

Biomarker approach for assessing chronic toxicity of Captan® herbicide using haematological, growth, endocrine and biochemical endpoints in air breathing catfish, *Clarias batrachus*

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Abstract

This study was conducted to determine the sub-lethal toxicity of Captan® on selected hematological (Hb, HCT, MCH) growth (K-factor, HSI, SGR), biochemical (serum glucose, protein), and endocrine parameters (growth hormone, T₃ and T₄) in *Clarias batrachus* under chronic exposures. Captan® was administered at predetermined exposure concentrations (0.53 and 1.06 mg/L) and monitored at day 15, 30 and 45 of the experimental periods. The experimental groups showed significantly lower values ($p < 0.05$) of haemoglobin content, haematocrit, MCH in Captan® exposed fish compared to control. Serum glucose was significantly higher ($p < 0.05$) in treated fish compared to the control group; reverse was the case for serum protein concentrations ($p < 0.05$). Assessment of growth parameters revealed significantly higher k-factor and SGR in control fish. HSI was however higher in treated fish which highlights the possibility of liver hypertrophy and hyperplasia of liver cells due to higher exposure and uptake of the herbicide. Endocrine responses (T₃ and T₄) emerged as the most sensitive biomarker category, depicting modulated responses between sub-chronic exposure at day-15 and chronic responses at day-45. In general, the study findings using these biomarkers indicate that Captan® exposures are capable of inducing stress-specific effects at the biochemical and physiological levels negatively impacting the overall health and longevity of such animals. The use of the IBR index provided a visual and easily comprehensible depiction of toxicity effects and biomarker responses in laboratory exposed fish and we anticipate a greater applicability in biomarker data from the wild which are largely heterogenous.

Introduction

The increase in human population and the proportional demand for food supply has in turn justified the massive use of technologies such as no-tillage, chemical and biological nutrition and intensive application of biocides (herbicides, insecticides and fungicides) (Pretty and Bharucha, 2015; Ogbeide et al., 2018; Ganie et al., 2021). Following the withdrawal of organochlorine pesticides like DDT and other chloro-organic herbicides (in the 1970s) other substitute pesticides, including herbicides still occur in high levels within agroecosystems, infiltrating air, soil, water and food matrices having adverse consequences for wildlife and human health (Rashid et al., 2010; Chukwuka et al., 2019). One such popular pesticide is Captan®, a non-systemic, general use pesticide (GUP) and phthalimide fungicide with predominant applications in vegetables, fruits and ornamentals farming (Müller et al., 2000). Despite a half-life of less than 24h in soil and water (half-life in lake water is 7h at 12°C and 1 h at 23°C) (Müller et al., 2000), environmental concentrations in surface water have ranged from 0.024–0.26 ug/L within aquatic environment not proximal to agrarian landscapes to 0.78 µg/L in surface water traversing agrarian landscapes (Filizola et al., 2005; Konstantinou et al., 2006).

Considering these environmental concentrations, and a low to medium bioaccumulation tendency, Captan® clearly constitutes a significant ecological risk to both target and non-target life forms of the aquatic ecosystem (Müller et al., 2000; Boran et al., 2012). Captan® exposure studies from fish toxicity studies have revealed deleterious pathological changes in liver, spleen and kidney, developmental toxicity and loss of vital functions like heart rate (Boran et al., 2012; Zhou et al., 2019). In human and rodents, it

has demonstrated significant carcinogenic, mutagenic, teratogenic and genotoxic potentials (Xu et al., 2011; Zhou et al., 2019). Other Captan® studies have recorded marked biochemical changes including decrease in the activity of microsomal cytochrome P-450, *in vivo* (Dalvi, 1988) and *in vitro* signs of oxidative damage and cytotoxicity in hepatocytes (Suzuki et al., 2004). Notwithstanding the few available studies on the Captan® toxicity on some vital physiological and biochemical markers in mammals (Dalvi, 1988), information on the chronic effects on fish are particularly sparse (WHO, 1990).

Various biomolecules, including glucose and proteins in serum proteins, antioxidant enzymes e.g., SOD, CAT and GST, endocrine parameters and hematological parameters have been used as biomarkers of measurable toxic effects of exposure to stressors (Adeogun et al., 2015; Adeogun et al., 2018; Jerome et al., 2020). However, biomarker usage in the field can be limited by data acquisition constraints which make the use of multivariate methods impracticable (Beliaeff and Burgeot, 2002). In such situations, a simple procedure that integrates and summarize biomarker responses into simplified interpretation is required. Thus, the complementary use of biomarkers with multiple interpretations to validate toxic responses is evolving into the use of a more simplified integrated biomarker response (IBR) index for stress response assessment in organisms under pollution regimes (Samanta et al., 2018; Paul et al., 2020).

Although the environmental impact of Captan® is expected to be limited by its high rate of hydrolysis (Müller et al., 2000) its continuous bioavailability implicates continuous and widespread human-usage and a resultant replenishment of environmental levels that increases risks of chronic exposures and effects (WHO, 1990). It is with this backdrop, that chronic toxic effects of Captan® was examined using critical endpoints i.e., haematological biomarkers (haemoglobin, total red blood cell count, total white blood cell count, mean corpuscular haemoglobin), growth biomarkers (condition factor, hepatosomatic index, specific growth rate), endocrine biomarkers (growth hormone, tri-iodothyronine, tetra-iodothyronine) and biochemical biomarkers (total serum protein, total serum glucose) in the air breathing catfish, *Clarias batrachus*. The responses of each endpoint were integrated into a single index of toxicity via the integrated biomarker response (IBR) protocol.

Materials And Methods

Fish maintenance and care

Adult specimens of the freshwater, air-breathing catfish, *Clarias batrachus* (n= 150) used in the study were purchased from an aquaculture farm at Basirhat, District North 24 Parganas, West Bengal. It was then transported to the Aquatic Toxicology laboratory, Barasat Government College, West Bengal [weight 108.4 ± 2.6 g (mean \pm SD); length 18.3 ± 0.9 cm (mean \pm SD)]. Flow-through outdoor tanks (capacity 6000 L) was used during fish acclimation in the laboratory for a total period of four weeks. They were maintained in chlorine free tap water (pH 7.5-7.8), 12:12 h light-dark cycle and natural temperature. A continuous oxygen supply to the water tanks was made available by means of air pumps (3-Watt Sobo 548A Aquarium Air Pump, China). A commercial fish diet having 30-35% crude protein was fed to the fish

ad libitum @ 3% of their body weight. Partial water renewal (30-35%) was done on every alternate day to ensure healthy water quality. The various experimental procedures that the fish were subjected to abided by the guidelines of Institutional Biosafety Committee, The University of Burdwan (approval no: BU/IBSC/20/Zo/34).

Test Chemical

Analytical grade Captan® (3aR,7aS)-2-[(trichloromethyl) sulfanyl]-3a,4,7,7a-tetrahydro-1H-isoindole-1,3(2H)-dione) C₉H₈Cl₃NO₂S, belonging to the phthalimide class of fungicides (molecular weight 300.59 g/mol; Jardine Distribution Inc.) was used as test solution.

Experimental design

Chronic toxicity study

Two sublethal concentrations of Captan® 0.53 and 1.06 mg/L, termed T1 and T2 respectively, were determined from fractions i.e., 1/10th, 1/20th respectively of predetermined Captan® 96h LC₅₀ (10.63 mg/l) (Saha, 2021). Captan® was measured using High Performance Liquid Chromatography (HPLC-Agilent 1260 Infinity, Agilent, Santa Clara, CA, USA) after an initial pre-treatment of the test solution. Since the difference between nominal and measured concentration was less than 5%, nominal values were used during the study. The fish were divided into three sets (ten fish per replicate): two Captan®-treated sets and control as per randomized design (Gomez and Gomez, 1984). There was a total of four replicates per set. Stock solution (1000 mg/L) of Captan® was made and stored in clean glass reagent bottles. An overhead tank filled with deep tube well water was used for dilution. The water and Captan® were entirely replenished after every three days in order to ensure the exact concentration and desired water quality during the entire period. Food was given to the fish 3-4 times a day until there was notable visual satiation. All the bioassays and water quality analysis were done following the protocols of American Public Health Association (APHA, 2012). The standard water quality parameters assayed throughout the experimental tenure are summarized in supplementary file.

