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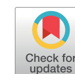
Endocrine disruptive action of diclofenac and caffeine on *Astyanax altiparanae* males (Teleostei: Characiformes: Characidae)



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ABSTRACT

Diclofenac (DCF) and caffeine (CAF) are persistent pharmaceuticals that occur in mixtures in the aquatic ecosystems causing effects in the reproductive physiology of aquatic organisms. This study evaluated the physiological reproductive responses of *Astyanax altiparanae* males exposed to nominal concentrations of DCF (3.08 mg L^{-1}) and CAF (9.59 mg L^{-1}) separately and combined, for 96 h. The steroids profile, estrogenic biomarker vitellogenin (*vtgA)*, testes and liver morphology, and also mortality of males were assessed. DCF and CAF degradation was 5% of the initial concentration for 24 h. The LC_{50} of the DCF and CAF were 30.8 mg L^{-1} and 95.9 mg L^{-1}, respectively. Males exposed to DCF and CAF exhibited a reduction of 17β-Estradiol (E_2) concentration compared to control (CTL). Similarly, testosterone (T) was also reduced in the DCF treatment, but this response was not observed in 11-Ketotestosterone (11-KT). Males exposed to DCF + CAF combined did not exhibit differences in T, E_2 and 11-KT steroids. The *vtgA* gene expression and the sperm concentration did not change among the treatments. Moreover, acute exposure revealed a hypertrophy of hepatocytes cells in the DCF and DCF + CAF treatments. In conclusion, DCF and CAF, isolated, exhibit an endocrine disruptive activity in *A. altiparanae* male, an opposite response observed with the mixture of both compounds that abolishes the endocrine disruptive effects. DCF seems to be more toxic for this species, altering also hepatocytes morphology.

1. Introduction

Pharmaceuticals compounds including antibiotics, analgesics, synthetic hormones and nonsteroidal anti-inflammatory drugs (NSAIDs) are currently found in surface, underground and drinkable waters worldwide (aus der Beek et al., 2016). Among the several pharmaceuticals in the aquatic environmental, diclofenac (DCF) and caffeine (CAF) are one most described in surface waters of Europe, North and South America, Asia and Africa (Ebele et al., 2017). Due to their main applications in human health, it is estimated that CAF world consumption rate continue to increasing to almost 9.7 million tons in 2018 (International Coffee Organization, 2019) and for DCF an annual world consumption of 1443 ± 58 tons (Acuña et al., 2015). According to aus der Beek et al. (2016) DCF maximum concentration found in surface waters were 1.52 μg L^{-1} (Africa); 4.4 μg L^{-1} (Asia-Pacific); 19.74 μg L^{-1} (Western Europe) and 6.0 μg L^{-1} (Latin America). Also, CAF was found in global waters, with maximum concentrations found in surface waters of 1.1 μg L^{-1} (Europe); 1.6 (North America) and in wastewater CAF maximum concentration was 94 μg L^{-1} (North America); 3.5 mg L^{-1} (Asia) (Quadra et al., 2019a).

Although pharmaceuticals like DCF (NSAID) and CAF are designed for specific mode of action in the animal physiology (Ribeiro and Sebastião, 2010; Scholer et al., 1986), they may display a disruptive endocrine activity in the reproductive physiology of fish including effects in steroidogenesis, *vtg* gene expression and fertility (Overturf et al., 2015). Studies conducted with the medaka *Oryzias latipes* exposed to 10 mg L^{-1} of DCF displayed a reduction in fertility and hatchability of embryos produced by second-generation (Lee et al., 2011). Estrogenic activity of DCF was observed in male frog *Xenopus laevis* exposed to 2.9 μg L^{-1} of DCF (Efosa et al., 2017) and also males of the Nile tilapia *Oreochromis niloticus* showing an *vtg* gene expression increased when exposed to 1 μg L^{-1} of DCF (Gröner et al., 2017). These reproductive

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impairments in fish were also described in the goldfish *Carassius auratus* males exposed to CAF exhibiting an increase of the Vtg plasma levels (Li et al., 2012). Pharmaceuticals like others substances that display an interference in the metabolism, binding, transport of natural hormones are classified as endocrine disrupting chemicals (EDCs) (Kavlock et al., 1996), biomarkers like *vtg* gene expression in males and the steroidal hormonal profile are useful indicators for the assessment of aquatic reproduction health (Hook et al., 2014).

All these pharmaceuticals in the natural environment occurs in mixtures and it could be an additional factor to impact the physiology of aquatic animals, especially the reproductive physiology of fish in polluted areas as observed in males showing androgens reduction in municipal waste water sites (Weber et al., 2019). Considering the presence of pharmaceuticals mixtures in the aquatic environment, an emerging concern of the interactive effects of these substances in the physiology of aquatic organisms has enhanced the risk assessment protocols in order to fulfill the gaps of knowledge in the toxicity of mixtures to non-target species (Vasquez et al., 2014). Since most part of pharmaceuticals investigations aims the effects of isolated compounds, the relevance mixtures studies are need in order to evaluate the distinct impacts of these combinations (Fent et al., 2006). Chemical mixtures can results in a stronger effect (synergism, potentiation, supra-additivity) or a low effect (antagonism, subadditivity, inhibition) (Groten et al., 1999).

Initial studies using pharmaceuticals mixtures in a bioassay approach with a small planktonic crustaceans *Daphnia* showed a necessity of evaluate different combinations due to the elucidation of the addition concept concentration of NSAIDs (Cleuvers, 2004). Recently investigations using minnow *Pimephales promelas* adults displayed an impairment in the egg production in additive manner after 21 days exposure to pharmaceuticals mixtures using synthetic steroidal pharmaceuticals (17α-ethinylestradiol, trenbolone, beclomethasone dipropionate, desogestrel and levonorgestrel), thus supporting the concept of the additional effects of pharmaceuticals in reproduction (Thrupp et al., 2018).

Pharmaceuticals mixtures also occur in South America, especially in Brazil with an increasing consumption and wrong disposal of several medicines (Quadra et al., 2019b). Studies showed the presence of NSAIDs including DCF and ibuprofen (Pereira et al., 2016), also DCF and CAF occurrence were pointed out in surface and drinkable waters of São Paulo State with a high population density (40 million of habitants) where CAF was found in 97% of 329 samples tested with the maximum concentration of 127 μg L^{-1} and DCF was found in average concentrations of 0.1 μg L^{-1}; both of these compounds were identified as potential risk for aquatic life (Montagner et al., 2018). Most of those contaminated areas contains native species like *Astyanax altiparanae*, thus the aim of the present study was to evaluate the endocrine disruptive effects of DCF and CAF on *A. altiparanae* males and the potential synergism of these pharmaceuticals in the physiological responses of the organisms.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). Diclofenac sodium salt (CAS: 15307-79-6) and caffeine (CAS: 58-08-2) with a purity of ≥98% were diluted in deionized water, since both pharmaceuticals presents a high solubility in water, then a vehicle was not necessary.

