect the function of the thyroid gland (Yu et al., 2015; Nugegoda and Kibria 2017). The fish thyroid hormones control important physiological functions including gametogenesis and ovulation (Yu et al., 2015). If the thyroid gland is not working as it should, this could result in reproduction problems including inhibition of gametogenesis and ovulation (Patino and Sullivan 2002; Nugegoda and Kibria 2017). It is, therefore, important to investigate the potential combined toxicity of mixtures of different types of pharmaceuticals detected in surface water on fish reproduction. Effects on fish reproduction may not only endanger ecological communities’ structure and sustainability (Comizzoli and Holt 2019) but also could hinder economies of communities that rely on income from fishing worldwide (Food and Agriculture Organization - FAO 2016).

The aim of the present study was to assess the effects of a mixture of three pharmaceuticals commonly detected in African surface waters (Madikizela et al., 2020), the ARV drug nevirapine (NVP) and the antibiotics SMX and TMP on fish reproductive organs. A semi-quantitative histological assessment protocol (Bernet et al., 1999; van Dyk et al., 2009) was used to evaluate, describe and estimate the damage to the fish gonadal tissue. Histopathological changes in tissues of organisms are considered valuable biomarkers in environmental toxicology as they give the advantage to evaluate the health of target organs affected by exposure to toxicants in the environment (Schlenk et al., 2008; van Dyk et al., 2009; Rašković and Poleksić 2017). The Southern African indigenous fish species O. mossambicus was used as the experimental organism in order to relate the risks of pharmaceutical exposure to local aquatic ecosystems.

2. Material and methods

2.1. Ethical clearance

This project was approved by the Faculty of Science Ethical Committee at the University of Johannesburg on the 20th of November 2015 (Protocol No. 201242617) before the commencement of the experiments.

2.2. Test pharmaceuticals

The test pharmaceuticals used in this study were the ARV drug NVP (C15H14N4O3; molecular weight: 290.3 g/mol; CAS- No. 129618-40-2; purity: ≥ 98%) and the antibiotics SMX (C10H11N2O3S; molecular weight: 253.28 g/mol; CAS-No. 723-46-6; purity: ≥ 98%) and TMP (C14H18N4O3, molecular weight: 290.3 g/mol; CAS-No. 738-70-5; purity: ≥ 98%). Dimethyl sulfide (DMSO) (solubility rate ≥ 22 mg/ml; purity: ≥ 99.7%) was used as a solvent for NVP and SMX (Hallare et al., 2006). Distilled water was used as solvent for TMP as it is easily soluble in water. All the test pharmaceuticals and DMSO were purchased from Sigma-Aldrich, South Africa.

The choice of exposure doses for each test pharmaceutical was guided by the environmental concentrations reported in accredited peer-reviewed journals in the period of 2015–2016. The lowest concentration of NVP (1.48 µg/L) selected for this study was detected in the Roodeplaat Dam, South Africa (Wood et al., 2015); the highest concentration (3.74 µg/L) was a value chosen between 1.48 µg/L (it was the highest concentration in South Africa in that period) and the highest concentration reported in the Nairobi river system (6 µg/L) in Kenya (K’Oreje et al., 2016). For SMX and TMP, their highest environmental concentrations (respectively 3.68 µg/L and 0.870 µg/L) detected in the Umgeni River System in Kwazulu Natal, South Africa, were used (Agunbiade and Moodley 2014; Matongo et al., 2015). The highest DMSO concentration used was 0.003 ml per 100 ml of water; this agrees with international guidelines which recommend a concentration under 0.01% v/v (Organisation for Economic Co-operation and Development - OECD 2018).

Stock solutions of the three test pharmaceuticals were prepared by mixing 10 mg of the pharmaceutical powder in a 100 ml of DMSO, thus obtaining a stock solution of 100 ppm concentration. To avoid the freezing of stock solutions containing DMSO, and at the same time to reduce the concentration of DMSO, the pharmaceutical was mixed with DMSO first, and a small amount of distilled water was added to make up to the volume. Nevirapine stock solution was kept in a freezer at -20 °C and SMX and TMP stock solutions were kept in the fridge at 4°C.