Blood collection and haematological assays

The anaesthetization of fish was done with 1% benzocaine solution in distilled water. Blood was collected at 15-day interval i.e., three times for the entire 45-day exposure period. Blood was drawn by caudal venipuncture of fish using a sterile 5 ml 22-gauge needle syringe (Dispovan, India), quickly transferred to EDTA vacutainer tubes (Becton Dickinson, USA) and tapped with fingers to prevent clotting of blood. A small portion of blood was collected without EDTA and spun in cooling microcentrifuge at 4000 rpm for 20 minutes at 4°C. A 20-200 µl capacity micropipette (Eppendorf, India) was used to draw the straw-coloured serum, poured to 1.5 ml microcentrifuge tubes and preserved at -20 °C. The analysis of Protein Content in Serum Samples (PCSS), Glucose Content in Serum Samples (GCSS), Growth Hormone (GH), Triiodothyronine (T₃) and Tetraiodothyronine (T₄) was done within 48 hours.

The blood Haemoglobin (Hb) level was measured using Sahli's haemoglobinometer and 0.1 N HCl. The endpoint was noted when the colour in the sample tube matched with that of the standard tube following continuous dilution of the sample with distilled water (Dacie and Lewis, 2016). Capillary tubes were used to draw blood, spun in a micro-haematocrit centrifuge for 3-5 minutes at 6000 rpm. Post centrifugation, a scale was used to measure the length of packed erythrocytes and the entire column of blood (Bain et al., 2016). For the estimation of Total Red Blood Cell Count (TRBCC) and Total White Blood Cell Count (TWBCC), 0.02 ml of blood was added to 3.98 ml of Hayem's fluid and Turk's fluid respectively in a small glass tube. It was then slightly shaken to maintain a uniform suspension of cells in the solution. The cells were then counted under Neubauer's haemocytometer (Rohem, India). Haematocrit (HCT), TWBCC, TRBCC and Mean Corpuscular Haemoglobin (MCH) were expressed as:

$$\text{HCT (\%)} = (\text{Length of packed erythrocytes} \div \text{total length of blood column}) \times 100$$

$$\text{TWBCC (10}^3 \text{ mm}^{-3}\text{)} = [\text{Total number of white blood cells counted in 4 squares of haemocytometer (N}_{\text{wbc}}\text{)} \times \text{dilution factor (D}_f \text{ of 50)}] \div [4 \times \text{volume factor (V}_f \text{ of 0.1)}]$$

$$\text{TRBCC (10}^6 \text{ mm}^{-3}\text{)} = [\text{Total number of red blood cells counted in 5 squares of haemocytometer (N}_{\text{rbc}}\text{)} \times \text{dilution factor (D}_f \text{ of 200)}] \div [5 \times \text{volume factor (V}_f \text{ of 0.1)}]$$

$$\text{MCH (pg)} = [\text{Haemoglobin (g dL}^{-1}\text{)} \times 10] \div \text{TRBCC (10}^6 \text{ mm}^{-3}\text{)}$$

Growth analyses of fish

The growth pattern in fish was measured on the basis of morphometric and organo-somatic parameters like Condition Factor (K), Hepatosomatic Index (HSI) and Specific Growth Rate (SGR). The assay was done after every 15d, 30d and 45 d. The length and weight of fish were recorded using a meter scale and electronic balance (Model: SF-400D). K, HSI and SGR were estimated by standard formula following the methods of Le Cren (1951) and Kaviraj et al. (2004):

$$\text{Condition Factor (K, g cm}^{-3}\text{)} = (\text{Fish mass, g}) \div (\text{Total length of fish, cm})^3 \times 100$$

$$\text{Hepatosomatic Index (HSI, \%)} = (\text{Weight of liver in fish, g}) \div (\text{Total weight of fish, g}) \times 100$$

$$\text{Specific Growth Rate (SGR, \% day}^{-1}\text{)} = [\text{Log natural of final body weight of fish (W}_f \text{, g)} - \text{Log natural of initial body weight of fish (W}_i \text{, g)}] \div \text{Time interim (t)} \times 100$$

Serum hormone analyses

The analysis of Growth hormone (GH) assay was done using heterologous competitive ELISA according to the method described by Lal and Singh (2005). The plasma T₃ and T₄ levels were determined by ELISA kits provided by Pishtaz Teb Co. (Tehran, Iran).

Analyses of serum protein and serum glucose

The Protein Content in Serum Samples (PCSS) and Glucose Content in Serum Samples (GCSS) was estimated following the method of Bradford (1976) and glucose colorimetric detection kit (Invitrogen, Carlsbad, USA) respectively.

Calculation of IBR

Integrated biomarker responses (IBRs) using various parameters (blood, growth, endocrine and biochemical) exposed to different concentrations of Captan® was estimated and multi-biomarker response in *Clarias batrachus* depicted as star plots. Multi-biomarker response was analyzed according to the methodology proposed by Beliaeff and Burgeot (2002) with modifications by Guerlet et al. (2010). Briefly, for each tissue, the general mean (m) and standard deviation (s) of a given biomarker was calculated (including data from all treatments), and subsequently standardized to obtain Y , i.e., $Y = (X - m) / s$, where X is the mean biomarker value of a given treatment. Thereafter Z was calculated as $Z = -Y$ or $Z = +Y$ according to the expected biological effect, with “-” representing an inhibition of a biological effect and “+” representing an induction (such evaluation was based on the average baseline biomarker values). Then, biomarker scores (S) were calculated as $S = Z + |\text{Min}|$, where $Z \geq 0$ and $|\text{Min}|$ is the absolute value of all Y calculated for a given biomarker (including all measurements). The scores (S) of all biomarkers measured in a given treatment and tissue were depicted as Star plots. The IBRs were calculated according to the following formulas:

$$A_i = S_i/2 \sin\beta (S_i \cos\beta + S_{i+1} \sin\beta)$$

where $\beta = \text{Arc tan} (S_{i+1} \sin\alpha / S_i - S_{i+1} \cos\alpha)$ and $\alpha = 2\pi / n$, $S_{n+1} = S_1$.

where, A_i is the area connecting the two scores (S), S_i and S_{i+1} are two consecutive clockwise scores (radius coordinates) of a given star plot, and n is the number of biomarkers used for calculations. The IBR index for each biochemical parameters were then standardized to calculate the mean value of each biomarker.

Statistical Analyses

The Shapiro-Wilk test examined the normality distribution of data and data transformation was applied in case of asymmetry to conform to normality while Levene's test was utilized to check homogeneity. The Graph Pad Prism 9.1.1 computer program (Prism, USA) was employed for data analysis. Comparisons between control and exposed fishes were performed by two-way ANOVA followed by Dunnett's Comparison Test to determine significant differences among the means ($p < 0.05$ - Gomez and Gomez 1984). Results are summarized as mean \pm standard deviation (SD). Principal component analysis (PCA), as multivariate test for association between parameters was performed using Statistica®. Difference in means across quantified biomarker responses was also depicted using Graph Pad Prism ®version 7. The Kaplan–Meier estimator, also known as the product limit estimator, is a non-parametric statistic used to estimate the survival of test organism during exposure period. For all analysis, statistical significance was ascertained at no less than $p < 0.05$.

