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Eco-endocrinological dynamics: Unraveling dexamethasone's influence on the interrenal axis in juvenile carp *Cyprinus carpio*

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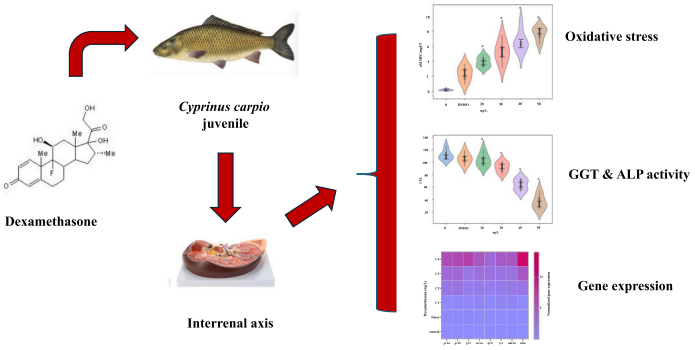
HIGHLIGHTS

- Unraveling the interrenal axis response to dexamethasone (DXE) exposure
- DXE escalates oxidative stress markers in *Cyprinus carpio*.
- Antioxidant enzyme activity increases post-DXE exposure.
- DXE alters ALP and GGT activity in *Cyprinus carpio*.
- Upregulation of stress-related genes in DXE-exposed *Cyprinus carpio*

GRAPHICAL ABSTRACT



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ABSTRACT

This study delves into the eco-endocrinological dynamics concerning the impact of dexamethasone (DXE) on the interrenal axis in juvenile carp, *Cyprinus carpio*. Through a comprehensive analysis, we investigated the effects of DXE exposure on oxidative stress, biochemical biomarkers, gene expression, and bioaccumulation within the interrenal axis. Results revealed a concentration-dependent escalation of cellular oxidation biomarkers, including 1) hydroperoxides content (HPC), 2) lipid peroxidation level (LPX), and 3) protein carbonyl content (PCC), indicative of heightened oxidative stress. Concurrently, the activity of critical antioxidant enzymes, superoxide dismutase (SOD), and catalase (CAT), significantly increased, underscoring the organism's response to oxidative insult. Notable alterations were observed in biochemical biomarkers, particularly Gamma-glutamyl-transpeptidase (GGT) and alkaline phosphatase (ALP) activity, with GGT displaying a significant decrease with increasing DXE concentrations. Gene expression analysis revealed a significant upregulation of stress and inflammation response genes, as well as those associated with sensitivity to superoxide ion presence and calcium

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signaling, in response to DXE exposure. Furthermore, DXE demonstrated a concentration-dependent presence in interrenal tissue, with consistent bioconcentration factors observed across all concentrations tested. These findings shed light on the physiological and molecular responses of juvenile carp to DXE exposure, emphasizing the potential ecological implications of DXE contamination in aquatic environments. Understanding these dynamics is crucial for assessing the environmental impact of glucocorticoid pollutants and developing effective management strategies to mitigate their adverse effects on aquatic ecosystems.

1. Introduction

The use of glucocorticoids, particularly dexamethasone (DXE), in treating chronic autoimmune and respiratory diseases has surged globally (Chalitsios et al., 2020). Recognized as a vital tool during the peak of the COVID-19 pandemic, DXE prominence led to its inclusion in the World Health Organization's Model List of Essential Medicines since 1995 and recognized as "major development" (Noreen et al., 2021). Amid ongoing concerns about new virus variants, the persistent presence of DXE in various environmental matrices remains a focal point for the scientific community.

As the pandemic evolves with the emergence of new variants, concerns persist for global health systems. Recent reports from the Center for Disease Control and Prevention of China (Liu et al., 2023) and the Ministry of Health in Mexico reveal an ongoing health emergency, amplifying the prevalence of DXE in various environmental matrices. Despite its classification as an emerging pollutant with historical documentation of low concentrations in the environment, specifically μg/L and ng/L, the damage inflicted on aquatic organisms remains inadequately explored (Weizel et al., 2018). In this context, this study explores the influence of realistic concentrations of DXE on the interrenal axis in juvenile specimens of the common carp (*Cyprinus carpio*).

Our investigation selects juvenile organisms of the species *C. carpio* as bioindicators due to the State of Mexico's substantial role as the primary producer of this species. Moreover, the extensive distribution of these fish for human consumption, coupled with their bottom-dwelling nature and diverse feeding habits, positions them ideally for assessing the impact on aquatic systems (Falfushynska and Stolyar, 2009).

Fish, being susceptible to endocrine disruptors in the aquatic environment (Schiller et al., 2014), face unique challenges with halogenated glucocorticoids, such as DXE, that resist elimination by wastewater treatment plants. These compounds persist in effluents, causing various effects on aquatic organisms, extending beyond reproductive issues to metabolic activity and signaling pathways (Runnalls et al., 2010; Macikova et al., 2014; Ammann et al., 2014; Shafwat-Yazdan et al., 2022).

As the biological significance of glucocorticoids unfolds, our study aims to contribute by investigating their impact on the interrenal axis. Previous research emphasizes the need for a deeper understanding of glucocorticoid effects, considering long-term side effects, variable age, and different exposure times (Wojnarowicz et al., 2014; Shafwat-Yazdan et al., 2022).

Alkaline phosphatase (ALP) and gamma-glutamyl-transpeptidase (GGT) are utilized to assess stress or toxicity criteria, offering insights into the magnitude of damage generated (Mazorra et al., 2002; Molina et al., 2005; Álvarez-Muñoz et al., 2006). Our study investigates the impact of DXE on the interrenal axis in juvenile carp, addressing critical gaps in understanding. We assess biomarkers such as alkaline phosphatase (ALP) and gamma-glutamyl-transpeptidase (GGT) to gauge stress and toxicity, offering insights into environmental impact. Additionally, we explore susceptibility and exposure biomarkers, providing a comprehensive assessment. Moreover, we delve into the intracellular mechanisms regulated by glucocorticoids, elucidating their role in genes associated with the calcium signaling pathway.

2. Material and methods

2.1. Ethics statement

The methodologies employed in this study adhere to the guidelines set forth by the Ethics and Research Committee of the Universidad Autónoma del Estado de México, with approval granted under ID RP. UAEM.ERC.065.2022.

2.2. Chemical and reagents

Dexamethasone (DXE), characterized by its CAS number 50-02-2, along with all other chemicals employed in this study, were procured from Sigma-Aldrich (St. Louis, MO). The preparation of the DXE stock solution involved dissolving the DXE powder in DMSO at a concentration of 25 mg/mL.

2.3. Organism procurement and maintenance

The Tiacaque Aquaculture Center, situated in the municipality of Jocotitlán, State of Mexico, provided the specimens. Acknowledged as the primary producer of common carp and grass carp in the country, the center served as a valuable source for our study. Juvenile fish from the same brood were selected at random for inclusion in this research project. Following their spawning, these specimens were nurtured in growth ponds filled with locally sourced well water until attaining a length of 8.0 ± 0.5 cm and an average weight of 7 ± 0.5 g, a developmental stage achieved approximately 10 weeks post-oviposition.