2.2. Animals and experimental design

A. altiparanae is a omnivorous teleost species that reproduces during the summer (de Jesus et al., 2017) and presents a sexual dimorphism (males exhibit roughness in the anal fin). One year old males (n = 350) were obtained from *Estação de Hidrobiologia e Aquicultura de Paraibuna* (CESP) (23°24′53.1″S 45°35′59.5″W) in Paraibuna City (São Paulo, Brazil). All mature fish (13.4 ± 6.1 g body mass and 9.9 ± 1.3 cm length) were safely transported to the laboratory facilities and maintained in black boxes (350 L) for 7 days, fed with extruded feed with 32% crude protein once per day. After this period the animals were transferred to aquariums (140 L each) involved in brown Ethylene-vinyl acetate (E.V.A.). Water parameters as O_2 levels, pH and temperature were measured under a 12:12 photoperiod, air supply was constant, and all animals were monitored during the experiments. The experimental design was carried out in 4 phases using 4 experimental groups (duplicate): Control (CTL), diclofenac (DCF), caffeine (CAF) and mixture (DCF + CAF). The first phase was the decay test, in which 3 groups (DCF, CAF and DCF + CAF) comprehending one aquarium with (n = 6) and one without fish in each group that received 1 mg L^{-1} of each compound. Water samples were collected at 0, 12 and 24 h to assess the real concentration of the compounds during the decay test. The Range Finding Test (RFT) (DCF: 500, 200, 100 mg L^{-1}; CAF: 200, 100, 50 mg L^{-1}) and Lethal concentration (LC_{50}) were carried out in this chronological sequence after the decay test. After RFT, the following concentrations were chosen for the LC_{50} test, that was carried out in duplicate: for DCF: 10, 20, 30, 40 e 50 mg L^{-1} and CAF: 7.5; 12; 30; 60 e 120 mg L^{-1} using 6 animals per aquarium. During the acute exposure (96 h) was used the nominal concentrations (10% of LC_{50}) of 3.08 and 9.59 mg L^{-1} for DCF and CAF, respectively. Each treatment received a total of 12 animals randomly distributed per aquarium (density: 1 fish/L). Water variables were monitored throughout the experimental period and did not differ among groups (pH: 7.4 ± 0.11; O_2: 6.34 ± 0.37 mg L^{-1}; Temperature: 25.4 ± 0.71 °C). The water was renewed (75% in 48 h) and the water physicochemical parameters were measured daily using an oximeter (YSI55) and a pH meter (Gehaka).

2.3. Liquid chromatography coupled to tandem mass spectrometry (LC – MS/MS)

The concentration of both pharmaceuticals was assessed using LC – MS/MS technique. All samples were collected on the same spot of the aquarium using 20 mL syringe attached to a 45 μm filter and transferred to an Ambar flask (10 °C) until chemical analysis. The pharmaceuticals were analyzed by a 1260 (Agilent Technologies, USA) Luna C18 HST (2) (100 × 2 mm; 2.5 μm) (Phenomenex) column at 35 °C combined with a 3200QTRAP mass spectrometer (MS-MS) (ABSciex). A sample of 5 μL was injected in the LC, the eluent flux rate was 220 μL min^{-1} and the mobile phase was 0.1% formic acid (Sigma-Aldrich LC–MS Grade) in ultrapure water (solvent A) and 0.1% formic acid in acetonitrile (J.T. Baker LC–MS Grade) (solvent B). The eluent equilibrium was 80% (solvent A) + 20% (solvent B) in 2.30 min run. Analytes were detected and quantified using ESI ionization and Multiple Reaction Monitoring (MRM) mode. The linearity for DCF and CAF were r = 0.99; the limit of detection for DCF and CAF were 1.5 μg L^{-1} and 0.054 μg L^{-1} respectively. The limit of quantification for DCF was 0.18 μg L^{-1} and for CAF was 5.1 μg L^{-1}. Data were recorded and processed using Analyst® 1.5.2 (ABSciex).

2.4. Sampling

Animals were anesthetized using a 0.1% benzocaine solution in water previously diluted in ethanol. Then, 1 μL of semen (n = 8/group) was collected using automatic pipette and stored in a buffer saline formaldehyde solution. Blood samples (n = 20/group) were collected by the caudal vein with disposable 1 mL heparinized syringes and 27G needles (Hepamax® S5000UI). Blood was centrifuged for 5 min at 600g at RT and plasma was distributed in aliquots and frozen at −80 °C until use. After blood and semen collection, males length (cm) and body mass (g) were recorded and the animals were euthanized by spinal cord section following the procedures of the National Council of

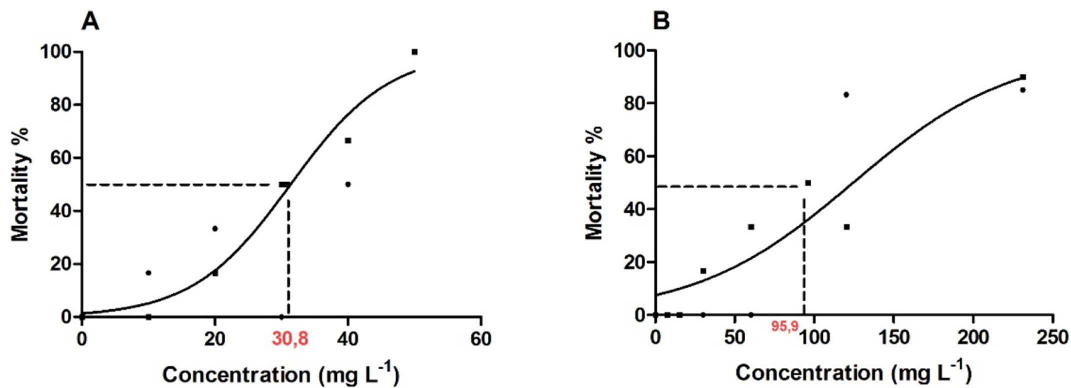

Fig. 1. The lethal concentration (LC_{50}) in *Astyanax altiparanae*. Diclofenac LC_{50} (30.8 mg L^{-1}) (A) and caffeine (95.8 mg L^{-1}) (B) in 96 h. Concentrations scale (X axis) and mortality (Y axis) are presented (n = 12).