Results And Discussion

Acute toxicity

The Kaplan–Meier curve demonstrates that Captan® had a concentration and duration-dependent adverse impact on the overall survival rates of *Clarias batrachus* compared with the control group (Mantel-Cox log-rank test; $p < 0.05$) (Figure 1a-b). From the 96h LC₅₀ curve, it is observed that higher concentration of Captan® is required to achieve 50% mortality within 24h. It further reveals that this concentration to achieve 50% mortality in exposed population decreased gradually with increasing duration. This is a strong indication that Captan® is more lethal with increased exposure duration. The relationship between the 24h LC₅₀ and 96h LC₅₀ indicates the toxicity of Captan® increases by a factor of 0.73 within 96 hours of exposure (Figure 1a). However, the wider error bar for 24-hour LC₅₀ indicates a wider possibility of responses that could lead to varied mortality patterns (higher or lower than 50% mortality). The smaller error bars for LC₅₀ at higher exposure duration i.e., 48, 72 and 96 h LC₅₀ indicate limited variations in mortality patterns (Figure 1a). From the survival curve, there was a 100% survival likelihood *Clarias batrachus* at control and at the lower exposure concentrations i.e., 9 mg/L (Figure 1b). The survival curve also depicts that the likelihoods of survival was higher at lower Captan® exposure concentrations for both shorter (< 50 hours) and longer exposure durations (>100 hours) (Figure 1b) (Mantel-Cox log-rank test; $p < 0.05$).

Hematological profiles and indices

Assessment of Hb concentration in serum of fish across exposure concentrations revealed that across exposure intervals, i.e., 15days, 30days and 45days revealed a linear decrease in Hb levels with increased exposure of Captan® from control to T2 exposure group (fig 2). Similar trends were observed for haematocrit (Hct) levels, TWBCC, TRBCC, and MCH levels in the control group and across exposure concentrations. Blood parameters are reliable indicators for fish health and physiology because they directly reflect the relationship between habitat quality and health status (Adeogun et al., 2015; Burgos-Aceves et al., 2019). This study showed significantly lower hemoglobin and hematocrit levels in all treatments after exposure to Captan® compared to the control group ($P < 0.05$). This reduction in hemoglobin may be due to the destructive effects of pesticides on tissues that produce hemoglobin, which could impact the transport and distribution of dissolved oxygen to the tissues for metabolism and induce respiratory distress in affected fishes (Saravanan et al., 2011).

Along with the hemoglobin (Hb) content and the leukocyte count, the hematocrit is regarded as a key indicator of the secondary stress response, and non-physiologically low hematocrit values are considered hallmarks of anemia, a specific pathophysiological stress response (Rebl et al., 2021). From this study, the changes in the levels of white blood cells following exposure to Captan® may be due to disturbances in the process of hematopoiesis and subsequent reduction or non-specific immune weakening in fish (Kumar et al., 2011). This study also revealed that the number of red blood cells (TRBCC) in all treatments after exposure to Captan® was less than the control group ($P < 0.05$). A decline in the number of red

blood cells primarily indicates the severe anemia caused by herbicide exposure, which reduces the total protein in blood plasma and has significant implications on the total energy balance of the body (Ramesh et al., 2009). The lower values of MCH in exposed fishes compared to the control group ($P < 0.05$) indicate a reduction in size and quantity of hemoglobin of red blood cells, which is diagnostic of anemia in fish (Rebl et al., 2021). The reduction of MCH could also reflect a large percentage of immature red blood cells in the bloodstream.

Growth biomarkers

Assessment of growth biomarkers and indices showed that irrespective of exposure intervals, condition factor (K) was significantly higher in control, followed by T1 and T2. HSI also differed in a linear pattern from control, followed by T1 and T2, however HSI was higher at highest Captan® exposure concentrations at T2 and least in control group. SGR on the other hand was significantly higher in control group and least in T2. The condition factor is an organism-level response, with factors such as nutritional status and toxic chemical exposure causing greater- than-normal and less-than-normal weights (Adeogun and Chukwuka, 2011; Ibor et al., 2019). However, the higher condition index values in control compared to the Captan® exposed fish is considered a reflection of depletion in energy reserves because this index is positively related to muscle and liver energy content (Stevenson and Woods Jr, 2006; Adeogun et al., 2013). The HSI on the other hand is one of the various organo-somatic indices and is often associated with contaminant exposure and response (Hismayasari et al., 2015; Pham and Nguyen, 2019). This is because the change in size of the liver is a reflection of its being a target organ for toxicity as well as a primary detoxification organ in fish (Velkova-Jordanoska and Kostoski, 2005; Feist et al., 2015). Thus, the higher HSI in Captan® exposed fish compared to fish in the control group reflects an increase in size (hypertrophy) or increased number of hepatocytes (hyperplasia) due to increased xenobiotic uptake and biotransformation (Adeogun et al., 2016).

The specific growth rate of fish is a tool mostly used in aquaculture for the estimation of fish food conversion rate over a certain period of time, and reduction in SGR can be related to the increase in pollutant-related stress (Sweilum, 2006). The significantly higher values of SGR in control group compared to Captan® exposure groups confirms that this herbicide impacts growth rate of exposed fishes possibly by disrupted metabolism and a resultant lowered food conversion rate.

Endocrine biomarkers

Expression of the pituitary growth hormones (GH) was significantly higher in control group and decreased linearly from T1 to T2. Patterns of decrease from control to highest exposure group was unchanged across exposure intervals (Figure 4a). However, irrespective of exposure intervals Tri-iodothyronine (T_3) did not differ significantly between control and T1 (intermediate exposure group), but marked difference was observed between the control group and highest exposure group. At day 15, thyroxine concentration in tissue of control fish was significantly lower than group T1 while levels in group T2 were significantly

higher than T1. At day 30 and 45, the trends reversed where T₄ showed highest levels in control group with decreasing levels from T1 group to T2 group.

The concentration-dependent down-regulation of the pituitary growth hormone in Captan® exposure groups is depictive of toxic effects. Studies have demonstrated negative effects of pollutants, including xenoestrogens and heavy metals on GH-mediated mechanisms, via interference with the GH receptor and/or GH transcription (Deane and Woo, 2009). Furthermore, the lower level of T₃ in fish exposed to Captan® compared to control group could be attributed to decrease in T₃ plasma concentration due to an inability of the organism to produce the optimal level T₃ or hypothalamus, pituitary and ovary interaction (Ruby et al., 1993). On day-15, the elevated levels of T₄ level recorded in exposure- group is consistent with other species exposed to pesticide (Liu et al., 2011). The increased activity of T₄ in exposure groups compared to control group on day 15 may be indicative of sub-chronic adaptive response in exposed fish, and deployment of growth factors for repair and turnover of somatic cells. Thyroid hormones play important roles in multiple physiological functions in aquatic animals including development, growth, and reproduction (Leatherland, 2000). Another possible explanation is the disrupted synthesis of the circulating thyroid hormones, or disrupted secretion and eventual conversion of T₄ to T₃ conversion in exposed fish (Movahedinia et al., 2018). T₄ generally represents >95% of the thyroid hormone output and it is typically present in higher quantities than T₃ in the blood circulation, with the higher T₄ concentrations serving as a pool of prohormone that can be converted into T₃ by 5-iodothyronine deiodinases in target tissues (Eales, 2006; Zoeller et al., 2007).