To maintain optimal conditions, the water quality in both the growth ponds and the exposure systems underwent daily monitoring, as detailed in Table 1.

Concurrently, stringent measures were taken to ensure environmental stability, with a consistent temperature of 23 ± 1 °C and regular light-dark cycles (12:12 h) maintained in the fish housing facilities. The daily diet of the fish consisted of protein-enriched food (35 %), supplemented with carbohydrates and essential nutrients, facilitating the desired growth rate among juvenile carp until the day they were introduced into the exposure systems.

2.4. Exposure system

The experimental setup comprised 100 L tanks, with 80 L of aerated water solutions containing varying concentrations of DXE (20, 30, 40, and 50 ng/L) prepared on-site. These concentrations were determined based on a study by Gutiérrez-Noya et al. (2023) and reflective of realistic environmental levels. Additionally, a tank devoid of DXE served as a control, while another contained only the solvent, DMSO, at a

Table 1
Measured water parameters in growth ponds and exposure systems.

Water parameter	Measured value
Dissolved oxygen	95 % saturation
Nitrite	0.032 ± 0.01 mg/L
Nitrate	2.7 ± 0.45 mg/L
pH	7.2 ± 0.15
Un-ionized ammonia	0.018 ± 0.01 mg/L

Values are expressed as mean ± standard deviation.

concentration of 0.01 %. Each tank housed 36 juvenile carp, as described in Section 2.3. Continuous aeration was provided to all tanks, and exposure time was 96 h. The systems were placed in sextuplicate.

2.5. Sampling procedure

The interrenal axis of *C. carpio* was obtained at 96 h. After completing exposure period, the carps were removed from the system and euthanized using hypothermic shock. Blood was drawn from the caudal vasculature with a 1 cc non-heparinized syringe; serum was separated and immediately proceeds to perform serum alkaline phosphatase and γ-Glutamyl-transferase determination. The pituitary gland and the cephalic kidney containing the interrenal tissue were quickly dissected (half of the tissue was used for gene expression evaluation) and the remaining half was fixed in Bouin's solution (for stress oxidative evaluation).

2.6. Stress oxidative determination

The evaluation of oxidative stress biomarkers was carried out after 96 h of exposure. The determination of HPC content employed the technique outlined by Jiang et al. (1992), LPX content was evaluated following the method described by Buege and Aust (1978), and the measurement of protein carbonyl content (PCC) was conducted according to the procedure introduced by Levine et al. (1994). The activities of SOD and CAT enzymes were assessed using the methodologies presented by Misra and Fridovich (1972) and Radi et al. (1991), respectively. Furthermore, PT content was quantified in relation to other biomarkers through the analytical approach described by Bradford (1976).

The samples underwent analysis using a spectrophotometer (Shimadzu, UV-2600). The experiments were replicated in sextuplicate with three readings performed for each replicate. To assess the reproducibility of the results, the precision was calculated using the following formula: C.V. = ($^{\mu s}$) 100 % (Table 2).

Table 2
The methodologies applied to determine oxidative stress biomarkers.

Biomarker	Sample amount	Reagents	Wavelengths	Method used
LPX	50 μL supernatant	450 μL Tris-HCl 150 nM 1 mL TCA-TBA	535 nm	Buege and Aust, 1978
HPX	100 μL supernatant	900 μL mixture ($FeSO_4$, H_2SO_4, dehydroxytoluene butylate, and xylenol orange).	560 nm	Jiang et al., 1992
POX	Precipitate	150 μL DNPH/HCl 10 nM 500 μL TCA 1 mL guanidine 6 M	366 nm	Levine et al., 1994
SOD	40 μL supernatant	260 μL CO_3 buffer (50 mM Na_2CO_3 and 0.1 mM EDTA) 200 μL adrenaline 30 mM	480 nm	Misra and Fridovich, 1972
CAT	30 μL supernatant	420 μL isolation buffer (0.3 M sucrose, 1 mM EDTA, 5 mM HEPES, and 5 mM KH_2PO_4) 300 μL H_2O_2 20 mM	240 nm	Radi et al., 1991
TP	13 μL supernatant	300 μL distilled water 1.25 mL Bradford reagent (Coomassie blue, Et-OH 96 %, H_3PO_4).	595 nm	Bradford, 1976

TCA: trichloroacetic acid. EDTA: Ethylenediaminetetraacetic acid. NADPH: Nicotinamide adenine dinucleotide phosphate. TCA-HCl: Tris hydrochloride. TCA-TBA: thiobarbituric-trichloroacetic acid. DNPH: 2,4-Dinitrophenylhydrazine.

2.7. Gene expression assessment

2.7.1. RNA extraction

Interrenal tissue segments designated for gene expression analysis were preserved in Qiagen's RNA later solution and promptly stored at −80 °C until total RNA isolation. The Qiagen RNeasy mini kit was employed for RNA isolation following the manufacturer's instructions. In summary, the interrenal tissue was homogenized in 600 μL of RLT lysis buffer. Subsequent steps included centrifugation, addition of reagents, and elution of RNA from the spin column with 30 μL of nuclease-free water.

The quantification of the isolated total RNA was performed spectrophotometrically at 260 nm using the THERMO Scientific NanoDrop 2000/2000c kit. RNA purity was estimated by calculating the UV absorbance ratios at 260 nm, 230 nm, and 280 nm, with the accepted purity value for each ratio being 2.0.

2.7.2. Reverse transcription

1 μg of total RNA was treated with gDNA Wipeout Buffer (Qiagen), then the reaction was carried out with Quantiscript Reverse Transcriptase and Quantiscript Reverse Buffer provided by Quantitect® Reverse Transcription Kit (QIAGEN, Hilden, Germany, REF 205313), the reaction was carried out according to the manufacturer's instructions. The reaction was incubated for 15 min at 42 °C followed by 3 min at 95 °C, after completion of reverse transcription, real-time PCR was performed as follows.

2.7.3. Real time PCR

RT product was diluted 1:3 in nuclease-free water, 2 μL were used in each reaction. Relative quantification of *gr1a*, *gr1b, gr2, stim*, *ryr, ip3r, cerca* and *ade1a* genes was done by real-time PCR using the Qiagen Rotor-Gene Q system, and QuantiNova Sybr® Green reagents, following the manufacturer's instructions. Primers were designed using Primer3 primer design software (Rozen and Skaletsky, no date; Untergasser et al., 2012) using the criteria for real-time PCR primer design described by Bustin (2000).

The sequences of the primers and the GenBank accession number of all genes are given in Table 3.

The concentration of the primers used in each reaction is 500 nM. The qRT-PCR cycling conditions started with an initial denaturation step at 94 °C for 15 s, followed by 35 cycles comprising denaturation at 94 °C for 15 s, hybridization at 60 °C for 30 s and extension at 72 °C for 30 s. To ensure uniform normalization, the internal β-actin gene was used

Table 3
Sequences of primers evaluated.