Table 1
DCF and CAF concentrations in water of the experimental groups. Real measured concentration A correspond to the initial experimental time (0 h) and real measured concentration B correspond to the renew period (48 h).

Experimental groups	Nominal concentration (mg L^{-1})	Real measured concentration (mg L^{-1}) A	Real measured concentration (mg L^{-1}) B
CTL	0	0	0
DCF	3.1	4.4 ± 0.57	4.3 ± 0.54
CAF	9.5	12.8 ± 0.78	10.5 ± 0.98
DCF + CAF	3.1|9.5	3.8 ± 0.28|13.4 ± 0.49	4.2 ± 0.36|11.1 ± 0.42

Table 2
Mean (± SEM) gonadosomatic index (% GSI; n = 10), hepatosomatic index (% HIS; n = 20) and sperm concentration (SPZ·mL^{-1}; n = 8) of *A. altiparanae* males exposed to DCF, CAF and DCF + CAF in 96 h. Lower case letters (a, b) in the same line correspond to significant difference between experimental groups ($p < 0.05$; ANOVA; SNK post hoc test).

	CTL	DCF	CAF	DCF + CAF
GSI	2.4 ± 0.3^{a}	2.8 ± 0.3^{a}	3.1 ± 0.2^{a}	3.6 ± 0.2^{b}
HSI	1.0 ± 0.1^{a}	1.0 ± 0.0^{a}	0.8 ± 0.0^{a}	$1.1 \pm 0,1^{a}$
SPZ·mL^{-1}	$4.5 \times 10^{9} \pm 1.1 \times 10^{9a}$	$4.5 \times 10^{9} \pm 1.3 \times 10^{9a}$	$3.1 \times 10^{9} \pm 9.9 \times 10^{8a}$	$2.7 \times 10^{9} \pm 5.7 \times 10^{8a}$

Experimental experimentation (NCR, 2019) and approved by the Ethics Committee on Animal Use, CEUA, Institute of Biosciences, USP, process n° 275/2017. Liver was collected and stored at −80 °C for gene expression and histology as well as testes for histology analysis.

2.5. Sperm concentration and somatic indexes

The sperm concentration was measured following the procedures described by Sanches et al. (2011) in a Neubauer's chamber (spermatozoa mL^{-1}). 1 μL of semen was obtained from the anesthetized animals (n = 8) after stripping, diluted into 4 mL of buffered saline formaldehyde solution, and each side of the chamber was counted, with a total of 3 counts were conducted. Somatic indexes (SI) of liver and gonads were calculated with the formula SI = (Tissue mass / whole body mass) ∗ 100.

2.6. Histology

Liver and testes were fixed in bouin solution for 24 h and stored in 70% ethanol. Testes were collected just to confirm the maturation stage and liver to observe potential abnormalities, as the presence of melanomacrophages centers (MMCs). According to routine histological procedures, samples were dehydrated in increasing concentrations of ethanol and xylene (dimethylbenzene), then embedded in Paraplast®. The blocks were sectioned at 5 μm thickness using a microtome (Leica RM2255), sections were mounted in glass slides and stained using hematoxylin and eosin (HE) or Masson's trichrome (Behmer et al., 1976). All sections were analyzed and documented using a computerized image capture system (microscope Leica DM1000, photographic camera Leica DFC295 and image capture Leica Application Suite Professional software, LAS V3.6).

2.7. Steroid analyses

Plasma levels of gonadal steroids were analyzed using ELISA immunoassay kits (Cayman Chemical®) following the manufacturer's guidelines. The analyses of testosterone (T), 11-ketotestosterone (11-KT) and 17β-estradiol (E_2) were carried out in a microplate reader (Spectra Max250, Molecular Devices) at a wave length of 405 nm. Samples were diluted 20 times for 11-KT, 10 times for T and 3 times for E_2 with ELISA buffer solution supplied also by Cayman Chemical ® following the protocol established and validated by Kida et al. (2016) with this same species. The lower limit of detection was 6 pg mL^{-1} (E_2), 3.9 pg mL^{-1} (T) and 0.78 pg mL^{-1} (11-KT). The minimum and maximum intervals of intraspecific coefficient of variation (CV) were 2.5–19.00, that was calculated considering the duplicates of the samples in the same plate. Values below 20% are recommended by Sink et al. (2008).

2.8. Molecular analysis

2.8.1. RNA extraction, cDNA synthesis and real-time qPCR

Total liver RNA was extracted using the Trizol reagent (Sigma®) according to the manufacturer's protocol. For quantification and analysis of the integrity of the material the Abs260/Abs280 ratio was evaluated in the Nanodrop™ Spectrophotometer (ND-1000), ratio between 1.7 and 2.2 was accepted. The purified RNA was stored at −80 °C.

The cDNA synthesis was performed using a commercial kit (SuperScript™ II Reverse Transcriptase). RNA (200 ng L^{-1}) samples were added in a 200 μL microtube, each tube received 1 μL of Randon