2.3. Exposure experiments

Fifty-two (52) sexually mature O. mossambicus were purchased from the Hartbeespoort Fisheries in Gauteng Province, South Africa. At arrival to the University of Johannesburg’s Research Aquarium Facility, the fish were kept for three months in quarantine tanks for observation. At the end of this quarantine period, the fish were moved into the environmentally controlled room. There, they were left to aclimate to the controlled conditions for 12 days before the start of the experiments.

The exposure of fish was conducted in 90 L glass tanks containing 75 L of water/exposure medium. The number of fish was kept at one fish per tank. Each tank was supplied with oxygen through a plastic pipe connected to a plastic filter in the tank to remove solid waste. A static renewal system was used to minimise the workload of changing the water manually (Mehler et al., 2018). The test media was renewed every 96 h to keep the test pharmaceuticals levels near the desired concentrations (OECD, 2019).

The fish were grouped in 5 exposure groups: control (4 fish), solvent control (4 fish), NVP low concentration designed as NVP L (6 fish), NVP high concentration designed as NVP H (6 fish), and the mixture of NVP, SMX and TMP referred to as NVP – antibiotic mixture in the text (6 fish). The experiment was repeated once, and the sex ratio was maintained at 1:1 when possible. Both the two exposures lasted 30 days and during this period, the fish were fed twice a day (Tilapia Grower Pellets 3 mm, AVI Products (Pty) LTD 2001/015923/07). Twenty-four hours before the end of the exposure, the feeding was stopped. As some chemicals affects fish behavior, every day the fish were checked twice, morning and evening, for any sign of stress (gasping at the surface; skin sores or white spots) or abnormal behavior in swimming (rubbing on tank surface).
walls, lying on its side, frantic swimming, etc.) and feeding (not eating). Any food waste or excrements were removed.

During the first week of exposure, one fish from the solvent control group jumped out of the tank and was found dead on the floor in the morning. After this incident, all the tanks were covered with a glass plate. This explains why the sample size in the result section is 51 and not 52 fish. The temperature of the water in tanks was kept at 26.5 ± 1.5°C; a day/night photoperiod cycle of 14:10 h was maintained to mimic summer conditions, and the dissolved oxygen was monitored regularly to ensure acceptable level between 80% and 120% (OECD, 2012). At the end of the experiment, water samples analysis showed that all physical and chemical water parameter were within the guidelines for the fish toxicity tests and for the O. mossambicus species optimal water parameters for reproduction (OECD 2012; Centre for Agriculture and Bioscience International - CABI 2019).

2.4. Histological assessment

At the end of the exposure experiment, the fish were weighed, the total length was measured, and the fish were killed by severing the spinal cord. A necropsy, external and internal, was performed, the gonads were removed and measured (weight and length). All the measurements were rounded to the nearest two decimals places. The gonadosomatic index (GSI) was then calculated using the weight and length of the gonads following the formula by Adams et al., (1993):

\[ \text{GSI} = \frac{\text{Gonad mass (g)}}{\text{Total body mass (g)}} \times 100 \]

The gonads from each fish were fixed as whole in Bouin’s solution for 24 h. The tissue processing was done following the standard histological processing technique (Humason 1979). After 24 h, the tissues were removed from the fixative and each gonad was cut into 4 to 6 small pieces (±5 mm long) depending on the length of the gonad. The tissues were then washed over-night in slow running water. The following morning, the tissues were dehydrated in increasing ethanol concentrations (30%–70%) and 95% ethanol mixture group. For data analysis, as no statistically significant difference was found in GSI of female and male fish (p = 0.157 and p = 0.881 respectively) and in ovary and testis indices (p = 0.119 and p = 0.285 respectively) between the control and the solvent control groups, the two groups data were combined to make one larger control group. The IBM SPSS Statistics software (version 25) was used for data analysis at a statistical significance level (p) < 0.05. The Shapiro-Wilk test was conducted to check for normality of the data distribution (small sample size < 55) and Levene’s test was used to test the homogeneity of variances. To compare fish organ indices between the different groups, the non-parametric test Kruskal–Wallis followed by the Mann-Whitney U test were used.