Biochemical biomarkers

The changes associated with GCSS and PCSS levels are summarised in Figure 5. Study findings revealed that GCSS increased linearly and significantly from control group to T1 to T2 across all exposure concentrations. However, PCSS increased linearly and significantly from control group to T1 to T2 across all exposure concentrations. The significant differences in glucose concentrations in plasma ($p < 0.05$) between the control and treatment fish, following the action of Captan®, may be considered to be the manifestation of stress. Reports show that marked glucose increase is a general response of fish experiencing pollutant effects, because fish challenged by environmental pollution may have a higher turnover of glucose, and more glucose may be produced from non-carbohydrate substrates (Levesque et al., 2002). Hepatic synthesis of detoxifying enzymes under stress conditions requires high energy levels that necessitates documented depletion of hepatic glycogen stores and increased deployment of glucose (Begum and Vijayaraghavan, 1995; Ceron et al., 1997). The observed decreased protein levels may be seen in starvation, and malabsorption or malnutrition (Pagana and Pagana, 1998) following extended exposures to stress. Concurrent decrease in total serum protein and a decrease in the haematocrit values implicates chronic exposures to pollutant toxicity (Adeogun et al., 2015). Change in protein synthesis is reflective of severity in cellular damage (Osman and Kloas, 2010) and may have dire implications on physiological activities and effectiveness of the immune system (Svoboda et al., 2001).

Integrated Biomarkers Response (IBR)

In order to compare the overall stress on *C. batrachus*, the IBR index was applied. From Table 1, control exposure group showed higher values of the IBR index for all biomarker parameters compared to T1 and T2 exposure groups. Reverse trends where IBR index was higher in Captan® exposure groups compared to fish in control group was observed for HSI and GCSS (Table 1). This is because, unlike the case for other biomarkers, higher IBR index values for HSI and GCSS indicate adverse reaction from toxicity. As such, evident from this index, the rank of toxic effects due to Captan® exposures can be ordered as: $T2 > T1 > T_0$ (control).

The transformed data of all the studied biomarkers are presented as star plot for each site in Figure 6(a-d). The star plots showing progressive increase in reduction or increase in size of shapes obtained for each experimental group inherently reflects a gradient in toxic responses. From the size and shape of the grey area within the star plot, it is clear that T2 Captan® exposure group with the smallest sized star plot and grey area was the most impacted group, followed by T1, while the control group with the largest shape and grey area was the least impacted. The reverse trends explained above where large grey areas for some biomarkers indicate adverse impact is also applicable.

The IBR approach provides a simple tool for a general description of the “health status” of populations, by integrating different biomarker signals (Leiniö and Lehtonen, 2005). Other studies that utilized this IBR index also demonstrated consistent indications of adverse toxicity impacts regardless of the considerable variability in the biomarker sets used for the index calculations (Broeg and Lehtonen, 2006; Damiens et al., 2007).

Table 1. Summary of IBR mean values of different biomarkers to different Captan® concentrations

Biomarkers	Control (T0)	T1	T2
Hb	4.75	0.0	0.0
HCT	5.09	1.47	0.0
TWBCC	5.11	0.87	0.0
TRBCC	4.51	0.12	0.0
MCH	5.04	0.89	0.0
K	4.53	0.13	0.0
HSI	0.0	2.01	4.92
SGR	5.02	0.60	0.0
GH	5.11	1.27	0.0
T₃	3.60	4.02	0.0
T₄	1.69	1.54	0.0
T₃:T₄	2.21	3.57	0.0
GCSS	0.00	0.97	5.15
PCSS	4.96	0.54	0.0

Multivariate analysis: PCA

The PCA presents the association between dependent variables (haematological, endocrine, growth and biochemical parameters) and independent or effect variables (exposure interval and exposure group) within ordination space (fig 7). Two principal components were extracted with PC 1 axis accounting for 64.7% of the total variance between parameters. From PC1, we infer that irrespective of exposure interval, that fish from the control group show significant positive association with hematological parameters (Hb, RBC, WBC, MCH, haematocrit), biochemical (total protein), growth (condition factor, K, SGR), and endocrine parameters (T₃, T₄, T₃:T₄ ratio and GH). This positive association implies that these parameters generally showed patterns of higher concentration in fish from the control group compared to fishes exposed to Captan®. On the negative axis of PC 1, the T2 exposure group showed a positive association with glucose and HSI. This indicates that these parameters showed significantly higher values in fish from the T2 exposure group than the T1 exposure group and fishes from the control group. These findings readily corroborate depictions from the difference in means of these parameters across exposure groups and control.

From PC 2, the T2 exposure group showed a negative association with the $T_3:T_4$ ratio and T_3 (fig 7). This indicates that $T_3:T_4$ ratio, and concentrations of T_3 were markedly lower in the highest Captan® exposure concentration compared to fishes in intermediate exposure concentrations (T1). The significant decrease in plasma T_3 and T_3/T_4 ratio in fish exposed at highest concentrations of Captan® suggests the inhibitory effect of this herbicide on extrathyroidal conversion of T_4 to T_3 (Eales et al., 1999; Brar et al., 2010). Quantifying T_4 and T_3 levels has been recommended as a good approach for detecting most xenobiotic effects on the peripheral thyroid system (Zoeller, 2010). The thyroid system plays a pivotal role in the body homeostasis and functioning of the nervous, cardiovascular and reproductive systems, and of body growth control (Danzi and Klein, 2012), and the effects of thyroid hormones mainly depend on intracellular concentrations of its biologically active and most circulated form T_3 (Buha et al., 2018). As such, the depleted levels of T_3 in the highest Captan® exposures portend significant impairments in physiological homeostasis.

Conclusion

From this study, the depiction of biomarker responses using haematological, growth, biochemical and endocrine parameters strongly reflect the toxic potential of Captan® at different levels of biological organization. The sensitivity and modulations of thyroid hormones T_4 and T_3 at sub-chronic and chronic exposure duration highlight its potential for the accurate depiction of toxicity in the wild, where toxic responses are rarely linear due to complex relationships and confounding factors in the environment. As such, the fish thyroid system may represent a valuable sentinel in aquatic ecosystems for predicting the actions of chemicals on other vertebrates, including humans. The study findings indicate that environmental applications of Captan® necessitate regulatory steps and public health awareness due to the risks it portends to aquatic biota and possibly humans.

Declarations

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Credit authorship contribution statement

Shubhajit Saha: Conceptualization, Investigation, Methodology, Data Curation, Formal Analysis, Visualization, Writing-Reviewing and Editing, Writing-Original Draft.

Dip Mukherjee: Conceptualization, Visualization, Writing-Reviewing and Editing, Writing-Original Draft.

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Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

References

1. Adeogun A, Babatunde T, Chukwuka A (2013) Evaluation of response patterns in somatic and otolith features of laboratory-reared and wild *Clarias* exposed to industrial effluent. *Res J Appl Sci Eng Technol* 5:626–634
2. Adeogun A, Chukwuka A (2011) Effect of textile factory effluent on otolith and somatic growth parameters in *Clarias gariepinus*. *Zoologist* 9:70–77
3. Adeogun A, Salami O, Chukwuka A, Alaka O (2015) Haematological and Serum Biochemical Profile of the Blue Crab, *Callinectes amnicola* from two Tropical Lagoon Ecosystems. *African Journal of Biomedical Research* 18(3):233–247
4. Adeogun AO, Ibor OR, Imiuwa ME, Omogbemi ED, Chukwuka AV, Omiwole RA, Arukwe A (2018) Endocrine disruptor responses in African sharptooth catfish (*Clarias gariepinus*) exposed to di-(2-ethylhexyl)-phthalate. *Comparative Biochemistry Physiology Part C: Toxicology Pharmacology* 213:7–18
5. Adeogun AO, Ibor OR, Onoja AB, Arukwe A (2016) Fish condition factor, peroxisome proliferator activated receptors and biotransformation responses in *Sarotherodon melanotheron* from a contaminated freshwater dam (Awba Dam) in Ibadan, Nigeria. *Mar Environ Res* 121:74–86
6. Bain BJ, Bates I, Laffan MA (2016) *Dacie and Lewis practical haematology e-book*. Elsevier Health Sciences
7. Begum G, Vijayaraghavan S (1995) Carbohydrate metabolism in hepatic tissue of freshwater catfish *Clarias batrachus* L. during dimethoate exposure. *Food chemical toxicology* 33(5):423–426
8. Beliaeff B, Burgeot T (2002) Integrated biomarker response: a useful tool for ecological risk assessment. *Environmental Toxicology Chemistry: An International Journal* 21(6):1316–1322
9. Boran H, Capkin E, Altinok I, Terzi E (2012) Assessment of acute toxicity and histopathology of the fungicide captan in rainbow trout. *Experimental Toxicologic Pathology* 64(3):175–179
10. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72(1–2):248–254
11. Brar NK, Waggoner C, Reyes JA, Fairey R, Kelley KM (2010) Evidence for thyroid endocrine disruption in wild fish in San Francisco Bay, California, USA. Relationships to contaminant exposures. *Aquat*