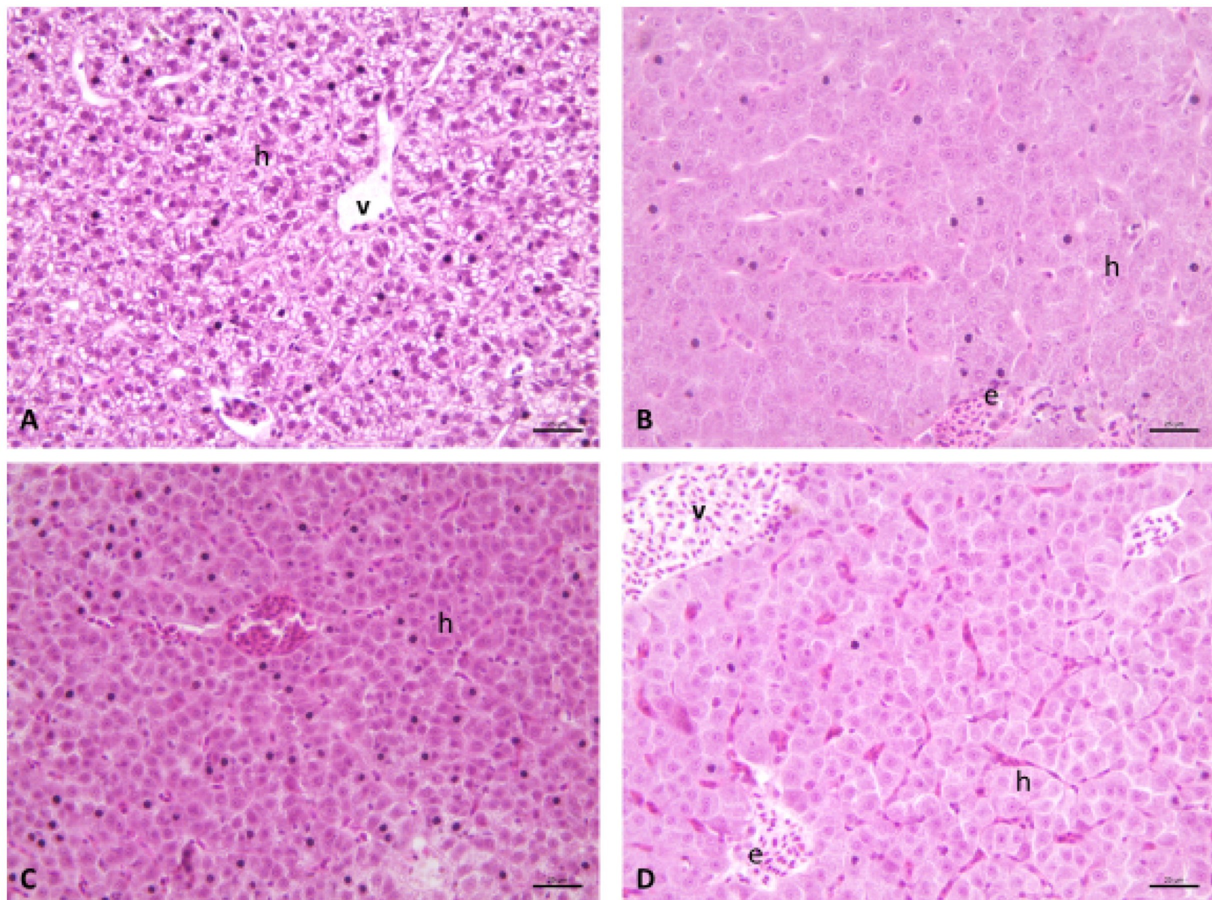

Fig. 2. Histological sections of *Astyanax altiparanae* liver exposed 96 h. CTL (A), DCF (B), CAF (C), DCF + CAF (D) (n = 6). Hepatocyte (h), erythrocyte (e) e vein (v).Bar 25 μm (H.E.).

hexamer (50 ng μL^{-1}) that was placed in a thermocycler for 10 min at 70 °C. Then samples were placed on ice for a thermal shock (4 °C), 8.75 μL MIX (5 μL buffer, 2.5 μL DTT and 1.25 μL dNTPs) was added and placed again in the thermocycler at 25 °C for 10 min. A new heat shock was given at 4 °C, and 0.6 μL of the enzyme was added and finally the samples were placed in the thermocycler at 42 °C for 50 min and at 70 °C for 10 min. After these procedures the samples were stored at −20 °C.

The RT qPCR reaction used *vtgA* primers (Forward: GCCTCTGCGT TGTTGATCTT; Reverse: AAACTCTGACCCTGCTGGAA) already designed by Tolussi et al. (2018) for the congeneric species *Astyanax fasciatus* and validated for *A. altiparanae* (Brambila-Souza et al., 2019). *β-Actin* gene (Forward: CCCAGTCCTTCTCAC; Reverse: ACCAGAAGCG TACAG) (Brambila-Souza et al., 2019) was used as a housekeeper gene. The real- time PCR reaction was performed using 12.5 μL of Power Sybr Green PCR Master Mix (Applied Biosystems), 2 μL cDNA and 0.5 μM primer (forward and reverse). The reaction was incubated for 2 min at 50 °C, 10 min at 95 °C followed by forty cycles of 15 s at 95 °C and 40 s at 60 °C. The melting curve was performed to test the specificity of the reaction that was made in duplicate in the Step One Real Time-PCR System (Applied Biosystems). The data was analyzed by the CT value using the housekeeper gene to normalize the ΔΔct (Livak and Schmittgen, 2001).

2.9. Statistical analysis

Normality and homogeneity variances were verified using Kolmogorov-Smirnov test and Bartlett's test, respectively. One-way analysis of variance (ANOVA) for parametric data was used following post hoc test SNK (Student-Newman-Keuls) for hormones, somatic index, *vtgA* gene expression and sperm concentration. LC_{50} data was estimated using Probit analysis. The non-parametric data were analyzed by Kruskal-Wallis followed by Dunn's test. Data were expressed as mean ± standard error of the mean (SEM) and the data were considered significance if $p < 0.05$. All the data were analyzed using GraphPad Prism 5.01 for windows (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Decay test and lethal concentration (LC_{50})

The results for the decay test were 5% for DCF and CAF, and the decay values were calculated based on the actual concentration ratio in 0 and 24 h (see Supplementary Fig. 1 of the Supplementary material). The LC_{50} results in 96 h for DCF and CAF were 30.8 mg L^{-1} and 95.9 mg L^{-1}, respectively (Fig. 1).

3.2. Acute exposure

Animals were monitored during the acute exposure, the survival rate in CTL and CAF was 100%, mortality was recorded in DCF and DCF + CAF treatments (16% and 12%, respectively). Nominal and measured concentrations are described in Table 1.