Gen	ID number		Sequence
gr1a	AJ879149.3	Forward	CACTTTGGGAGACATGGGCG
		Reverse	TTCCTCGAACCCAGCCATCA
gr1b	AM697886.1	Forward	ATGCAGGAGATGGTGGAGGG
		Reverse	CCGCCAGCATCTCAGGAAAC
gr2	AM183668	Forward	TACCTGACACCTCCATCCGC
		Reverse	GAACCCTGGCAGAGCTTTGG
serca	XM_042729421	Forward	GCGACAACAAAGGCACGGCT
		Reverse	TGCAAAGCAGCGAGCACCCA
stim1	XM_42739941	Forward	CTCTTAACCATGCCCCTGCT
		Reverse	TGGTGGCTTTAGTTTGGCACT
ip3r	XM_042726217	Forward	TGGACATGTACACTCACTCAAGG
		Reverse	GGTGCAAAGAAATCCAGAGGG
ryr	M_042743870	Forward	GAACTTCTGCGAGGACACCA
		Reverse	GTGCTGCTTCTCCATTCCCT
ade1a	XM_042729716	Forward	ATCTGCCACAGAACTGGAGAC
		Reverse	CACAGATGTGAATGCCGGGT
β-actina	JQ619774.1	Forward	AGACCTGTATGCCAACACCG
		Reverse	AGGGCCAGACTCATCGTACT

consistently in all samples.

At the end of the q-PCR cycles, a dissociation curve was generated to differentiate specific from non-specific products. This was achieved using the following conditions: 10 s at 95 °C, 10 s at 65 °C and 10 s at 95 °C with 0.2 °C increments, with data collection at each increment of the melting curve.

Following the q-PCR reaction, the melting curves were analyzed to validate the specificity of the PCR amplicons. For each gene evaluated, the relative transcript level was determined and normalized by β-actin housekeeping. The transcript levels of each gene at the different exposure concentrations were normalized to the respective at that exposure concentration. The relative fold change for each concentration was calculated by the equation: fold change = 2-ΔΔΔCt (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008), where ΔCt = Ct (target gene) - Ct(β-actin gene) and ΔΔΔCt(concentrations) = ΔCt(concentrations) - ΔΔCt(concentration1). For the gene expression experiment, we used the remaining interrenal axis from the oxidative stress experiment. The experiments were replicated in sextuplicate with three readings performed for each replicate.

2.8. ALP & GGT analysis

Biochemical analysis was conducted by centrifuging the red-capped microtubes at 2500 rpm for 10 min. The resulting serum was utilized for quantifying alkaline phosphatase (ALP) and gamma glutamyl transpeptidase (GGT) levels using the VetScan VS2 system from Abaxis. The experiments were replicated in sextuplicate with three readings performed for each replicate.

2.9. Measuring the concentration of DXE and assessing bioconcentration factors

To analyze DXE concentrations in both exposure water and the interrenal axis of *C. carpio* exposed to realistic levels of DXE, the following methodology was employed:

In brief, the determination of DXE in water samples involved a two-step filtration process, utilizing an 8 μm membrane followed by a 2.5 μm membrane. Solid-phase extraction (SPE) cartridges, specifically Phenomenex Strata XL from Torrance, CA, were utilized. These cartridges were conditioned with 10 mL of methanol followed by 10 mL of milli Q® water. The water samples, spike with DXE, were introduced into the SPE cartridges at a flow rate of 5 mL/min and subsequently eluted with 6 mL of methanol. The solvent was then evaporated to dryness using N-Evap 112 nitrogen from Organomation, Haverhill, MA. The dried samples were reconstituted with 1 mL of methanol:water (50:50) and passed through a 0.2 μm syringe filter before injection.

In the process of determining DXE levels in the interrenal axis of carp, the tissue underwent a water wash and subsequent drying. A 0.50 g portion of carp tissue was spiked with DXE solution. The DXE-enriched sample underwent a 30-second vortexing using a Jeho Tech VM-96B vortexer from Korea and was then stored at −20 °C for 30 min. To initiate extraction, 20 mL of methanol: milli Q® water (1:1) was added, and the sample underwent a 10-minute extraction using a Nexul NXP 1002 ultrasonic cleaner from Japan. Following this, the sample was centrifuged at 24000 rpm for 10 min to ensure effective separation of the organic phase.

The solid-phase extraction (SPE) cleanup process involved conditioning the cartridge with 5 mL of methanol, 5 mL of ultrapure water (UPW), and 5 mL of methanol: ultrapure water (1:1). After loading the sample, the cartridge underwent a rinse with 5 mL of ultrapure water and was then vacuum-dried for 15 min. For sample elution, 10 mL of MeOH followed by 5 mL of acetone:methanol (1:1) was employed, and the cartridge was vacuum-dried for 3 min. The eluent was initially concentrated using a rotary evaporator and further concentrated under a gentle flow of nitrogen (N2) until nearly dry. The concentrated eluent was reconstituted to 1 mL using acetonitrile: ultrapure water (3:7) and filtered through a 0.2 μm PTFE membrane filter.

The quantification and identification of DXE in water and the intrarenal axis were conducted using LC-MS/MS (liquid chromatography-mass spectrometer) equipment manufactured by Waters in Mildford, MA, US. An Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm) was employed for the analysis. The mobile phases consisted of 0.1 % (v/v) formic acid in water (A) and 0.1 % (v/v) formic acid in methanol (B). The column temperature was maintained at 40 °C, with an injection volume of 10 μL and a flow rate of 0.3 mL/min. The total run time was set at 10 min, starting with 90 % A at 0.1 min, transitioning to 10 % A at 8 min, and returning to 90 % A at 10 min. The identification and quantification of DXE in water and intrarenal axis samples, were conducted in a quattro Premier XE triple quadrupole mass spectrometer electrospray ionization, operating in the positive mode (Waters, Mildford, MA, US).

For the determination of limits of quantification (LOQ) and detection (LOD), calculations were based on Brubaker's (1999) formula:

$$\mathrm{LOD} = t_{0.99} \times \mathrm{S} \text{ and } \mathrm{LOQ} = 3 \times \mathrm{LOD},$$

where $t_{0.99}$ represents the one-tailed statistic at the 99 % confidence level for n replicates, and S denotes the standard deviation of recovery results from n samples fortified at the estimated LOQ.

The mass spectrometer parameters employed for the quantification of DXE were configured as follows: positive ionization mode, parent ion (m/z) = 363.2, product ion (m/z) = 147.1, cone voltage (V) = 20. The detection limit (LOD) for water was determined to be 1.2 ng/L, with a quantification limit (LOQ) of 3.15 ng/L. In the case of interrenal axis carp, the detection limit (LOD) was measured at 0.15 ng/g, while the quantification limit (LOQ) was established at 0.4 ng/g.

Utilizing the concentration of DXE in both interrenal axis of *C. carpio* and the exposure water, the bioconcentration factor (BCF) was computed using the formula: BCF = [concentration in interrenal axis] / [concentration in exposure water] (García-Medina et al., 2022).