Toxicol 96(3):203–215

12. Broeg K, Lehtonen KK (2006) Indices for the assessment of environmental pollution of the Baltic Sea coasts: integrated assessment of a multi-biomarker approach. *Mar Pollut Bull* 53(8–9):508–522
13. Buha A, Matovic V, Antonijevic B, Bulat Z, Curcic M, Renieri EA, Tsatsakis AM, Schweitzer A, Wallace D (2018) Overview of cadmium thyroid disrupting effects and mechanisms. *Int J Mol Sci* 19(5):1501
14. Burgos-Aceves MA, Lionetti L, Faggio C (2019) Multidisciplinary haematology as prognostic device in environmental and xenobiotic stress-induced response in fish. *Science of the total environment* 670:1170–1183
15. Ceron J, Sancho E, Ferrando M, Gutierrez C, Andreu E (1997) Changes in carbohydrate metabolism in the eel *anguilla anguilla*, during short-term exposure to diazinon. *Toxicological Environmental Chemistry* 60(1–4):201–210
16. Chukwuka A, Ogbeide O, Uhunamure G (2019) Gonad pathology and intersex severity in pelagic (*Tilapia zilli*) and benthic (*Neochanna diversus* and *Clarias gariepinus*) species from a pesticide-impacted agrarian catchment, south-south Nigeria. *Chemosphere* 225:535–547.
doi:<https://doi.org/10.1016/j.chemosphere.2019.03.073>
17. Dalvi R (1988) Involvement of glutathione in the reduction of captan-induced in vivo inhibition of monooxygenases and liver toxicity in the rat. *Journal of Environmental Science Health Part B* 23(2):171–178
18. Damiens G, Gnassia-Barelli M, Loquès F, Roméo M, Salbert V (2007) Integrated biomarker response index as a useful tool for environmental assessment evaluated using transplanted mussels. *Chemosphere* 66(3):574–583
19. Danzi S, Klein I (2012) Thyroid hormone and the cardiovascular system. *Medical Clinics* 96(2):257–268
20. Deane EE, Woo NY (2009) Modulation of fish growth hormone levels by salinity, temperature, pollutants and aquaculture related stress: a review. *Reviews in fish biology fisheries* 19(1):97–120
21. Eales J (2006) Modes of action and physiological effects of thyroid hormones in fish. *Fish endocrinology* 2:767–808
22. Eales JG, Brown SB, Cyr DG, Adams BA, Finnson KR (1999) Deiodination as an index of chemical disruption of thyroid hormone homeostasis and thyroidal status in fish, *Environmental Toxicology and Risk Assessment: Standardization of Biomarkers for Endocrine Disruption and*, 8th Volume. ASTM International, Environmental Assessment
23. Feist S, Stentiford G, Kent M, Santos AR, Lorance P (2015) Histopathological assessment of liver and gonad pathology in continental slope fish from the northeast Atlantic Ocean. *Mar Environ Res* 106:42–50
24. Filizola H, Ferracini V, Abakerli R, Gomes MA (2005) Monitoramento de agrotóxicos e qualidade das águas em área de agricultura irrigada. *Current Agricultural Science Technology* 11(2)
25. Ganie ZA, Obrigawitch T, Kang IH, Copeland D, Gutteridge S, May J, Schrage B, Albright R, Chandi A (2021) An outlook of FMC's current and future herbicide-resistance management strategies. *Pest*

management science 77(4):1559–1563

26. Guerlet E, Vasseur P, Giambérini L (2010) Spatial and temporal variations of biological responses to environmental pollution in the freshwater zebra mussel. *Ecotoxicology environmental safety* 73(6):1170–1181
27. Hismayasari IB, Marhendra APW, Rahayu S, Saidin SD, Supriyadi D (2015) Gonadosomatic index (GSI), hepatosomatic index (HSI) and proportion of oocytes stadia as an indicator of rainbowfish *Melanotaenia boesemani* spawning season. *International Journal of Fisheries Aquatic Studies* 2(5):359–362
28. Ibor OR, Adeogun AO, Regoli F, Arukwe A (2019) Xenobiotic biotransformation, oxidative stress and obesogenic molecular biomarker responses in *Tilapia guineensis* from Eleyele Lake. *Nigeria Ecotoxicology environmental safety* 169:255–265
29. Jerome FC, Hassan A, Chukwuka AV (2020) Metalloestrogen uptake, antioxidant modulation and ovotestes development in *Callinectes amnicola* (blue crab): A first report of crustacea intersex in the Lagos lagoon (Nigeria). *The Science of the total environment* 704:135235
30. Kaviraj A, Bhunia F, Saha N (2004) Toxicity of methanol to fish, crustacean, oligochaete worm, and aquatic ecosystem. *Int J Toxicol* 23(1):55–63
31. Konstantinou IK, Hela DG, Albanis TA (2006) The status of pesticide pollution in surface waters (rivers and lakes) of Greece. Part I. Review on occurrence and levels. *Environ Pollut* 141(3):555–570
32. Le Cren ED (1951) The length-weight relationship and seasonal cycle in gonad weight and condition in the perch (*Perca fluviatilis*). *The Journal of Animal Ecology*:201–219
33. Leatherland JF (2000) Contaminant-altered thyroid function in wildlife. *Environmental endocrine disruptors: an evolutionary perspective*:155–181
34. Leiniö S, Lehtonen KK (2005) Seasonal variability in biomarkers in the bivalves *Mytilus edulis* and *Macoma balthica* from the northern Baltic Sea. *Comparative Biochemistry Physiology Part C: Toxicology Pharmacology* 140(3–4):408–421
35. Levesque H, Moon T, Campbell P, Hontela A (2002) Seasonal variation in carbohydrate and lipid metabolism of yellow perch (*Perca flavescens*) chronically exposed to metals in the field. *Aquat Toxicol* 60(3–4):257–267
36. Liu S, Chang J, Zhao Y, Zhu G (2011) Changes of thyroid hormone levels and related gene expression in zebrafish on early life stage exposure to triadimefon. *Environmental toxicology pharmacology* 32(3):472–477
37. Movahedinia A, Salamat N, Kheradmand P (2018) Effects of the environmental endocrine disrupting compound benzo [a] pyrene on thyroidal status of abu mullet (*Liza abu*) during short-term exposure. *Toxicol Rep* 5:377–382
38. Müller F, Ackermann P, Margot P (2000) Fungicides, Agricultural, 2. Individual Fungicides. *Ullmann's Encyclopedia of Industrial Chemistry*
39. Ogbeide O, Chukwuka A, Tongo I, Ezemonye L (2018) Relationship between geosorbent properties and field-based partition coefficients for pesticides in surface water and sediments of selected