3.3. Somatic indexes and sperm concentration

The gonadosomatic index (GSI) was higher in males from

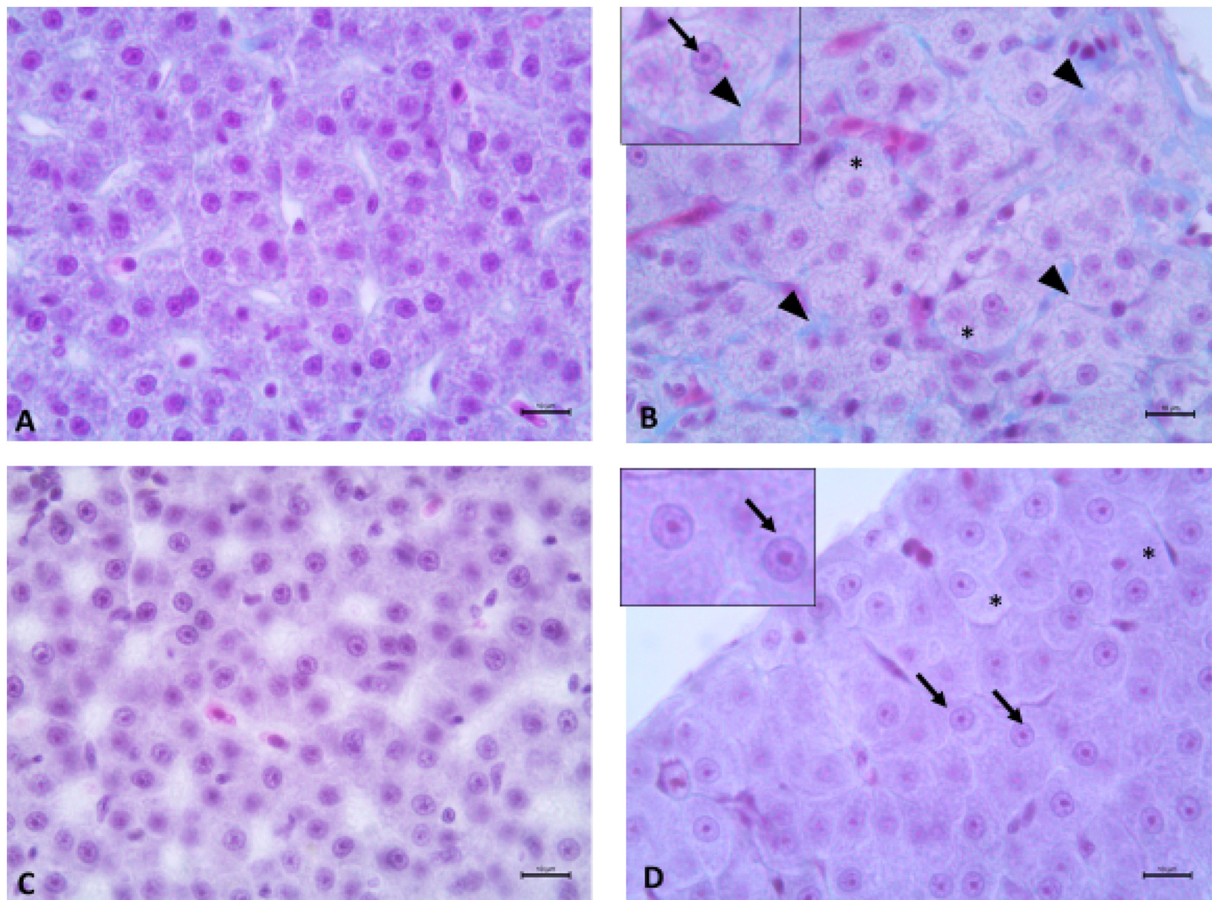

Fig. 3. Histological sections of *Astyanax altiparanae* liver exposed 96 h. CTL (A), DCF (B), CAF (C), DCF + CAF (D) (n = 3). Picnotic nuclei (arrow), connective tissue (head arrow), hypertrophy (*). Bar 10 μm. (Masson's Trichrome).

DCF + CAF group than CTL (P= 0.02). Nonetheless, the acute exposure to DCF, CAF and DCF + CAF did not alter the hepatosomatic index (HSI) (P= 0.16). The sperm concentration (SPZ mL^{-1}) of *A. altiparanae* males exposed to the experimental groups DCF, CAF e DCF + CAF also did not change compared to the CTL group (P=*0.51*) (Table 2).

3.4. Histological analysis

Males exposed to DCF and DCF + CAF treatments displayed hypertrophy of hepatocytes cells exposed to DCF isolated and combined (Fig. 2B, D; Fig. 3B, D), a thickened connective tissue deposit was observed in the DCF treatment (Fig. 3B), and an abnormalities in liver including picnotic nuclei (Fig. 4D). Those pathologies were not observed in the CTL and CAF treatments (Fig. 3A, B), neither MMCs in all slides among the treatments. Testes histology showed that all animals were mature (Fig. 5).

3.5. Plasma steroid concentration

The steroid hormone analysis showed an alteration in the hormonal concentration in males exposed to the pharmaceuticals. 17β-estradiol (E_2) levels were reduced in animals exposed to DCF and CAF compared to the CTL (P= 0.02) (Fig. 6A). Besides, testosterone (T) levels were lower in males exposed to DCF compared to the CTL (P= 0.013) (Fig. 6B). 11-ketotestosterone (11-KT) levels did not change (P= 0.059) (Fig. 6C). The exposition to the mixture (DCF + CAF) did not alter plasma steroid levels.

3.6. *vtgA* gene expression

The relative gene expression of vitellogenin (*vtgA*) of *A. altiparanae* was not altered (P=*0.08*) in any of the experimental treatments (DCF, CAF e DCF + CAF) compared to the CTL group (Fig. 7).

4. Discussion

The present study evaluated the toxicity of both pharmaceuticals DCF, CAF, isolated and combined on the reproductive physiological responses of *A. altiparanae* males. The pharmaceuticals DCF and CAF exhibited an endocrine disruptive activity in the hormonal profile of exposed males, with a reduction in E_2 and T plasma concentrations in DCF treatment, together with the reduction of E_2 in the CAF group. Interestingly, these alterations were not observed in the mixture group. The *vtgA* gene expression was not altered in the treatments compared to CTL group showing that both compounds, in these experimental conditions, are not estrogenic for this species, as stated for *Xenopus laevis* (Efosa et al., 2017) and *Oreochromis niloticus* (Gröner et al., 2017). Moreover, under acute exposure, these pharmaceuticals did not alter the sperm concentration, but altered hepatocytes morphology of *A. altiparanae* males.

In our investigations, DCF showed a low degradation rate of 5% in 24 h as described similarly by Lee et al. (2011). Although DCF was included in the *Watch list* 2013/39/EU by the European Union (Ribeiro et al., 2015) and more recently removed from this list (Loos et al., 2018), it has been classified as a persistent compound that can cause physiological alterations in different animals (Bonnefille et al., 2018). CAF also presented a decay of 5% based on the initial concentration