2.5. Pharmaceutical analysis from the exposure media

To confirm that the exposure mediums were kept at the concentration values near the expected concentrations of the test pharmaceuticals during the whole experiment, water samples were collected for analysis at three separate times from different tanks: at the beginning of the exposure, after 96 h and after each water renewal. The samples were analysed in an ISO 17025 accredited laboratory at the North-West University Potchefstroom Campus, South Africa. The nominal concentrations of NVP (1.48 μg/L and 3.74 μg/L) chosen for the present study are from previously published studies (Wood et al., 2015; K’Oreje et al., 2016). The analysis of NVP from water samples showed a mean recovery rate of 79% with an average recovery standard deviation (RSD) of 10%. This confirmed the presence of NVP in the water samples collected at the start of the exposure, after 96 h and after the renewal of the exposure media. The average concentrations of NVP L (1.48 μg/L) at the start of the experiment was 1.78 μg/L, 1.46 μg/L after 96 h and 2.16 μg/L 30 min after renewal of the test media. For NVP H (3.74 μg/L) the average concentrations were 3.85 μg/L, 4.24 μg/L and 3.94 μg/L. Nevirapine was below the instrument detection limits in the control water samples.

2.6. Data analysis

Five exposure groups were used for this study; those were the control group (bore whole water only), the solvent control group (diluted DMSO), NVP L group, NVP H group, and NVP - antibiotic mixture group. For data analysis, as no statistically significant difference was found in GSI of female and male fish (p = 0.157 and p = 0.881 respectively) and in ovary and testis indices (p = 0.119 and p = 0.285 respectively) between the control and the solvent control groups, the two groups data were combined to make one larger control group. The IBM SPSS Statistics software (version 25) was used for data analysis at a statistical significance level (p) < 0.05. The Shapiro-Wilk test was conducted to check for normality of the data distribution (small sample size < 55) and Levene’s test was used to test the homogeneity of variances. To compare fish organ indices between the different groups, the non-parametric test Kruskal–Wallis followed by the Mann-Whitney U test were used.

3. Results

3.1. Necropsy and gonadosomatic index (GSI)

Macrosкопically, the gonads showed a few abnormalities; these included swollen ovaries from a female fish in the NVP H group and shrunken ovaries from another female fish from the NVP – antibiotic mixture group.

The highest GSI (5.25) in female fish was from the NVP – antibiotic mixture group and so was the highest GSI for male fish (0.67). Statistical analysis (Kruskal-Wallis test) indicated no significant difference in GSI values across the groups for both female (p = 0.571) and male fish (p = 0.072) (see Fig. 1).
3.2. Ovary histology

*Oreochromis mossambicus* ovarian tissue consists of multiple ovarian follicles in different stages of development. The ovary is enveloped by a thin connective tissue, the tunica albuginea (Fig. 2A). The younger germinal cells, oogonia, are smaller and they are grouped in oogonia nests between the more developed follicles (Fig. 2G). The mature follicles, oocytes, are larger. Using Wallace and Selman (1981) and van Dyk (2006) classification of oocytes developmental stages in teleost fish, five different oocyte development stages were observed in this study. Firstly, stage 1 oocyte (O I) which appear oval-shaped with a dark red or purple cytoplasm (H&E stain), a large nucleus and nucleolus (Fig. 2B). They are found in groups between mature follicles. Secondly, stage 2 oocyte (O II) or peri-nucleolar, they are slightly larger with multiple nucleoli at the periphery of the nucleus (Fig. 2B). Thirdly, stage 3 oocyte (O III) or cortical alveolar are visibly larger compared to stage 2 and stained light purple (H&E stain); numerous lipid vesicles (cortical alveoli) are present close to the periphery (Fig. 2A). The chorion or zona radiata and perifollicular cells (forming a three-layered envelope of the oocyte) are visible at this stage. Fourth, stage 4 oocyte (O IV) or vitellogenic oocytes which are larger than stage 3 oocytes; their nucleus is in the center, and small yolk globules are present in the periphery of the cytoplasm. In the late vitellogenic stage, the yolk globules or vitellogenic granules are increased and almost fill the cytoplasm. The thick chorion (CH), pale eosinophilic (H&E), is