To assess the matrix effect, the slopes derived from the calibration curves fitted to the matrix were compared with those obtained from solvent standards. Matrix effects (ME%) were determined by subtracting 1 from the ratio of the slope of the matrix-matched calibration curve (B) to the slope of the DXE standard solution curve (C), and then multiplying by 100:

$$\mathrm{ME}\ (\%) = (\mathrm{CB} - 1)\ 100\%.$$

A positive value indicates signal enhancement, while a negative value suggests signal suppression. An acceptable signal enhancement or suppression effect is defined when matrix effect values fall within the range of −20 % to +20 %.

2.10. Statistics

Homogeneity of variance and normal distribution were assessed using the Bartlett and Shapiro-Wilk tests, respectively. To identify differences between means, a Tukey test was conducted with a significance level set at $p < 0.05$. Group differences were evaluated using a one-way ANOVA test ($\alpha = 0.05$) with Sigma Plot 12.3 software. Pearson correlation analyses were carried out to determine the strength of the relationship between oxidative stress and gene expression, as well as the correlation between biochemical findings. Significance was set at $p < 0.05$, and the analyses were performed using R software. Additionally, the resulting correlations were illustrated using chord correlations.

3. Results

3.1. Oxidative stress

The cellular oxidation biomarkers, namely 1) HPC, 2) LPX, and 3) PCC, exhibited a concentration-dependent escalation in the interrenal

axis of *Cyprinus carpio* following 96 h of exposure to all DXE treatments (Fig. 1A–C). Furthermore, the activity of the antioxidant enzymes, superoxide dismutase (Fig. 1D), and catalase (Fig. 1E) displayed statistically significant increases compared to the control and DMSO groups. These results indicate a notable impact of DXE on cellular oxidative stress markers and antioxidant enzyme activity in the interrenal axis of *Cyprinus carpio.*

3.2. Biochemical biomarkers

Fig. 2A illustrates the GGT activity, revealing a notable and statistically significant decrease with increasing concentrations of DXE compared to both the control group and the DMSO group. Similarly, the alkaline phosphatase enzyme exhibited a consistent pattern, with the most significant decrease observed at the concentration of 50 ng/L. These findings indicate a concentration-dependent impact of DXE on GGT activity and alkaline phosphatase enzyme levels.

3.3. Gene expression

All concentrations of DXE examined, excluding DMSO, demonstrated a significant elevation in the expression of genes associated with stress and inflammation response, sensitivity to superoxide ion presence, and calcium signaling in the interrenal axis of *Cyprinus carpio* (Fig. 3). It is noteworthy that the highest concentration of DEX exhibited a more pronounced expression of genes such as *gr1a, gr1b, gr2, serca, ryr, ade1a,* and *stim.*

3.4. Bioconcentration factor

The analysis of interrenal tissue indicated a concentration-dependent presence of DXE at all four tested concentrations. Moreover, consistent bioconcentration factors were observed, remaining within the range of 0.5 to 0.6 across all concentrations. These results suggest a proportional uptake of DXE by the interrenal tissue, demonstrating a consistent pattern of bioaccumulation across the various concentrations tested (Table 4).

3.5. Pearson correlation

Pearson's correlation analysis unveiled robust and positive correlations among all biomarkers and both oxidative stress markers, GGT and ALP enzyme activities. Notably, the analysis highlighted a significant and positive correlation between stress-related genes, the inflammatory response sensitive to the presence of the superoxide ion, calcium signaling, and biomarkers of oxidative damage. These findings underscore the interconnectedness and mutual influence of various biological indicators, emphasizing the comprehensive impact of DXE exposure on the studied physiological and molecular parameters (Fig. 4).

4. Discussion

Increasing exposure to pharmaceutical contaminants, particularly glucocorticoids such as DXE, poses significant environmental health challenges, especially in aquatic organisms such as the common carp *Cyprinus carpio*. Given the clinical relevance of DXE in the treatment of diseases such as Covid-19, its presence in water has raised concerns about potential impacts on aquatic life and surrounding ecosystems. Previous research by Hidasi, 2016; Stolte et al., 2008a, highlights the ability of glucocorticoids to interfere with stress response and corticosteroid receptor expression in carp tissues. Furthermore, these compounds have been shown to induce oxidative stress in various biological systems (Deng et al., 2019; Hira et al., 2020), but unknowns remain as to how they specifically affect interrenal tissue and key biomarkers such as hydroperoxide (HPC), protein carbonyl (PCC) and lipoperoxide (LPX) content. This manuscript will discuss the findings obtained, to explain the mechanisms underlying the effects of DXE on the eco-endocrinology of juvenile carp interrenal tissue.

In this work, we decided to work with the interrenal tissue. This is located at the kidney head of teleost's and is the main site of steroid hormone and catecholamine production and triggers the stress response in these organisms (Hontela, 2005; Takahashi and Sakamoto, 2013). Because dexamethasone, a synthetic glucocorticoid, is similar in structure to the endogenous natural hormone cortisol, it could interfere with the stress response axis (Stolte et al., 2006). Cortisol binds to glucocorticoid receptors (GCRs) to regulate the expression of genes involved in a variety of crucial physiological processes, such as development, glucose metabolism, osmoregulation, immune response, and behavior. Therefore, the presence of synthetic glucocorticoids in wastewater can have a significant impact on multiple physiological processes in a variety of aquatic species (Hidasi, 2016).

Studies in phagocytes obtained from the interrenal tissue of the common carp (*Cyprinus carpio L.*) have demonstrated the expression of four corticosteroid receptors, including three glucocorticoid-related receptors (GCRs): GCR1, which has two splice variants (GCR1a and GCR1b), as well as GCR2, and a mineralocorticoid receptor, MR. These receptors begin to be expressed as early as four days post-fertilization and play a critical role in the development and functionality of the adult organism. Therefore, the interrenal tissue is a highly relevant site to study the effects of exposure to DXE and other synthetic glucocorticoids on the physiology and development of aquatic organisms (Stolte et al., 2008b).

Exposure to DXE has significant effects on mitochondrial function and calcium homeostasis, which may lead to increases in biomarkers of cellular oxidation and antioxidant enzymes, as observed in this study. Glucocorticoids, such as DXE, play a biphasic role in mitochondrial function, showing trophic effects at low concentrations and damaging effects at high concentrations (Du et al., 2009). Overstimulation or understimulation of the glucocorticoid signaling pathway can have a negative impact on cellular function (Lee et al., 2013).

The connection between glucocorticoids and mitochondrial function is established by mitochondrial oxidation, membrane potential and calcium retention capacity. (Du et al., 2009). These compounds may have adverse actions on mitochondrial integrity, as glucocorticoid response elements (GERs) in the mitochondrial genome and glucocorticoid receptors (mtGCRs) reside in this organelle (Lee et al., 2013). Mitochondrial regulation of gene expression can occur directly on mitochondrial DNA (mtDNA) and oxidative phosphorylation genes (OXPHOS), as well as indirectly on nuclear genes responsible for the synthesis of proteins essential for mitochondrial function (Lee et al., 2013; Kokkinopoulou and Moutsatsou, 2021).

DXE induces dysregulation of oxidative phosphorylation and reactive oxygen species (ROS) formation, as evidenced by the biomarkers evaluated in Fig. 1, including hydroperoxides (HPC), protein carbonylated (PCC) and lipoperoxides (LPX). In addition, antioxidative enzymes such as catalase and superoxide dismutase show a significant increase on exposure to dexamethasone, which is supported by studies such as that of Hira et al. (2020). These biomarkers indicate antioxidant response to ROS generation and dysregulation of mitochondrial function.