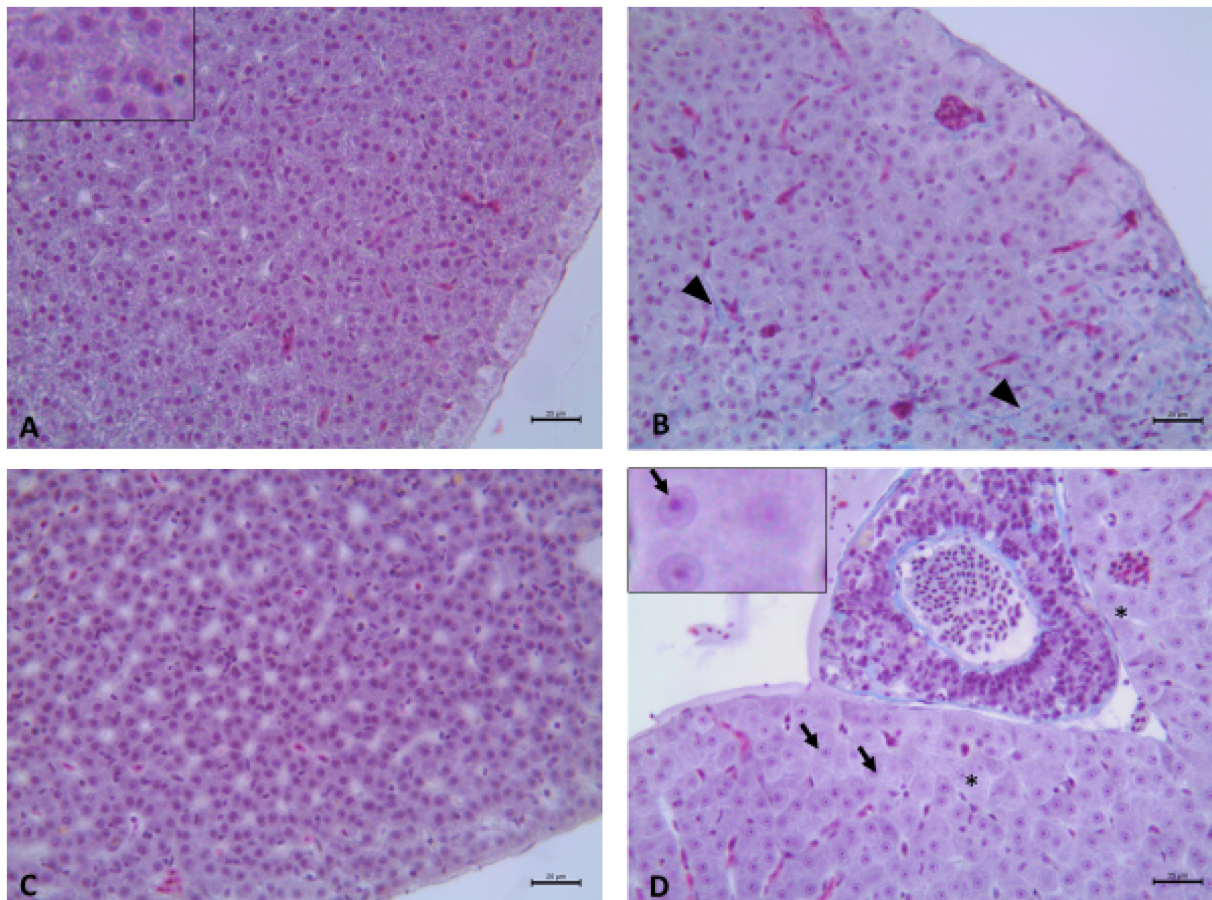

Fig. 4. Histological sections of *Astyanax altiparanae* liver exposed 96 h. CTL (A), DCF (B), CAF (C), DCF + CAF (D) (n = 3). Picnotic nuclei (arrow), connective tissue (head arrow), hypertrophy (*). Bar 25 μm. (Masson's Trichrome).

(1 mg L^{-1}) in 24 h, and this compound is also described as a persistent substance in marine and freshwater environments (Verenitch and Mazumder, 2008), commonly used as an indicator of anthropogenic activities (Buerge et al., 2003). Some studies reported that CAF has a short half-life of 1 day (Lam et al., 2004), however others investigations showed that CAF is not degraded by photolytic factors (Marques et al., 2013), indeed our results showed that CAF has a persistent characteristic in water. The decay of different compounds in the natural environment depends on several variables like pH, light, temperature and microbiota as well (Getoff, 2002). The persistence of pharmaceuticals is an issue for wild life and humans in general as a result of absence of efficiency in many treatments plants that cannot remove these substances and their ubiquitous presence has been noted in natural waters (Machado et al., 2016).

The mortality data assessed using LC_{50} (in 96 h) was 30.8 mg L^{-1} and 95.9 mg L^{-1} for DCF and CAF, respectively, therefore both pharmaceuticals can be considered harmful to *A. altiparanae,* since European Union Directive 93/67/EEC classified the toxicity of substances based on the LC_{50} values, where $LC_{50} < 1$ mg L^{-1} (very toxic), 1–10 mg L^{-1} (toxic), 10–100 mg L^{-1} (harmful). The result of LC_{50} depends on several aspects of the organism development including the life stage, physiology and also the method adopted, as exposure period (Finney, 1985). Thus, mortality data can support the impact of different compounds, and in our study DCF can be considered more harmful compared do CAF. Similar ranges were described in the literature for DCF, as 70.98 mg L^{-1} for *Cyprinus carpio* in 96 h (Islas-Flores et al., 2013), 167 mg L^{-1} for *Danio rerio* in 96 h (Memmert et al., 2013), 10 mg L^{-1} for *Oryzias latipes* (Nassef et al., 2009).The value of LC_{50} for CAF was not described so far for a tropical fish species. LC_{50} data for CAF (100 mg L^{-1}) is available only for *Pimephales promelas* (Moore et al., 2008).

The toxicity of both compounds was observed in the biological responses of the males, more specifically in the liver. According to the protocols of Bernet et al. (1999), this histopathology could be classified as progressive changes (eg. hypertrophy) and regressive changes (nuclei abnormalities). The biological effects caused by DCF and DCF + CAF treatments in the acute exposure revealed abnormalities in the liver of the males, including hypertrophy of hepatocytes exposed to DCF isolated and combined, picnotic nuclei and an earlier conjunctive tissue deposit was observed in the liver of animals from DCF treatment, but MMCs were not observed in the animals from all treatments. Despite the alterations in the hepatocytes of exposed animals, our study did not observe an HSI change in all treatments, instead a physiological response in the cellular level. The individuals exposed to DCF and DCF + CAF displayed those alterations, but these pathologies were not observed in the CAF treatments showing that most part of the toxicity was caused by the presence of DCF, a result that could explain the lower concentrations of DCF compared to CAF to induce different LC_{50} data for this species.

The acute hepatic response to the presence of DCF can be a potential indicative of the impacts that could occur in sub chronical exposures, using environmental concentrations. These responses were described in liver of the trout *Salmo trutta f. fario* exposed chronically to 100 μg L^{-1} of DCF, displaying an increase of irregularly shaped and vesiculated hepatocytes and degenerating nuclei (Schwarz et al., 2017). Despite the toxicity of CAF in the LC_{50}, our investigations did not show changes in hepatic histology, neither the presence of MMCs. The absence of biological hepatic alterations after CAF exposure was also described in sábalo *Prochilodus lineatus* using biochemical and genotoxic parameters (Santos-Silva et al., 2018).