Table 1  
Percentage prevalence of observed histopathological changes in the gonads of both female and male fish in all the groups.

|                      | Control | NVP L | NVP H | Mixture | Total |
|----------------------|---------|-------|-------|---------|-------|
| **Histopathological changes in the ovaries** |         |       |       |         |       |
| Haemorrhage           | 0       | 0     | 0     | 1       | 4     |
| Ovary                | 0       | 0     | 0     | 1       | 2     |
| Inhibition of oogenesis | 6      | 5     | 5     | 7       | 24    |
| Oocytes              | 0       | 0     | 0     | 1       | 2     |
| Atresia              | 6       | 5     | 5     | 7       | 24    |
| Necrosis             | 0       | 0     | 0     | 1       | 2     |
| **Histopathological changes in the testes** |         |       |       |         |       |
| Vascular congestion  | 4       | 5     | 4     | 6       | 14    |
| Interstitial tissue  | 6       | 7     | 6     | 5       | 20    |
| Necrosis             | 0       | 1     | 0     | 0       | 1     |

* The percentage prevalence expresses the number of fish that showed the histopathological change not the intensity or severity of the histological change. The severity of the histopathological change is shown by the gonad index.

**Fig. 1.** Comparison of mean gonadosomatic indices (GSI) (%) of fish from the different groups. No statistically significant difference was found between the groups for both female and male fish ($p > 0.05$).
clearly visible surrounded by the oocyte envelope; and the cortical alveolar material is still visible, pushed at the periphery of the cytoplasm (Fig. 2A). Lastly, the maturation stage, stage 5 oocyte (OV) are slightly larger than stage 4 with many large yolk globules distributed throughout the cytoplasm; the germinal vesicle breaks down and is inexistent in fully mature oocytes (Fig. 2B). Although all the above five oocyte stages were observed in this study, stage 4 (vitellogenic) and 5 (mature) oocytes were more frequent in the ovaries of most of the fish. Degenerating oocytes or atretic oocytes (AO) were also observed in almost all the fish; they are

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**Fig. 2.** A - B. Organisation of *O. mossambicus* ovarian tissue from a control fish. The tunica albuginea (TA); follicles in different stages of development: stage 1 oocytes (OI); stage 2 oocytes (OII); stage 3 oocytes (OIII); stage 4 oocytes (OIV); stage 5 oocyte (OV) and atretic oocyte (AO). Note the envelope with an irregular shape. The perifollicular envelope (OM) surrounding the chorion (CH). C. Increased oocytes atresia (AO) in a fish exposed to NVP H. Note the younger oocytes stages still intact next to the atretic ones. D. A necrotic ovary (NO) in a fish exposed to NVP H. Note the folded tunica albuginea. E. A fish from the Mixture group showing a dissolved follicle envelope of the atretic oocytes (AO, arrows). Note the intact stage 4 oocytes near the atretic mass. F. Vacuolation (stars) of the oogonial (OG) nest tissue in a fish exposed to NVP. G. Oogonial nest (OG) from a control fish showing small primordial germ cells; oogonia (arrows). H. A necrotic ovary (NO) from a fish exposed to the mixture of pharmaceuticals. Most of the tissue is necrotic.
Using McDonald et al. (2000) the testes cysts contain mostly spermatogonia and spermatocytes with a large, dark nucleus. In sexually mature male cells have a faintly vacuolated cytoplasm and a round oval, dense nucleus stained with H&E (Fig. 2C and E). The observed atretic oocytes were in stage 4 (vitellogenic). Post-ovulatory follicles characterised by a collapsed oocyte envelope (the three-layered perifollicular envelope) and the absence of yolk material inside were not observed in the fish from the present study.