- agrarian catchments: Implications for risk assessment. *J Environ Manage* 217:23–37.
doi:<https://doi.org/10.1016/j.jenvman.2018.03.065>
40. Osman AG, Kloas W (2010) Water quality and heavy metal monitoring in water, sediments, and tissues of the African Catfish *Clarias gariepinus* (Burchell, 1822) from the River Nile, Egypt. *Journal of Environmental Protection* 1(04):389
 41. Pagana KD, Pagana TJ (1998) *Mosby's manual of diagnostic and laboratory tests*. St. Louis, MO. Mosby, Inc.
 42. Paul T, Kumar S, Shukla S, Pal P, Kumar K, Poojary N, Biswal A, Mishra A (2020) A multi-biomarker approach using integrated biomarker response to assess the effect of pH on triclosan toxicity in *Pangasianodon hypophthalmus* (Sauvage, 1878). *Environ Pollut* 260:114001
 43. Pham HQ, Nguyen AV (2019) Seasonal changes in hepatosomatic index, gonadosomatic index and plasma estradiol-17 β level in captive reared female rabbit fish (*Siganus guttatus*). *Aquac Res* 50(8):2191–2199
 44. Pretty J, Bharucha ZP (2015) Integrated pest management for sustainable intensification of agriculture in Asia and Africa. *Insects* 6(1):152–182
 45. Rashid B, Husnain T, Riazuddin S (2010) Herbicides and pesticides as potential pollutants: a global problem, Plant adaptation and Phytoremediation. Springer, pp 427–447
 46. Rebl A, Seibel H, Baßmann B (2021) Blood Will Tell: What Hematological Analyses Can Reveal About Fish Welfare. *Frontiers in Veterinary Science* 8:194
 47. Ruby SM, Idler DR, So YP (1993) Plasma vitellogenin, 17 β -estradiol, T3 and T4 levels in sexually maturing rainbow trout *Oncorhynchus mykiss* following sublethal HCN exposure. *Aquatic toxicology* 26(1–2):91–101
 48. Samanta P, Im H, Na J, Jung J (2018) Ecological risk assessment of a contaminated stream using multi-level integrated biomarker response in *Carassius auratus*. *Environ Pollut* 233:429–438
 49. Saravanan M, Kumar KP, Ramesh M (2011) Haematological and biochemical responses of freshwater teleost fish *Cyprinus carpio* (Actinopterygii: Cypriniformes) during acute and chronic sublethal exposure to lindane. *Pesticide Biochemistry Physiology* 100(3):206–211
 50. Stevenson R, Woods WA Jr (2006) Condition indices for conservation: new uses for evolving tools. *Integrative comparative biology* 46(6):1169–1190
 51. Suzuki T, Nojiri H, Isono H, Ochi T (2004) Oxidative damages in isolated rat hepatocytes treated with the organochlorine fungicides captan, dichlofluanid and chlorothalonil. *Toxicology* 204(2–3):97–107
 52. Svoboda M, Luskova V, Drastichova J, Žlabek V (2001) The effect of diazinon on haematological indices of common carp (*Cyprinus carpio* L.). *Acta Veterinaria Brno* 70(4):457–465
 53. Sweilum MA (2006) Effect of sublethal toxicity of some pesticides on growth parameters, haematological properties and total production of Nile tilapia (*Oreochromis niloticus* L.) and water quality of ponds. *Aquac Res* 37(11):1079–1089

54. Velkova-Jordanoska L, Kostoski G (2005) Histopathological analysis of liver in fish (*Barbus meridionalis petenysi* Heckel) in reservoir Trebeništa. *Natura Croatica* 14(2):147–153
55. WHO, W. H. O (1990) Captan: health and safety guide, Captan: health and safety guide. p. 20–20
56. Xu, J-n., LDONG, Q.-k. WANG JHU, -f. X LI (2011) Transformation of Human Bronchial Epithelial Cells Induced by Captan. *Agrochemicals*:04
57. Zhou Y, Chen X, Teng M, Zhang J, Wang C (2019) Toxicity effects of captan on different life stages of zebrafish (*Danio rerio*). *Environmental toxicology pharmacology* 69:80–85
58. Zoeller RT, Tan SW, Tyl RW (2007) General background on the hypothalamic-pituitary-thyroid (HPT) axis. *Crit Rev Toxicol* 37(1–2):11–53
59. Zoeller TR (2010) Environmental chemicals targeting thyroid. *Hormones* 9(1):28–40

Figures

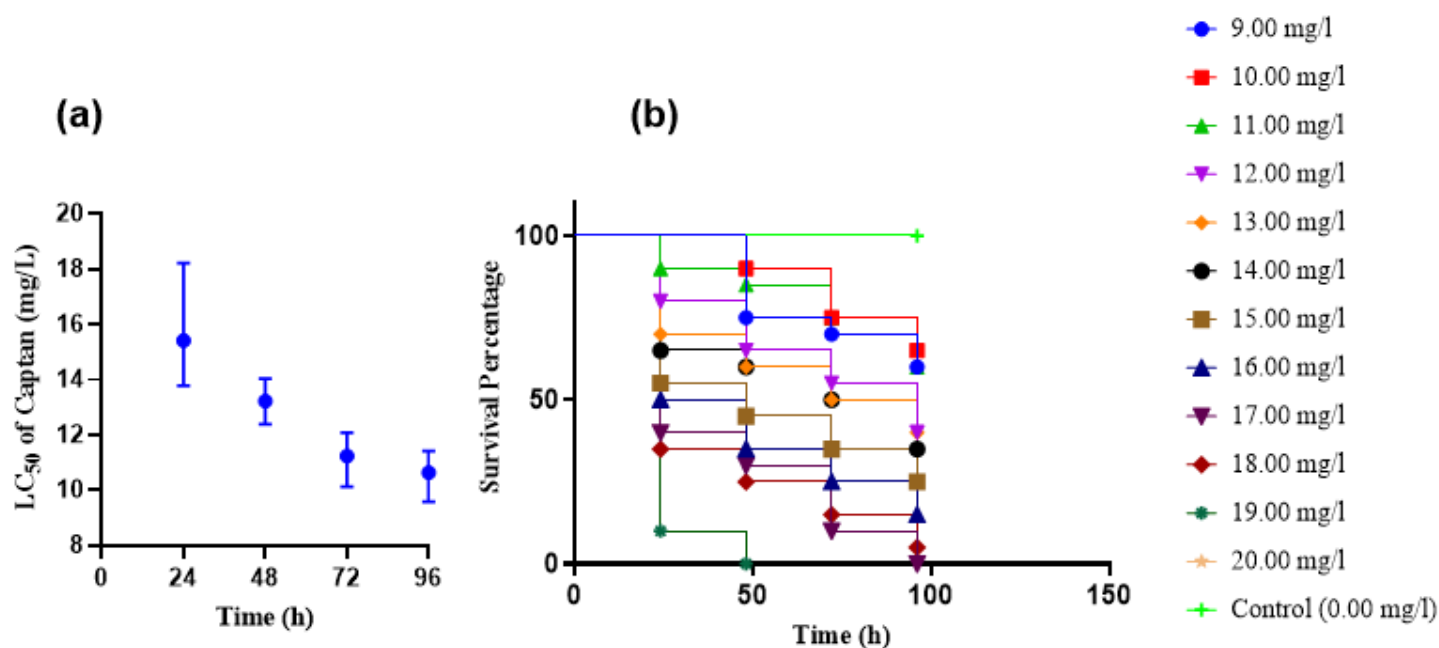


Figure 1

(a) 24, 48, 72 and 96 h LC₅₀ values associated with 95% confidence intervals of Captan® and (b) Kaplan–Meier survival curves of *Clarias batrachus* exposed to Captan® (Log-rank (Mantel-Cox) test (recommended); Chi square- 122.3; df 11; P value: <0.0001; P value summary: ****; Are the survival curves sig different: Yes).