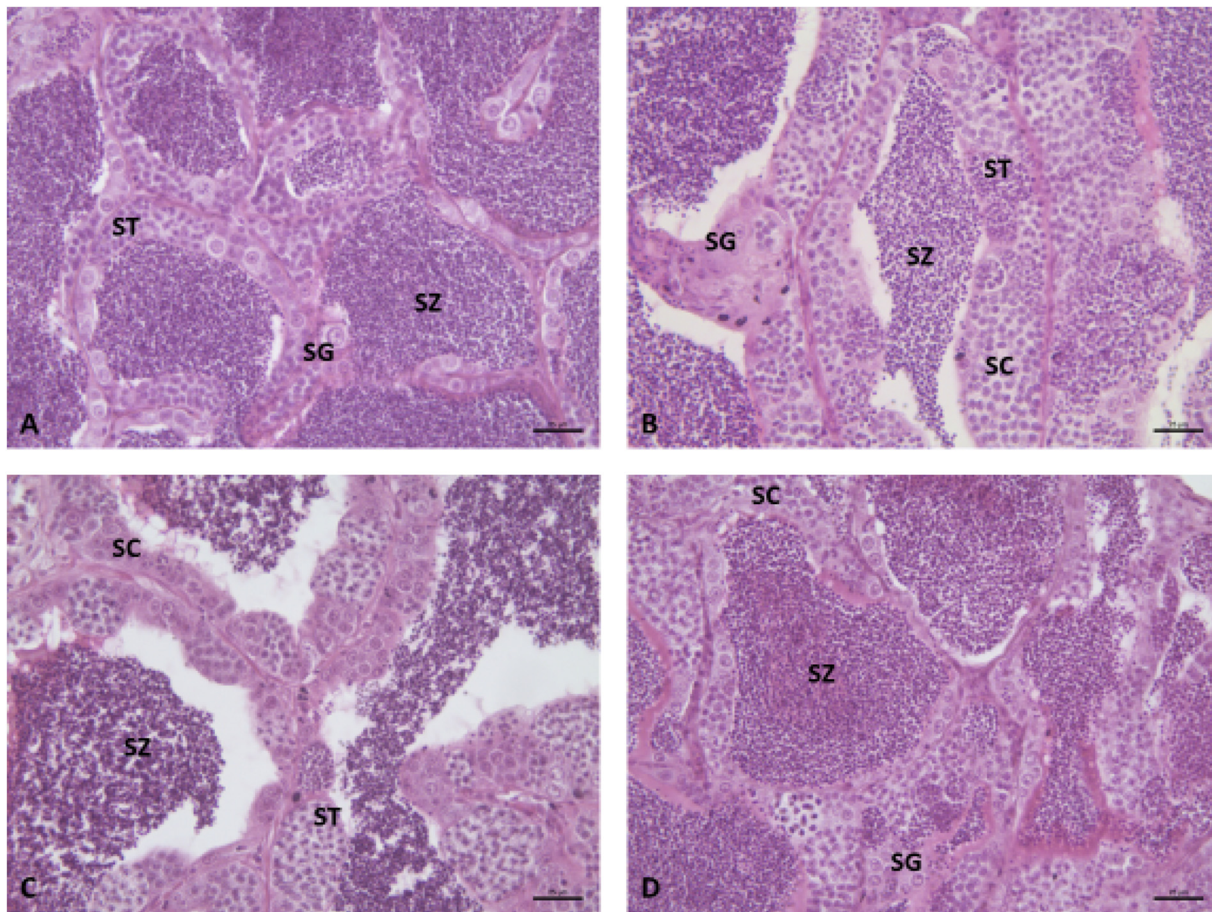

Fig. 5. Histological sections of *Astyanax altiparanae* testes exposed 96 h. Control (A), diclofenac (B), caffeine (C), diclofenac and caffeine (D). Spermatogonia (SG), spermatocytes (SC), spermatids (ST), spermatozoa (SZ). Bar 25 µm (H.E.).

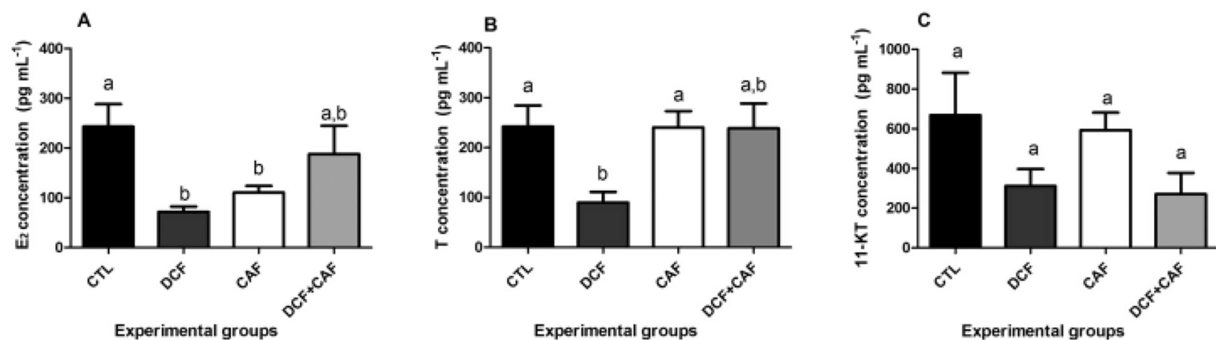

Fig. 6. Mean (± SEM)) (n = 10) plasma concentration (pg mL^{-1}) of: A. 17β-estradiol (E_2), B. Testosterone (T) and C. 11-Ketotestosterone (11 - KT) in *Astyanax altiparanae* exposed for 96 h to diclofenac (DCF), caffeine (CAF) e diclofenac and caffeine (DCF + CAF) and control (CTL) . Different lower case letters (a, b) correspond to significant difference among experimental groups ($p < 0.05$; ANOVA; SNK post hoc test).

Regarding the testes, all animals were mature and sperm concentration was not affected by the pharmaceuticals tested. Although, the impacts of pharmaceuticals in hormones profile and *vtg* gene expression are related to reduction of somatic index (Guiloski et al., 2015; Ji et al., 2013) in our investigations was observed an increase in the GSI of DCF + CAF treatment compared to CTL group. This GSI increase was also observed in adult females of *Oryzias latipes* exposed to DCF, but with a low fertility rate, and the authors described a potential compensatory response of the animals (Lee et al., 2011).