The prevalent histological changes observed in ovaries were atresia or degeneration of vitellogenic oocytes in 88.89% of all fish and vacuolation of the oogonial nests also in 88.89% of all fish. Vacuolation of the oogonial nest was characterised by the presence of big clear and empty vacuoles in the germinal epithelium (Fig. 2F). Although most of the fish, exposed and the control, presented oocyte atresia, it was increased in the fish exposed to NVP H and NVP − antibiotic mixture. While control fish ovaries showed one or two atretic oocytes (Fig. 2A), in the fish exposed to NVP H and to NVP − antibiotic mixture the number of atretic oocytes was high (more than two atretic oocytes) (Fig. 2C and E). In many cases, the envelope of the atretic oocytes in the exposed fish had disintegrated and the content of the follicles (yolk material) was spread in the ovaries. Other observed changes in ovaries were haemorrhage (14.81%) and necrosis (11.11%) in two fish exposed to NVP H (Fig. 2D) and to NVP − antibiotic mixture (Fig. 2H). Necrosis in one fish (from the NVP − antibiotic mixture group) had taken over the whole ovary tissue visibly resulting in inhibition of oogenesis in the ovary. For this fish, inhibition of oogenesis was scored as the final endpoint (Fig. 2H). The percentage prevalence (number of fish presenting the pathology) of all the observed histopathological changes in ovaries is given in Table 1.

### 3.3. Testis histology

The O. mossambicus adult testis tissue consists of multiple sacs/cysts or seminiferous lobules containing different stages of development of male reproductive cells. All the seminiferous lobules are enclosed by an envelope, the tunica albuginea. Each stage of male reproductive cells forms a distinctive group inside the lobule. All developmental stages of male reproductive cells (spermatogenesis stages) were observed in this study. Those included spermatogonia (SG), spermatocytes (SC), spermatids (SD), and spermatozoa (SZ) (Fig. 3A). The young immature cells, spermatogonia, are normally found towards the periphery of the lobule. Spermatogonia are large, and oval shaped with a large clear nucleus and cytoplasm. In contrast, the mature cells, spermatozoa, are small with a dark blue nucleus stained with H&E (the cytoplasm is not visible). Spermatogonia are found in the lumen of the seminiferous lobule or spermatogonial cyst. The space between the seminiferous lobules (interlobular area) contains blood vessels and Leydig cells responsible for producing male reproductive hormones (Fig. 3B). Leydig cells have a faintly vacuolated cytoplasm and a round oval, dense dark nucleus. In sexually mature male fish testes cysts containing spermatozoa were predominant while for sexually immature fish, the testes cysts contain mostly spermatogonia and spermatocytes. Using McDonald et al. (2000) fish gonads sexual maturity classification, most of the testes of male fish in this study were classed in stage 2 (mid-spermatogenic) and stage 3 (late spermatogenic). In stage 2, mid-spermatogenic, there is an equal mix of all developmental stages, while in stage 3, mature cells, late spermatogenic, spermatozoa were the most predominant.

The most prevalent histopathological change in the testis tissue of most of the fish was vacuolation (clear vacuoles) observed in the testis interstitial tissue around Leydig cells (in 83.33% of all fish, Fig. 3E) as well as vacuolation of spermatocytes (75%, Fig. 3D) and spermatids (79.17%, Fig. 3F). Other observed changes were vascular congestion (58.33%) and intersex, characterised by the presence of oocytes inside the testis tissue (41.67%, Fig. 3C). These histological changes were present in the exposed fish as well as in the control groups. However, compared to the control fish, the fish exposed to the test pharmaceuticals presented a slight increase in changes. Table 1 shows the percentage prevalence (number of fish presenting the pathology) of observed histopathological changes in the testis tissue.

#### 3.4. Semi-quantitative histological analysis

In general, female fish exposed to the test pharmaceuticals recorded higher ovary indices compared to the control fish (Fig. 4A). The NVP H group recorded the highest mean ovary index (19.43 ± 8.38) followed by NVP − antibiotic mixture group with a 17.25 ± 9 mean ovary index, then the NVP L and the control groups with a mean ovary index of 13.20 ± 30.3 and 8 ± 5.3, respectively. Statistical analysis showed a significant difference in female gonad index across the groups (p = 0.025). Post-hoc analyses revealed significant differences between the control fish and the fish exposed to NVP higher concentration (p = 0.004) and between the control fish and fish exposed to NVP − antibiotic mixture of pharmaceuticals (p = 0.024).