Calciotropic hormones, such as cortisol, aldosterone and DXE, participate in pathways with calcium as a second messenger, generating transduction signals (Lin and Hwang, 2016). Mitochondrial products, such as ROS and cellular messengers such as cAMP, ADP, Ca^{+2}, NAD^{+}, have the ability to regulate nuclear gene expression (Liu and Butow, 2006).

In summary, exposure to dexamethasone triggers complex responses in mitochondrial function, resulting in ROS generation and activation of antioxidant enzymes as an adaptive response. These findings have important implications for understanding eco-endocrinological mechanisms and should be considered in the context of cellular health and survival in organisms such as the fish *Cyprinus carpio.*

In reference to biochemical biomarkers, DXE can generate the

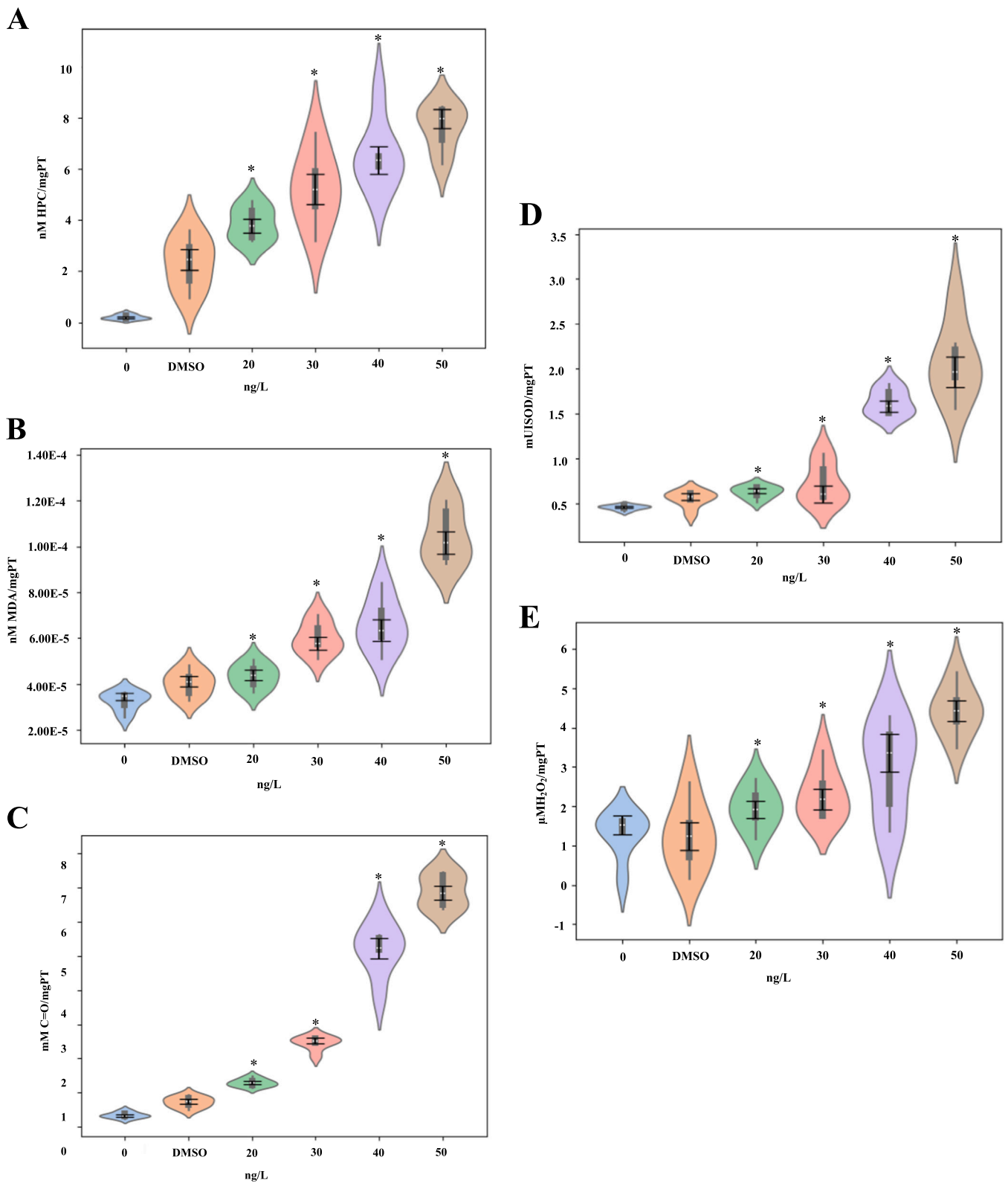

Fig. 1. Oxidative stress induced by DEX in the interrenal axis of *Cyprinus carpio.* Asterisks (*) denote a significant difference compared to the control group. The data presented represent the mean values ± standard deviation from six independent experiments. Each letter corresponds to a specific oxidative stress biomarker (A: HPC, B: LPX, C: PCC, D: SOD, E: CAT). HPC [F (5,30) =44.146]; LPX [F (5,30) =60.794]; PCC [F (5,30) =34.054]; SOD [F (5,30) =29.372]; CAT [F (5,30) = 14.21].

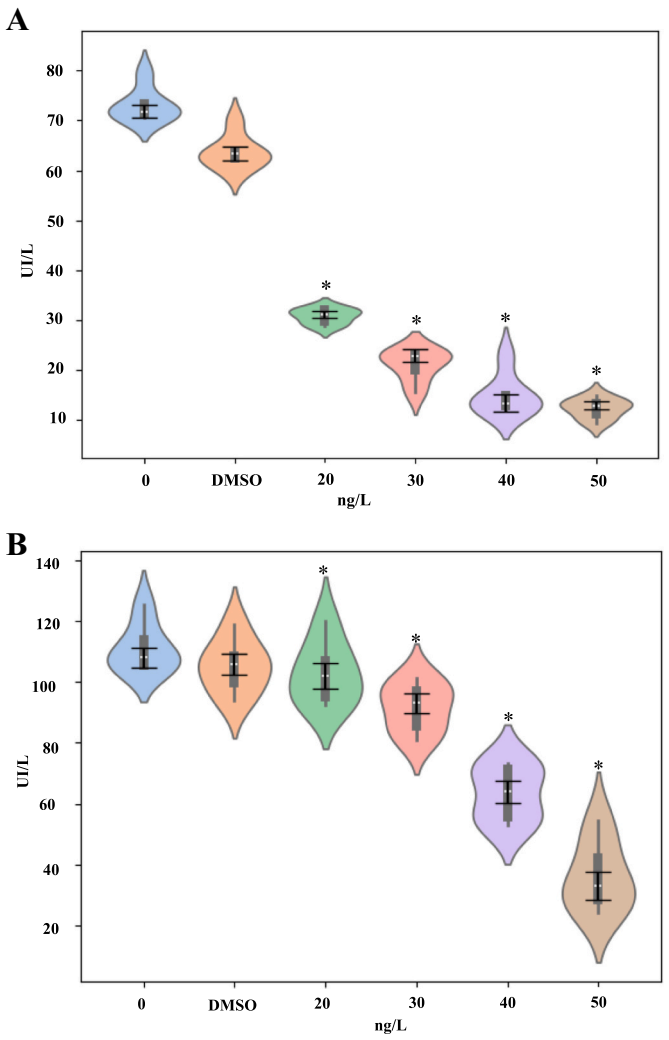

Fig. 2. DXE-induced alterations of Gamma-glutamyl-transpeptidase (GGT) and alkaline phosphatase (AP) in the interrenal axis of *Cyprinus carpio*. Asterisks (*) denote a significant difference compared to the control group. The data presented represent the mean values ± standard deviation from six independent experiments. A represents Gamma-glutamyl-transpeptidase activity and B alkaline phosphatase activity. GGT [F (5,30) =32.754]; ALP [F (5,30) =58.792].