Considering the potential effects of pharmaceuticals on the reproductive physiology of aquatic species, biomarkers of effect were analyzed in *A. altiparanae* males. The decrease in plasma T concentrations triggered by DCF can be related to the capacity of this anti-inflammatory to interact with steroidogenesis pathway altering the enzymes activity. In vitro studies using the carp *Cyprinus carpio* testes exposed to DCF, showed a reduction of 56% in the enzymatic activity of CYP17, an enzyme that converts 17a-hydroxyprogesterone (17P4) to androstenedione (AD), which is the substrate to produce T (Fernandes et al., 2011). Since androgens are essential for the maintenance and regulation of spermatogenesis and spermiogenesis allowing the maturation and development testes and differentiation of germ cells (Schulz and Miura, 2002), the alteration observed in the present studied could lead to disturbances in fertility of animal exposed to DCF.

In fact, chronical studies using DCF showed an influence in the fertility of the second generation of eggs of parental females and males of the medaka *O. latipes* exposed to 10 mg·L^{-1} of DCF (Lee et al., 2011).

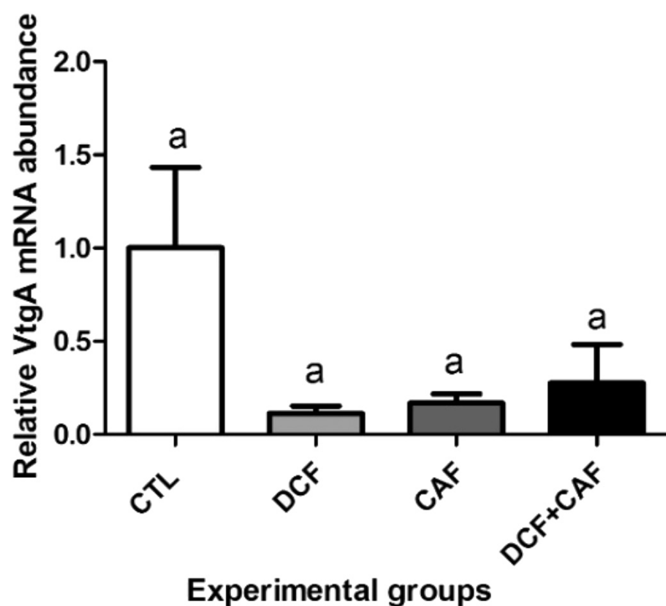

Fig. 7. Mean (± SEM) (n = 6) relative gene expression of *vtgA* in *Astyanax altiparanae* males exposed 96 h to diclofenac (DCF), caffeine (CAF) e diclofenac and caffeine (DCF + CAF) and control (CTL). The same lower-case letters (a) show no statistical difference among experimental groups ($p < 0.05$; ANOVA; SNK post hoc test).

The acute exposure also showed of interference in the E_2 plasma concentration in *A. altiparanae* males exposed to DCF and also CAF. Since E_2 is the product of the T aromatization, catalyzed by P450 aromatase (Lubzens et al., 2010; Nagahama, 1994), a reduction of T could lead to a reduction of the estrogen E_2 in DCF treatment. Moreover, these alterations in hormones can impact pituitary genes as observed in Nile tilapia *Oreochromis niloticus* exposed to 100 $\mu g \cdot L^{-1}$ DCF chronically where LH expression was statistically significant reduced (Gröner et al., 2017), in vitro studies using pituitary explants an reduction of *fshb lhb* expression were also observed in *A. altiparanae* exposed to DCF (Branco et al., 2019), therefore, alterations in this regulation could impact negatively the HPG axis and the fish reproduction.

Regardless of the effects of isolated DCF and CAF in the hormonal profile of *A. altiparanae* males, similar alterations were not observed in the DCF + CAF treatment in the steroid analyzed. This result suggests that the mixture could elucidate an antagonist effect compared to the isolated pharmaceuticals in hormonal profile and more interestingly these responses could be masking the endocrine disruptive effects of pharmaceuticals in the reproduction of this species. The investigations of pharmaceuticals mixtures in a laboratory scale, applying the addition concept, were firstly conducted in studies using the crustacean *Daphnia* and anti-inflammatories drugs, and the concept was highlighted in order to understand the additional effects of mixtures (Cleuvers, 2004). Yet, recent studies have showed different physiological responses of fish exposed to pharmaceuticals mixtures. Juvenile females of trout *O. mykiss*, exposed to environmental relevant concentrations of paracetamol, carbamazepine, naproxen and ibersatan for 42 days displayed an increase of 11-KT e E_2 plasma levels, but none alterations on plasma Vtg (Schmitz et al., 2018). Likewise, in zebrafish *Danio rerio* larvae, exposed to relevant concentrations of mixtures (caffeine, ibuprofen, carbamazepine and tamoxifen) did not exhibit any alterations in *vtg* gene expression (Aguirre-Martínez et al., 2017). These different responses in the reproductive parameters of species reveal the necessity of chronical studies in a relative long scale of the reproductive life cycle of the species. Our study demonstrated that in a short period the mixtures studied seem to inhibit their effects when isolated, however it is necessary to access the same mechanism in different periods of exposure to evaluate the risk of different interactions of pharmaceuticals in reproduction of aquatic organisms.

The estrogenic capacity of DCF and CAF to induce *vtgA* gene expression in *A. altiparanae* males in an acute exposure was not observed. Opposite results were described in other fish males including *O. latipes* exposed to 8 $mg \cdot L^{-1}$ of DCF (Hong et al., 2007), *O. niloticus* exposed to 1 $\mu g \cdot L^{-1}$ of DCF (Gröner et al., 2015) and *C. auratus* exposed to 2 mg L^{-1} of CAF that exhibited an increase of serum Vtg (Li et al., 2012). The estrogen receptors (ERs) are classified in different subtypes including ERα and ERβ that respond to several endocrine disruptor chemicals (Shanle and Xu, 2011), thus the molecular signaling to an estrogenic activity could be observed or not in different species exposed to xenoestrogens compounds.

5. Conclusion

In conclusion, DCF and CAF exhibit an endocrine disruptive activity in *A. altiparanae* males, with a suggested antiestrogenic action. However, the mixture of both compounds abolishes the endocrine disruptive effects. DCF seems to be more toxic for this species, altering also hepatocytes morphology.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2020.108720.

Declaration of competing interest

We have no conflict of interest.

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