Exposed male fish showed slightly higher mean testis indices compared to the control fish. The NVP H group recorded the highest mean testis index (11.60 ± 4.56); the NVP − antibiotic mixture group mean testis index was 11 ± 7.4 while it was 9.43 ± 3.21 for the NVP L group and 8 ± 4.41 for the control group. There was no statistically significant difference in male fish testis indices across the groups (p = 0.563; Fig. 4B).

#### 4. Discussion

Studies on the occurrence of human pharmaceuticals in African aquatic environments are emerging from different parts of the continent; however, studies on the potential effects of those pharmaceuticals on aquatic organisms are scarce. High levels of some types of pharmaceuticals including ARVs and antibiotics were detected in African surface waters. Scientific publications exist on mixtures of different types of xenobiotics associated with impairment of hormonal functions in fish (Yu et al., 2015; Nugegoda and Kibria 2017). In addition, other studies have discussed that mixtures of previously known non-steroid compounds may mimic steroid hormones properties in aquatic environments (Thienpont et al., 2011; Kwon et al., 2016; Archer et al., 2017). Thus, it is important to investigate the risk of mixtures of non-steroid pharmaceutical compounds in African surface waters on indigenous fish species reproduction. In this laboratory exposure study, we assessed the effect of a mixture of environmental concentrations of three pharmaceuticals commonly detected in African surface waters (the antiretroviral drug NVP and the antibiotics SMX and TMP) on adult O. mossambicus gonadal tissue. Significant histopathological responses were observed in the ovaries of female O. mossambicus exposed to the high concentration of NVP (3.74 μg/L) and to NVP − antibiotic mixture. Histopathological changes in exposed male fish testis tissue were not significantly different from the control fish. However, in both male and female fish, the mean testis and ovary indices of the fish exposed to NVP (3.74 μg/L) and to NVP − antibiotic mixture were higher meaning that the gonadal tissue was reacting to those pharmaceuticals. The most concerning histological change observed in the exposed female fish ovaries was increased atresia of vitellogenic and mature oocytes. Oocyte atresia is considered as a normal physiological process in fish which happens to oocytes that have failed to mature or ovulate; this normally
happens at the end of the spawning period (Blazer 2002; Smith and Walker 2004; Lubzens et al., 2010; Brown-Peterson et al., 2011). Nevertheless, many studies have found that exposure to steroid compounds or other xenobiotic compounds exhibiting endocrine disrupting properties can lead to an abnormal high rate of oocyte atresia in female fish (Kime 1995; Van Den Belt et al., 2002; Maack and Segner 2003; Madureira et al., 2011). Since atresia consists in degeneration of vitellogenic oocytes before they mature or mature oocytes before ovulation, if it happens at a high rate it can affect the fertility of the fish (Miranda et al., 1999). A reduction in female fish reproductive activity is a great danger to the fish species survival and will unavoidably affect the whole fish population (Schoenfuss et al., 2016).

There is insufficient information on the effects of non-steroid human pharmaceuticals such as ARVs, antibiotics and their mixtures in surface waters on fish reproduction. In Spain, data published by Madureira et al. (2011) on the exposure of male and female zebrafish to a mixture of five non-steroid pharmaceuticals (SMX, TMP, carbamazepine, fenofibrate, propranolol hydrochloride) at their environmental concentrations showed a decrease in mature gametes in both male and female fish. Exposed female fish in the same study also showed a high frequency of oocyte atresia in the ovaries. Based on these findings, Madureira et al. (2011) stated that non-steroid pharmaceutical mixtures may have the potential to disrupt the reproductive system of fish. The same study stressed the importance to assess the health risk of different mixtures of pharmaceuticals present in surface waters on fish reproduction.

In humans, there is mixed research data on the safety of the use of the antibiotic combination SMX-TMP during pregnancy. Some studies have reported potential risks of abortion and physical...
deformation of the foetus in the first semester of the pregnancy (Richardson et al., 2000; Czeizel et al., 2001; Straub 2016). Other studies have reported that the risk of birth defects and malformation of the foetus in pregnant women is low and that the benefit of the medication outweighs its potential health risks (Ford et al., 2014).