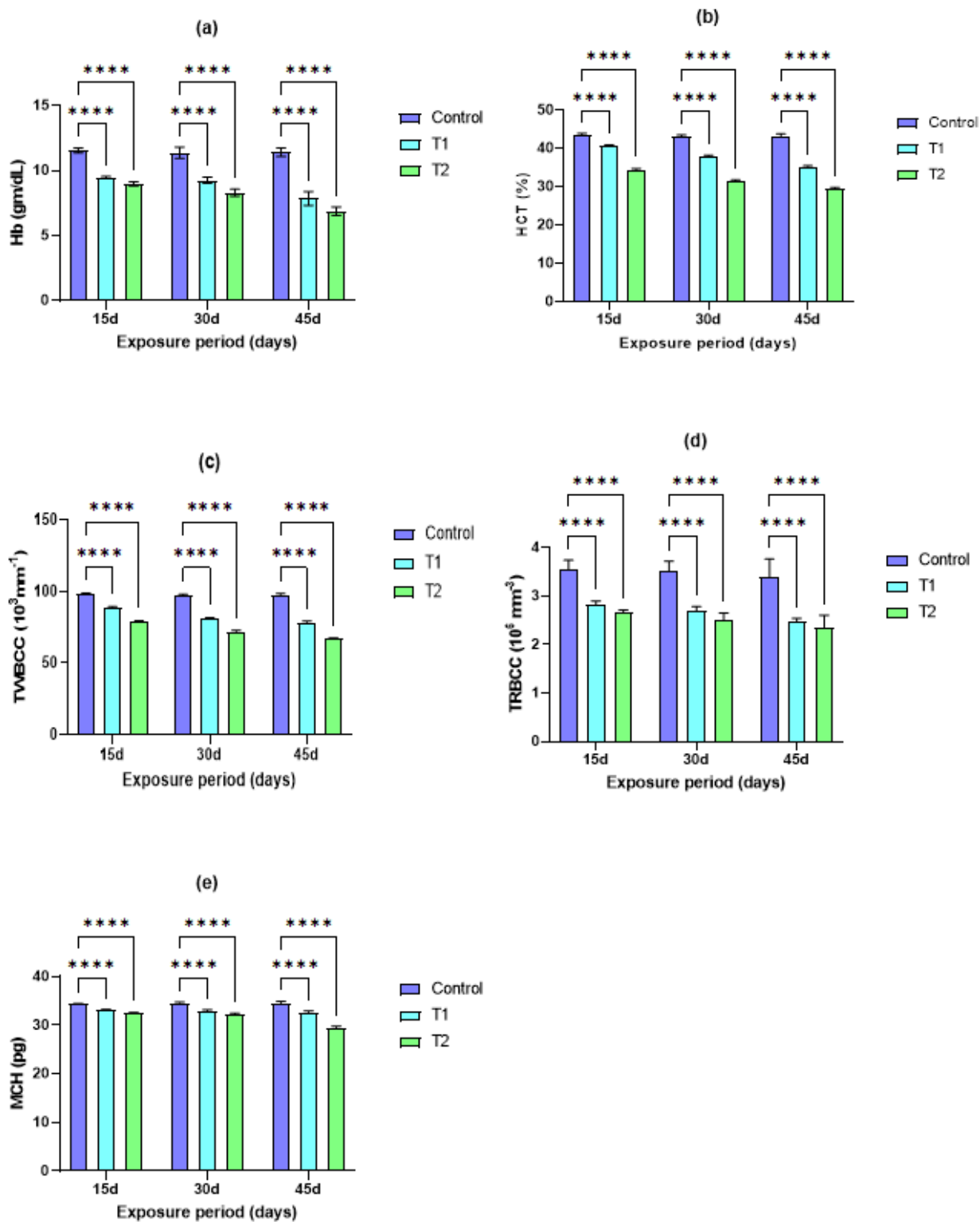


Figure 2

(a-e): Difference in mean values of haematological parameters (a) Haemoglobin (b) Haematocrit (c) Total white blood cell count (d) Total red blood cell count (e) Mean corpuscular haemoglobin in *Clarias batrachus* exposed to sublethal concentrations of Captan® (0.53 and 1.06 mg/L) for 15, 30 and 45 days. Where error bars= SD, * denotes significant differences to control within the same exposure time (p<0.05)

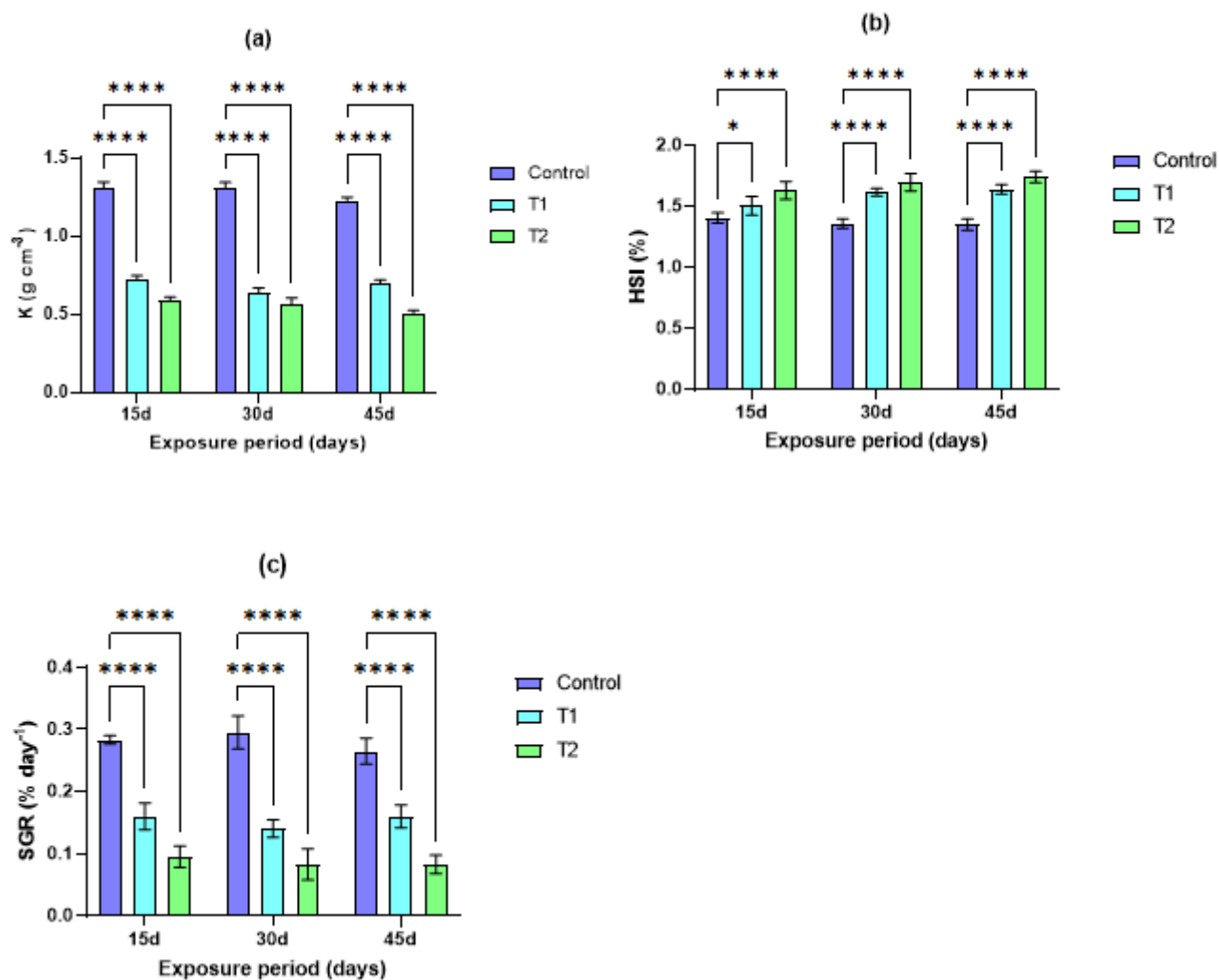


Figure 3

(a-c): Difference in mean values of growth endpoints and indices (a) Condition Factor (K); (b) Hepatosomatic Index (HSI) (c) Specific Growth Rate (SGR) in *Clarias batrachus* exposed to sublethal concentrations of Captan® (0.53 and 1.06 mg/L) for 15, 30 and 45 days. Where error bars= SD, * denotes significant differences to control within the same exposure time (p<0.05)

