Effects of 17α-ethinylestradiol-based contraceptives on ovarian tissue using histopathological examination and FSH, LH, CYP1A, and CYP3A expressions using qRT-PCR in the ovaries and brain tissues of common carp (Cyprinus carpio L.)

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ABSTRACT
In this study, the main aim was to identify the effects of exposure to 17α-ethinylestradiol-based contraceptives on the ovaries and brain tissues of common carp. For this purpose, histopathological changes were evaluated in ovaries after 21 days of contraceptive exposure. In addition, the expression levels of the CYP1A, CYP3A, FSH, and LH genes were measured in contraceptive-exposed brain tissue and ovaries respectively, using qRT-PCR (p<0.05, p<0.01, and p<0.001, respectively). No histopathological lesions, including inflammation, degeneration, or necrosis in the evaluated ovarian tissue of common carp were found after both high and low levels of exposure to contraceptives. Therefore, it is concluded that these contraceptives were highly dangerous to the reproduction system of common carp; the accumulation of contraceptive waste in the water supply could adversely affect and threaten the common carp population. This may indirectly also affect other animals.

Key words: Brain, Ovary, Contraceptive, Common carp, FSH, Histopathology, LH, qTR-PCR, 17α-ethinylestradiol.

INTRODUCTION
The endocrine systems of animals in nature, such as fish, can be disrupted by certain chemicals in the environment (León-Olea et al., 2014; Dirican et al., 2015; Karataş 2016; Karataş and Albayrak 2018). It has been reported that large amounts of estrogenic chemicals have contaminated the environment in recent years (Hu et al., 2017). These chemicals cause sexual disorders in fish (Jobling et al., 1998; Vajda et al., 2008; Lange et al., 2009; Ziyalan and Ince, 2011) and a decrease in population in many animal species (Thorpe et al., 2009). 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) are among the most commonly used contraceptives. E2 and EE2 attract the most attention among the millions of estrogenic chemicals in the environment. Contraceptives are commonly used by humans; however, they pose a potential risk for aquatic organisms since they reach aquatic environments via human wastes.

MATERIALS AND METHODS
Chemical: Contraceptives were purchased from pharmacy as a commercial product. Reginon 0.020 mg/0.075 mg tablet (Koçak Farma Istanbul/Turkey) was used for this purpose. It contains 0.02 mg ethynylestradiol for each pill.

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Fish: 48 female fish for toxic group, and 24 female fish for control group were obtained from Atatürk University, Faculty of Fisheries and the Inland Water Fish Breeding and Research Center. Fish were fed ad-libitum with pellet feed twice a day for 60 days in a stock pond to provide their acclimatization to the environmental. Each aquarium (volume 70 L) contained 12 fish and totally 72 fish were used in the present study.

The fish were exposed to the contraceptive pills for periods of 21 days in static aquarium systems. EE2 exposure concentrations for common carp were selected according to previous study (Huang et al., 2015). The fish in group I and II were the control. The fish in groups III and IV were given dose of 0.6 µg/L, V and VI were given dose of 0.3 µg/L concentrations of EE2 for 21 days. At the end of the exposure period, all the fish were immediately sacrificed by decapitation. The half of ovarium tissues were taken for histopathological examination, also another half of ovarium tissues and brain were quickly removed and stored at -80 °C for the total RNA isolation.

At the end of the experiment, ovarium tissue samples were stored for 1 day being fixed in a 10% buffered formalin solution for histopathology. Tissue samples were washed with tap water before routine serial treatment of samples with graded alcohol and xylene were performed in Shandon Citadel 2000 tissue system. After routine histopathological processing, ovarium samples were embedded in paraffin block and 5 µm sections were prepared using a rotary microtome. All sections were stained with haematoxylin and eosin (H&E) for standard histopathological evaluation. Slides were examined under the light microscopy.

For the evaluation of mRNA expression of CYP1A, CYP3A, FSH and LH, real-time PCR analysis was performed in CFX96 TouchTM Real-Time PCR Detection System. Total RNA was isolated from ovarium and brain tissues using TRIZOL reagent according to the manufacturer’s instructions. The RNA samples were treated with RNase-free water with DEPC and then cDNA synthesis was performed using QuantiTect Reverse Transcription from 1µg of the treated RNA according to manufacturer’s instructions. 1µg each cDNA was used as templates for amplification using SYBER Green Master Mix and gene specific primers. Real Time PCR primers (GAPDH, CYP1A, CYP3A, FSH and LH) were designed according to the sequence of common carp (Cyprinus carpio L.) using the primer design program Oligo 6.0 and Primer 5.0. These primers and their PCR conditions are given in Table 1. GAPDH was used as an internal control for qRT-PCR. Each PCR reaction which was performed in triplicate. The specificity of PCR amplification was confirmed by agarose gel electrophoresis and melting curve analysis. Relative fold of expression of genes was determined with the 2^-ΔΔCT method (Livak and Schmittgen, 2001)(Table 1).