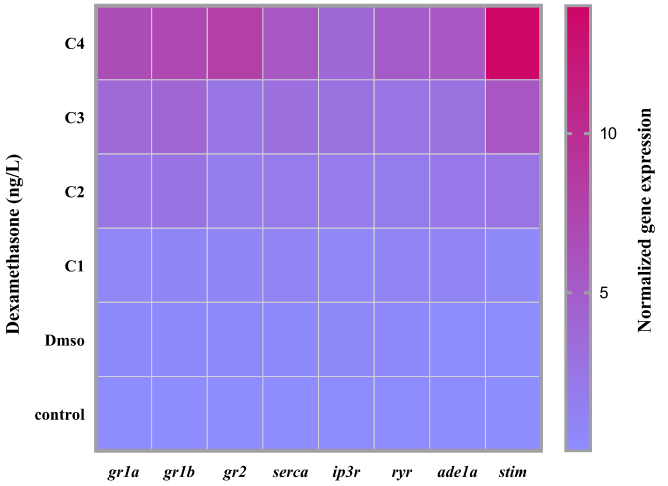

Fig. 3. Normalized expression levels of genes associated with stress and inflammation response (*gr1a, gr1b & gr2*); sensitive to the presence of superoxide ion (*ip3r & ryr*) and, calcium signaling (*serca, ip3r, ryr, ade1a & stim*) following acute exposure of interrenal axis of *Cyprinus carpio* to DXE. The presented data depict the mean values ± standard deviation derived from six distinct experiments. *gr1a* [F (5,30) = 0.484], *gr1b* [F (5,30) =0.529], *gr2* [(5,30) =0.426], *serca* [F (5,30) =0.240], *ip3r* [F (5,30) =0.691], *ryr* [F (5,30) =0.381], *ade1a* [F (5,30) =0.250], *stim* [F (5,30) =0.156]. ***gr1a:*** *Cyprinus carpio* mRNA for glucocorticoid receptor *GR1a (nr3c1 gene);* ***gr1b:*** *Cyprinus carpio mRNA for putative glucocorticoid receptor (nr3c1 gene), variant GR1b;* ***gr2:*** *Cyprinus carpio* glucocorticoid receptor *type 2;* ***serca:*** *Cyprinus carpio sarcoplasmic/endoplasmic reticulum calcium ATPase 2-like;* ***ip3r:*** *Cyprinus carpio inositol 1,4,5-trisphosphate receptor type 1-like;* ***ryr:*** *Ryanodine receptor;* ***ade1a:*** *Adenosine receptor;* ***stim:*** *Cyprinus carpio stromal interaction molecule 1-like.*

Table 4
DXE bioconcentration factor in *Cyprinus carpio* interrenal tissue.

Nominal concentration	DXE quantification		
	DXE in water of exposure (ng/L)	DXE in interrenal tissue (ng/g)	Bioconcentration factor (BCF)
CONTROL	ND	ND	ND
DMSO 0.01 %	ND	ND	ND
20 ng/L	8.4	3.9	0.46
30 ng/L	9.7	5.6	0.58
40 ng/L	10.3	6.4	0.62
50 ng/L	12.5	7.2	0.58

Values are the mean of three replicates ± S.D. LOQ-DEX for water: 3.15 ng/L and LOQ-DEX for interrenal tissue: 0.4 ng/g.

decrease of alkaline phosphatase (ALP) and gamma-glutamyl transpeptidase (GGT) in *Cyprinus carpio* due to its effects on enzymatic activity and cellular homeostasis. ALP, a widely distributed and thermostable enzyme, catalyzes the hydrolysis of phosphoric acid monoesters under alkaline conditions and is dependent on calcium for its function. In studies with osteoblast cells, dexamethasone has been shown to promote the formation of reactive oxygen species (ROS) and cause a significant decrease in ALP activity (Deng et al., 2019). The results obtained in this study reveal a decrease of >50 % in ALP activity at concentrations of 40 and 50 ng/L DXE.

Likewise, GGT is essential for glutathione homeostasis and the detoxification of xenobiotics. GGT catalyzes the breakdown of extracellular glutathione into cysteine, a key amino acid for intracellular glutathione synthesis. Under conditions of exposure to xenobiotics, such as DXE, GGT activity decreases rapidly, reaching levels up to 30 % below baseline at 20 ng/L DXE. This decrease in enzyme activity becomes most evident at 40 and 50 ng/L, where GGT activity is minimal. This phenomenon has been observed previously in comparative studies between rodents and fish, where it was shown that fish have a lower glutathione-dependent detoxification capacity during exposure to oxidants and electrophiles (Kendall, 1989).

DXE induces a decrease in ALP and GGT activity in *Cyprinus carpio*, suggesting an alteration in cellular homeostasis and detoxification capacity in response to exposure to this synthetic glucocorticoid.

As previously mentioned, at high concentrations DXE can induce alterations in the physicochemical properties of both plasma and mitochondrial membranes. When DXE intercalates in the membrane, it can modify the function of the associated proteins, generating lipoperoxidation as observed previously in this study and changes in their permeability (Itagaki et al., 2010). These events are associated with a rapid reduction of calcium stored in the endoplasmic/sarcoplasmic reticulum. In addition, DXE also generates a decrease in ATP synthesis, because of the uncoupling of oxidative phosphorylation accompanied by an increase in proton leakage from the mitochondria (Buttgereit and Scheffold, 2002; Stahn and Buttgereit, 2008).