Evidence from previous studies have shown that exposure to mixtures of different types of xenobiotic compounds may affect the function of the thyroid gland in fish (Yu et al., 2015; Nugegoda and Kibria 2017). The thyroid gland, which is part of the hypothalamic-pituitary-thyroid axis, is involved in the synthesis, release or regulation of different hormones that control important physiological activities including reproduction, growth, and development (Nugegoda and Kibria 2017; Leemans et al., 2019).

The role of the thyroid gland in fish reproduction has been studied extensively; thyroid hormones control gametogenesis and ovulation (Patino and Sullivan 2002; Yu et al., 2015; Nugegoda and Kibria 2017). Most of these studies have stressed that the presence of different types of xenobiotic compounds in surface water is a threat to fish communities as those chemicals interaction in mixtures can inhibit the thyroid gland function. A dysfunctional thyroid gland in fish may result in the inhibition of gametogenesis, delayed gonad development and reduced egg fertilization rate (Nugegoda and Kibria 2017). In sexually mature female fish particularly, a dysfunctional thyroid gland may inhibit ovulation and increase the development of ovarian cysts (Patino and Sullivan 2002; Mendola et al., 2008).

Although limited information is available on the possibility that antibiotics (such as SMX in mixture with other pharmaceuticals) may affect fish reproduction through their effect on the hypothalamic-pituitary-thyroid axis (Thienpont et al., 2011; Kwon et al., 2016), there is no information available on ARV compounds alone or in mixture with other pharmaceuticals affecting reproduction in fish. In humans, however, research data are available on the combined ARVs therapy effects on the reproductive system. Many studies have mentioned that the combination of ARVs in the highly active antiretroviral therapy (HAART) used in the HIV treatment may have negative effects on the male reproductive system (Da Silva et al., 1990; Kushnir and Lewis 2011; Azu 2012). The negative effects observed in male HIV patients on HAART regimen include histopathological changes such as seminiferous tubules atrophy, and alteration in semen parameters including reduced sperm motility and altered sperm mitochondrial DNA (Pavili et al., 2010; Savasi et al., 2019). Conflicting results have been reported on female HIV patients receiving the HAART; some studies reported no negative effects either on the reproductive system or the foetus (Patel et al., 2005; Myer et al., 2010; Kushnir and Lewis 2011) while other studies reported impaired oocyte mitochondrial DNA that may lead to reduced fertility (Lopez et al., 2008; Kushnir and Lewis 2011; Hernandez et al., 2017). Research data on rats treated with ARVs and their combinations have reported testicular alterations including degeneration of seminiferous tubules as well as a decrease in sperm motility in male rats (Azu et al., 2014; Awodele et al., 2018; Oyeyipo et al., 2018). In female rats, effects in oocytes including mitochondrial DNA depletion, decreased number of follicles, absence of oocyte maturation and increased oocyte apoptosis were observed (Awodele et al., 2018; Tang et al., 2018).

The mechanism of how the combined ARVs or their combination in the HAART therapy affect the reproductive system is still not well understood, but some studies have proposed that it may be through the sperm and oocyte mitochondrial DNA toxicity which can lead to changes in sperm parameters, absence of oocyte maturation and high rate of oocyte apoptosis (Pavili et al., 2010; Kushnir and Lewis 2011; Morén et al., 2014; Hernandez et al., 2017). As mitochondrial DNA is inherited from the mother to the embryo; the consequences of a decrease in oocyte mitochondrial DNA due to toxicity, would be inhibition of oocyte maturation, decreased fertilization, embryo development delay or failure to get pregnant (Osellame et al., 2012; Morén et al., 2014; Hernandez et al., 2017).