Statistical analysis of all data was performed using SPSS 16. One-way ANOVA was used to analyse the mRNA levels of CYP1A, CYP3A, FSH and LH between treatment and control groups. This is followed by Tukey’s post-hoc test was performed using the Graph pad prism software. qRT-PCR results are expressed as mean ± SEM. Statistically differences were considered to be significant at p<0.05, p<0.01 and p<0.001. The number of mature oocytes were classified according to developmental stages of oogenesis (Kagawa et al., 2013). In total 10 randomly selected microscopic areas at 20X magnification from each slides of fish exposed to EE2 were examined and mature oocytes were counted. Obtained scores

### Table 1: Primer sequences and qRT-PCR conditions

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5’-3’</th>
<th>Length (bp)</th>
<th>Accession no</th>
<th>Reaction Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F: TCCGTCTTGAAAGAACCCTGTG&lt;br&gt;R: ATTCACCTACACTGACCCA</td>
<td>212</td>
<td>AJ870982.1</td>
<td>94°C 15 s / 55°C 30 s / 72°C 30 s (40 cycles)</td>
</tr>
<tr>
<td>CYP1A</td>
<td>F: TTCTTCTTTTCACCATTCC&lt;br&gt;R: TCAAGATTGAAGCTTGATGG</td>
<td>149</td>
<td>AB048939.1</td>
<td>94 °C for 15 s, 59 °C 30 s / 72 °C 30 s. (40 cycle)</td>
</tr>
<tr>
<td>CYP3A</td>
<td>F: CACAAGAAGAAGCGAGTGGA&lt;br&gt;R: TTCAGAGATTGAAGCTTGATGG</td>
<td>146</td>
<td>GU046696.1</td>
<td>94 °C for 15 s, 61°C 30 s / 72 °C 30 s. (40 cycle)</td>
</tr>
<tr>
<td>FSH</td>
<td>F: GGTATGTTGATGCTGTCC&lt;br&gt;R: TGGTGCTCATTTGATGCAG</td>
<td>127</td>
<td>AB003583.1</td>
<td>94 °C for 15 s, 58 °C 30 s / 72 °C 30 s. (40 cycle)</td>
</tr>
<tr>
<td>LH</td>
<td>F: CATTAGCGGATGACTCTTGG&lt;br&gt;R: ATGAAGCATGCATTACCCA</td>
<td>195</td>
<td>X59889.1</td>
<td>94 °C for 15 s, 56 °C 30 s / 72 °C 30 s. (40 cycle)</td>
</tr>
</tbody>
</table>
were analyzed by One-way ANOVA statistical methods. Value of results $p<0.05$ was considered statistically significant.

**RESULTS AND DISCUSSION**

No histopathological lesions were observed including inflammation or degeneration, in either the control or the application groups. However, we detected the main differences between these groups according the oogenesis stages of follicles. The oogenesis stages in control groups were normal. Immature and mature oocytes at various stages were detected during microscopic evaluations (Fig. 1A, B, C). In the application groups, the number of mature follicles was lower than the number in control groups. There were statistically significant differences between control and application groups ($p<0.05$). In low-dose exposure to contraceptives, folliculogenesis was more active than with high-dose exposure and there were more mature follicles in the low-dose group ($p<0.05$).

**Table 2:** Showing changes of CYP1A, CYP3A, FSH, and LH gene expression levels in high and low dose EE2-based birth control pills exposed to brain and ovarium tissues.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Dosage</th>
<th>Cont</th>
<th>21 days exposure</th>
<th>Levene sig</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CYP1A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low dose</td>
<td>$0.82\pm0.01$</td>
<td>$1.64\pm0.01^*$</td>
<td>0.959</td>
<td></td>
</tr>
<tr>
<td>High dose</td>
<td>$0.82\pm0.01$</td>
<td>$2.32\pm0.08^{**}$</td>
<td>0.959</td>
<td></td>
</tr>
<tr>
<td><strong>CYP3A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low dose</td>
<td>$0.64\pm0.01$</td>
<td>$1.42\pm0.1^*$</td>
<td>0.683</td>
<td></td>
</tr>
<tr>
<td>High dose</td>
<td>$0.64\pm0.01$</td>
<td>$1.93\pm0.01^{**}$</td>
<td>0.683</td>
<td></td>
</tr>
<tr>
<td><strong>FSH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low dose</td>
<td>$1.44\pm0.01$</td>
<td>$0.65\pm0.1^{***}$</td>
<td>0.657</td>
<td></td>
</tr>
<tr>
<td>High dose</td>
<td>$1.44\pm0.01$</td>
<td>$0.42\pm0.8^{***}$</td>
<td>0.657</td>
<td></td>
</tr>
<tr>
<td><strong>LH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low dose</td>
<td>$1.24\pm0.01$</td>
<td>$2.41\pm0.01^{**}$</td>
<td>0.747</td>
<td></td>
</tr>
<tr>
<td>High dose</td>
<td>$1.24\pm0.01$</td>
<td>$3.17\pm0.2^{***}$</td>
<td>0.747</td>
<td></td>
</tr>
</tbody>
</table>