The uncoupling of oxidative phosphorylation leads to increased superoxide ion ($\bullet O_2^-$) formation, which flows into the cytoplasm through the voltage-dependent anion channel (VDAC). This increase in anions causes calcium to flow from the storage into the mitochondria through

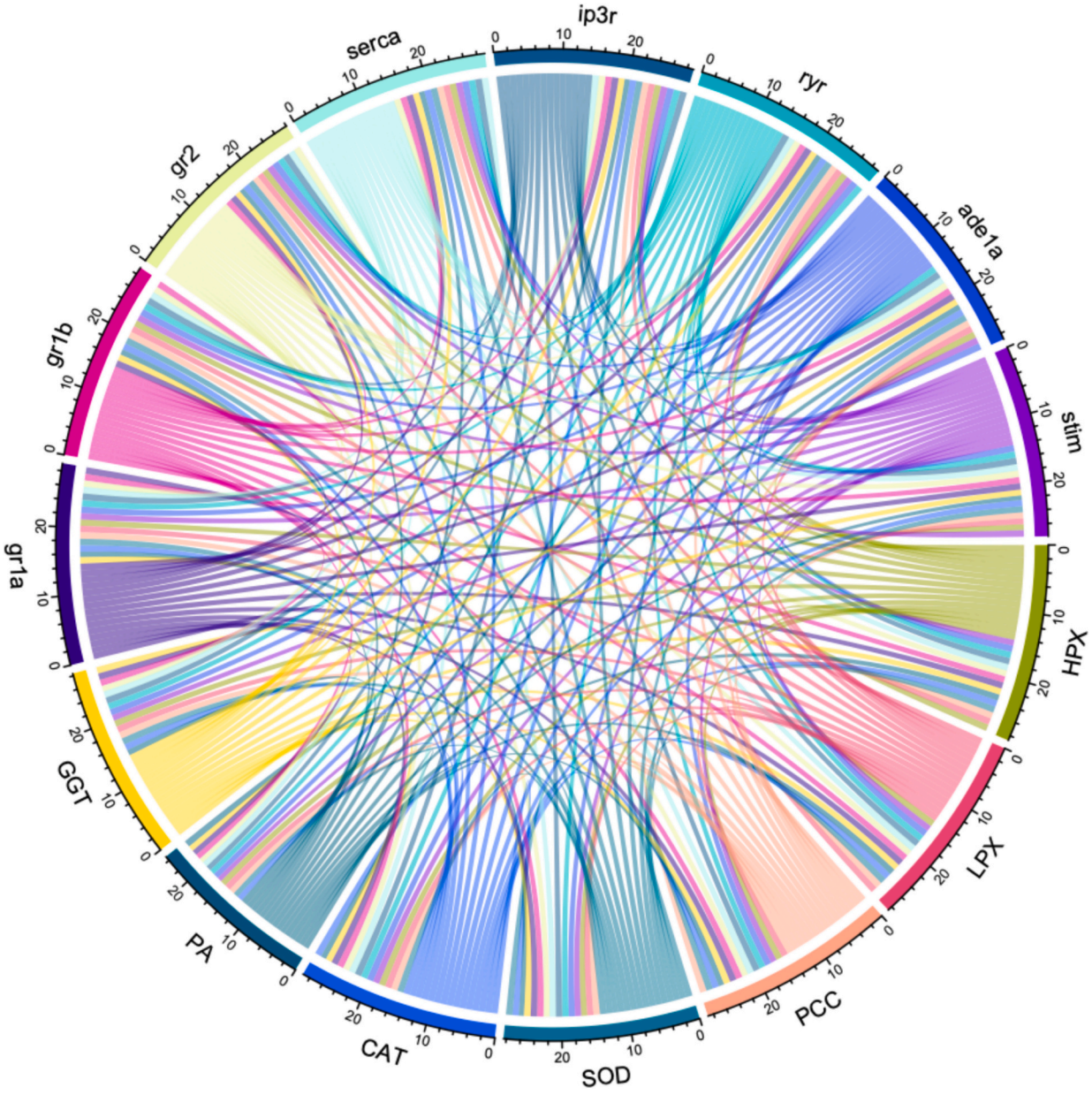

Fig. 4. Pearson correlation between oxidative stress, biochemical and gene expression biomarkers in *Cyprinus carpio*.

transporter channels: the ryanodine receptor (*ryr*) and the inositol triphosphate receptor (*ip3r*), which are anchored in the endoplasmic/sarcoplasmic reticulum. Calcium transport requires energy, which is generated by the ATPase, *serca* (sarco-endoplasmic reticulum Ca^{+2} ATPase) (Csordás and Hajnóczky, 2009).

In the outer membrane of the mitochondrion, VDAC forms a multimolecular complex with *ryr* and *ip3r*, with the aim of significantly increasing the concentration of calcium in the mitochondrial matrix. In particular, the presence of *ip3r* seems to be concentrated at the interface between the endoplasmic reticulum and the mitochondrion, being a potential target for the formation of reactive oxygen species, in particular superoxide ion. The decrease in stored calcium is detected by the ORAI sensor, which is very sensitive to low concentrations of this cation, which is coupled to *stim* (stromal interaction molecule) which form the structure of a calcium release-activated Ca^{+2} channel (CRAC), located in the plasma membrane, increasing the calcium entry towards the storage in the endoplasmic/sarcoplasmic reticulum, trying to compensate the decrease generated in the latter (Báthori et al., 2006; Csordás and Hajnóczky, 2009).

This series of findings agree with our results, since DXE shows an increase in gene expression of the glucocorticoid receptor isoforms *gr1a*, *gr1b* and *gr2* in *C. carpio*, mainly at the highest concentration (50 ng/L). This concentration causes an elevated gene expression (up-regulation) of these receptors in the following order: *gr2* > *gr1a* > *gr1b*. With respect to genes related to intracellular calcium dynamics, the results obtained show a correlation between the concentration of dexamethasone and the expression of *stim, serca, ryr, ip3r* and *ade1a* genes.

Based on the findings of this study, we propose a mechanism by which dexamethasone (DXE) exerts its effects on the interrenal axis of *Cyprinus carpio*. Glucocorticoids, including DXE, exert their biological activity through the glucocorticoid receptor (GCR), which becomes active upon binding to its ligand, either natural (cortisol) or analogs such as DXE. In common carp, the GCR is expressed in three isoforms: *gr1a, gr1b*, and *gr2*. DXE can enter cells either through G protein-associated receptors (GPR) or by diffusing across the plasma membrane. Once inside the cells, DXE triggers protein degradation by increasing intracellular calcium (Ca^{+2}) levels through store-operated

calcium entry (*SOCE*). *SOCE* acts via channels that constitute the calcium-release-activated calcium (CRAC) channel, which relies on a protein complex comprising a calcium sensor from the endoplasmic reticulum, known as stromal interaction molecule (*stim*), and the channel pore subunit (*orai*), both located in the plasma membrane and sensitive to intracellular calcium fluctuations.

Moreover, due to its lipophilic nature, DXE can easily diffuse across various membranes, including the plasma and mitochondrial membranes. By intercalating into these membranes, DXE can alter the structure of membrane-associated proteins, leading to lipid peroxidation (LPX) and changes in membrane permeability. These alterations are accompanied by a rapid depletion of calcium stored in the endoplasmic/sarcoplasmic reticulum (Buttgereit and Scheffold, 2002).