In addition to mitochondrial DNA toxicity of germ cells, the HAART is also suspected to affects the hypothalamic-pituitary-thyroid axis resulting in the high rate of thyroid disorders observed in HIV patients on the HAART (Calza et al., 2002; Beltran et al., 2003; Madeddu et al., 2006; Hoffmann and Brown 2007; Nelson et al., 2009; Joshi et al., 2016; Ji et al., 2016; Ibrahim et al., 2019). Few studies have also discussed that ARVs and their combinations may have negative effects on reproductive hormones in both male and female rats (Awodele et al., 2018). However, the mechanism by which ARVs may affect reproductive hormones production and metabolism is still to be investigated (Beltran et al., 2006).

Considering the available data on the potential effects of the HAART regimen on the reproductive system in humans and rats, it would not be wrong to state that NVP and its mixture with the antibiotics SMX and TMP in the present study were responsible for the increased oocyte atresia observed in exposed female fish ovaries. This is supported by the mean ovary indices which were

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**Fig. 4.** Comparison of mean gonad indices (summed histopathological scores for ovary and testis) from male and female fish in the different groups. Significant differences were found in ovary indices between groups ($p = 0.025$). The arrows show the groups which were significantly different from the control group.
higher in the exposed fish compared to the control group. According to Bernet et al. (1999) and as confirmed by several fish histopathological studies, a high organ index (the summed histopathological score for a specific target organ) usually corresponds to a high degree of damage to the tissue (van Dyk et al., 2009; Rasković and Poleksić 2017). There are very few published data on the effects of ARV drugs on fish reproductive organs; Robson et al. (2017) found no significant difference in gonad indices of male and female *O. mossambicus* exposed to the HIV drug efavirenz (10.3 ng/L and 20.6 ng/L) for 96 h, but the exposed fish had higher gonad indices compared to the control fish. Available studies on male and female rats treated with EFV and NVP as single toxicants for four to six weeks observed alterations in the testis and ovary tissues as well as a reduction in the level of reproductive hormones (Adramoye et al., 2012; Awodele et al., 2018).

Although the ovaries of female fish exposed to NVP 3.74 μg/L and those exposed to the mixture of NVP (1.48 μg/L) and antibiotics showed increased atresia, no significant change was observed in the GSI. This is not unheard of as Madureira et al., (2011) also reported strong evidence of pharmaceuticals mixtures that can disrupt the reproductive system by causing a change in the histology of the gonads without affecting the GSI. For some reason, not clear, the presence of NVP and the antibiotics (SMX and TMP) triggered atresia of vitellogenic oocytes and vacuolations of interstitial tissue in exposed female fish. These pharmaceuticals may have acted as EDCs and induced atretic oocytes at an abnormally high rate; this which may lead to impaired fish reproduction as observed by Diniz et al. (2005) and Madureira et al. (2011). More studies are needed to investigate further the mechanism by which these pharmaceuticals have induced the observed effects in female fish ovaries.

### 5. Conclusion

In this study, adult *O. mossambicus* were exposed to a mixture of the antiretroviral drug NVP and the antibiotics SMX and TMP at environmental relevant concentrations in South African surface waters. It was found that the chronic exposure to the mixture of the three pharmaceuticals may cause increased atresia of vitellogenic oocytes in female fish ovaries. This increased incidence of atresia of vitellogenic oocytes may lead to reduced fertility; consequently, it may result in a reproduction problems. It was also found that the concentration of NVP (1.48 μg/L) reported in South African surface waters during the period of 2015–2017 had no significant effects on the fish gonad histology. However, the NVP concentration reported from surface waters in Kenya in the same period (>3.74 μg/L) may have chronic effects on female fish ovaries. As this was a semi-quantitative histological assessment on gonad fish, we recommend a full quantitative study using reproductive biomarkers and stereological techniques to quantify the effects of the mixture of these pharmaceuticals on fish reproduction and reproducitivity. In addition, a study on effective methods of pharmaceuticals waste disposal and wastewater disinfection to reduce the concentrations of pharmaceutical compounds as well as their metabolites in the African aquatic environments is needed.

### Credit statement

GM Wagenaar: Conceptualization of the research project; Data curation; Funding acquisition; Methodology: Project administration; Resources; Supervision; Validation; Visualization; Review & editing; Corresponding author. UMC Nibamureke: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Writing the original draft; Review & editing.

### Declaration of competing interest

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.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.129900.

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