The results of CYP1A, CYP3A, FSH, and LH gene expressions are expressed as Mean ± SE. (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Levene significance $P>0.05$.

A qRT-PCR was conducted to measure the FSH and LH mRNA transcription levels in the ovaries of common carp that had been exposed to EE2-based contraceptives for a long time. The results showed a decrease in FSH mRNA transcript levels in fish exposed to low and high doses ($p<0.01$ and $p<0.001$, respectively) (Fig. 2A). On the other hand, both doses showed an important increase in the LH mRNA transcript level ($p<0.01$ and $p<0.001$, respectively) (Fig. 2B). These findings show that EE2-based contraceptives down regulate FSH gene expression in common carp while it up regulates LH gene expression. Gene expression data normality and homogeneity for FSH and LH are shown in Table 2.

A qRT-PCR was performed to measure the CYP1A and CYP3A mRNA transcription levels in the brains of common carp that had been exposed to EE2-based contraceptives for a long time. The results showed that both low and high doses of contraceptive exposure upregulated the level of CYP1A and CYP3A mRNA expression in the brains of common carp ($p<0.05$ and $p<0.01$, respectively) (Figs. 3A and 3B). Gene expression, data normality, and homogeneity for CYP1A and CYP3A are shown in Table 2. Founded that the application of both low- and high-dose EE2-based contraceptives resulted in a statistically significant reduction in FSH gene expression in common carp ovaries. Harris et al. (2001) showed that FSH expression and plasma FSH content decreased in female rainbow trout that were exposed to estrogenic contaminants. Although any histopathological lesions were not observed in all ovaria, we detected serious diminish in the number of mature oocytes. In addition we observed that contraceptives exposing down regulates the FSH mRNA transcript levels. The decreasing number of mature oocyte confirmed that transcriptional results. This result indicated that exposing to this contraceptives drug suppressed the oogenesis in the ovary tissues of common carp. In addition, we showed in this study that long-term exposure to EE2-based contraceptives statistically increased the level of LH mRNA expression in common carp. Harding et al. (2013) showed

![Fig 1: Ovarium tissues of common carp. A) Normal histology. Mature follicles (arrows) and early stage follicles (arrow head). Control group. B) Mature follicles (arrows) and early stage follicles (arrow head). Low dose group. C) Mature follicles (arrows) and early stage follicles (arrow head). High dose group. H&E. 100 x.](image-url)
that LH mRNA expression increases in coho salmon that are exposed to long-term EE2. Cytochrome P450s play a pivotal role in responses to different pesticides. Data on the effects of the EE2-based contraceptives on CYP1A and CYP3A gene expression in common carp are therefore limited. In the current study, we found that chronic contraceptive exposure up regulated both CYP1A and CYP3A in the brains of common carp. Previous studies have reported that cytochrome P450s are related to oxidative stress (Jeon et al., 2016; Dzul-Caamal et al., 2012; Xing et al., 2014). Several studies showed that some drugs, chemicals, and pesticides induced the expression of cytochrome P450s genes. Obtained results from this study indicated that CYP1A and CYP3A could be used as biomarkers for EE2 exposure in the brains of common carp.

In conclusion, exposure to chemical for 21 days causes severe diminish in the number of mature oocytes in the ovaries of common carp. Long-term contraceptive exposure leads to significant up regulation of CYP1A and CYP3A gene expressions in the brain and down regulation of FSH and up regulation of LH gene expressions in the ovaries. These findings showed that EE2 could promote reproductive dysfunction. It is, therefore, suggested that contraceptive exposure is highly dangerous to the health of common carp. Based on this knowledge, aquatic systems should be monitored for EE2 pollution. Furthermore, when pharmaceutics like contraceptives reach the water supplies, they could disrupt the reproductive systems of aquatic animals, thus, this situation adversely affects the food chain and indirectly the ecosystem (Fig 1).

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