Furthermore, high concentrations of DXE in the cellular environment result in decreased ATP synthesis due to increased proton leakage associated with uncoupling of oxidative phosphorylation. Simultaneously, there is a notable increase in superoxide anion ($\bullet O2^-$) generation, which can migrate into the cytoplasmic space via the voltage-dependent anion channel (VDAC). This mitochondrial increase in anions ($\bullet O^{2-}$ and H_2O_2) activates calcium transporter channels, namely the ryanodine receptor (*ryr*) and the inositol triphosphate receptor (*ip3r*), anchored to the endoplasmic/sarcoplasmic reticulum, the primary storage site of calcium. Calcium release from these storage sites occurs only in the presence of energy provided by the ATPase *serca* (sarcoplasmic/endoplasmic reticulum Ca^{+2} ATPase).

To facilitate calcium dynamics between the mitochondria and the storage site, VDAC on the mitochondrial outer membrane forms a multiprotein complex with *ryr* and/or *ip3r*, ensuring a significant increase in calcium flux to the mitochondrial matrix. Notably, *ip3r* appears to be concentrated at the interface between the endoplasmic/sarcoplasmic reticulum and the mitochondria and may serve as a potential target for reactive oxygen species, particularly superoxide ions (Fig. 5).

5. Conclusions

Exposure of *Cyprinus carpio* to DXE resulted in a concentration-dependent increase in cellular oxidation biomarkers (HPC, LPX, PCC) within the interrenal axis. Moreover, the activity of key antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT) exhibited significant elevation compared to control groups. This exposure also induced noticeable changes in biochemical indicators, particularly in Gamma-glutamyl-transpeptidase (GGT) and alkaline phosphatase (AP) activity. GGT activity notably decreased with rising DXE concentrations, with the most significant decline observed at 50 ng/L. Additionally, DXE exposure led to heightened expression of genes associated with stress and inflammation response, sensitivity to superoxide ions, and calcium signaling in the interrenal axis of *Cyprinus carpio*. Notably, the highest DXE concentration prompted pronounced upregulation of stress-related genes such as *gr1a, gr1b,* and gr2, along with those associated with calcium signaling. Analysis of interrenal tissue revealed a concentration-dependent presence of DXE, with consistent bioconcentration factors observed across all tested concentrations. This consistent bioaccumulation pattern suggests uniform DXE uptake by interrenal tissue regardless of concentration. Overall, these results highlight how DXE exposure induces oxidative stress, alters biochemical markers, influences gene expression linked to stress and inflammation response, and exhibits consistent bioaccumulation within the interrenal axis of *Cyprinus carpio*. These findings offer valuable insights into the physiological and molecular responses of fish to DXE exposure, emphasizing

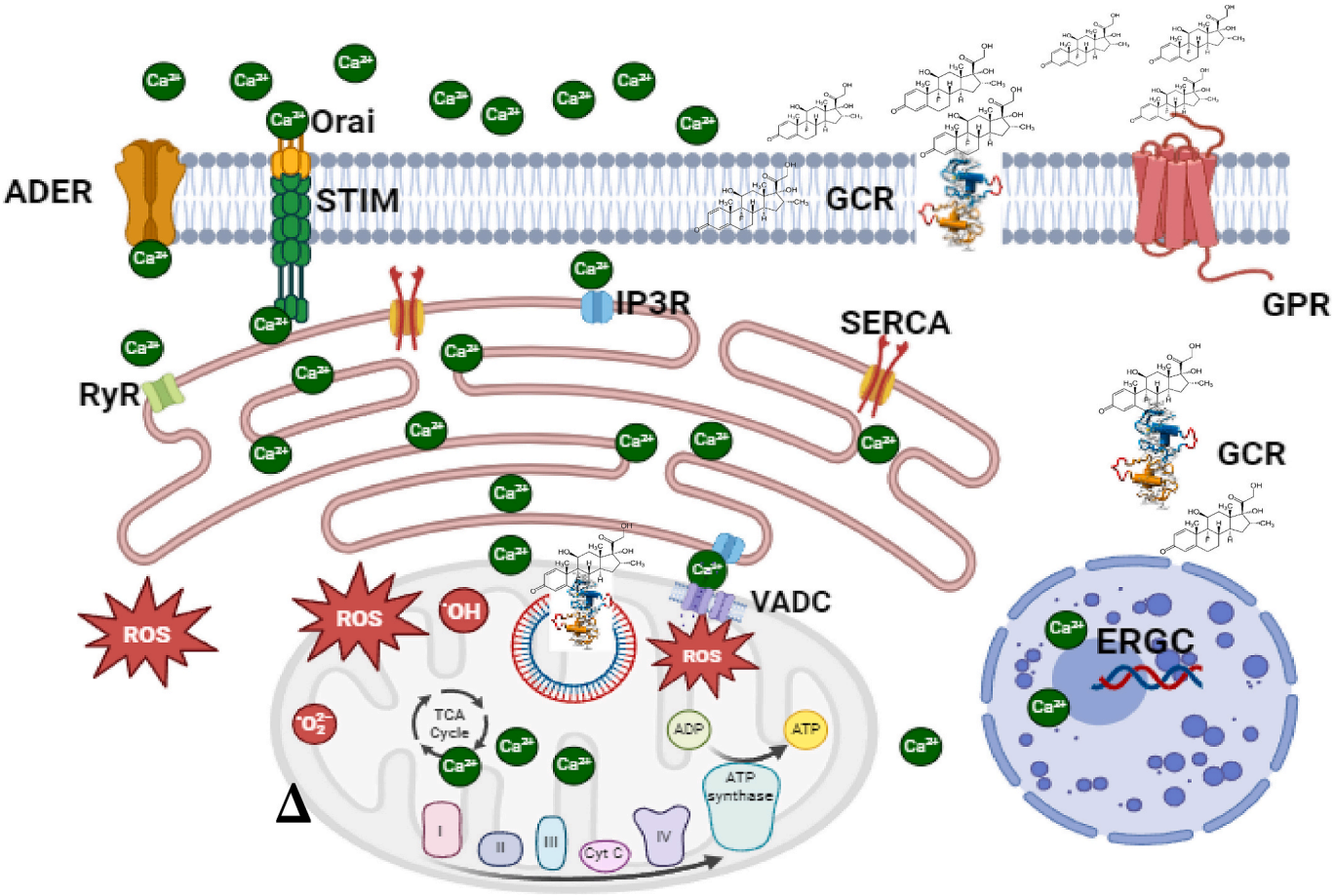

Fig. 5. Proposed toxicity mechanisms for DXE in the interrenal axis of *Cyprinus carpio*.

the potential ecological consequences of DXE contamination in aquatic ecosystems.

CRediT authorship contribution statement

Veronica Margarita Gutierrez-Noya: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Leobardo Manuel Gómez-Oliván:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **José Manuel Orozco-Hernández:** Software, Investigation, Formal analysis, Data curation. **Karina Elisa Rosales-Pérez:** Software, Methodology, Formal analysis. **Idalia Casas-Hinojosa:** Methodology, Investigation, Data curation. **Gustavo Axel Elizalde-Velázquez:** Software, Investigation, Formal analysis, Data curation. **Sandra Gracía-Medina:** Writing – original draft, Software, Formal analysis. **Marcela Galar-Martínez:** Writing – original draft, Validation, Supervision, Conceptualization. **Luis Alberto Orozco-Hernández:** Investigation, Funding acquisition, Formal analysis.

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.

Data availability

Data will be made available on request.

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