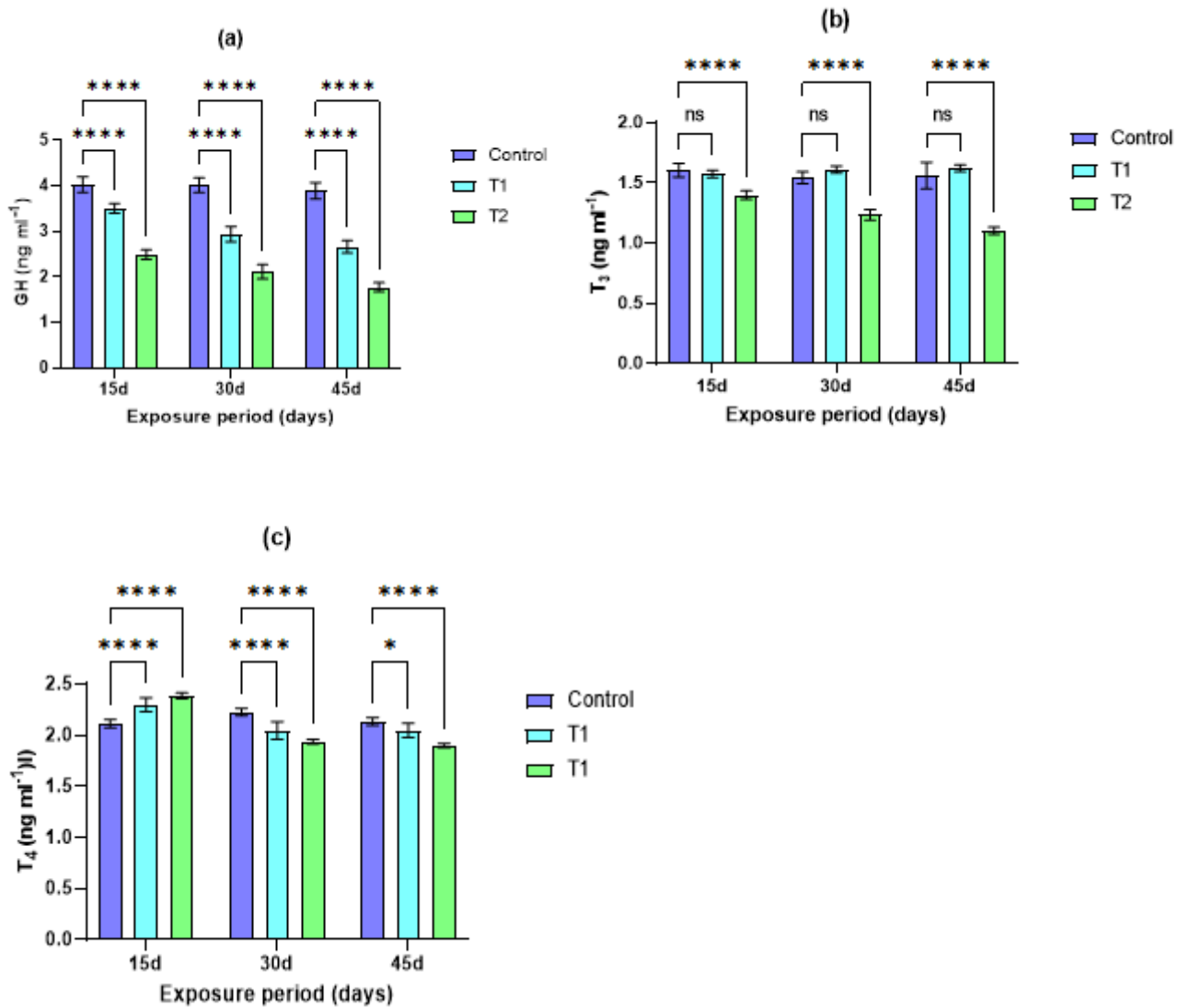


Figure 4

(a-c): Difference in mean values of endocrine biomarkers (a) GH - Growth hormone (b) T₃- Tri-iodothyronine (c) T₄- tetra-iodothyronine (thyroxine) in *Clarias batrachus* exposed to sublethal concentrations of Captan® (0.53 and 1.06 mg/L) for 15, 30 and 45 days. Where error bars= SD, * denotes significant differences to control within the same exposure time (p<0.05) Biochemical biomarkers

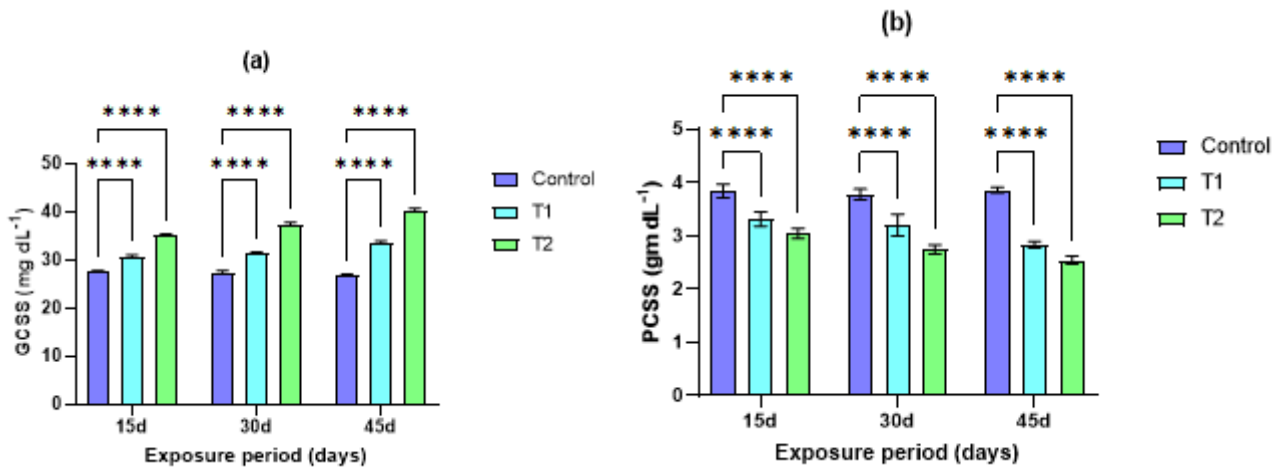


Figure 5

(a-b): Difference in mean values of biochemical parameters (a)GCSS- Glucose Content in Serum Samples, (b) PCSS- Protein Content in Serum Samples of *Clarias batrachus* exposed to sublethal concentrations of Captan® (0.53 and 1.06 mg/L) for 15, 30 and 45 days. Where error bars= SD, * denotes significant differences to control within the same exposure time ($p < 0.05$)

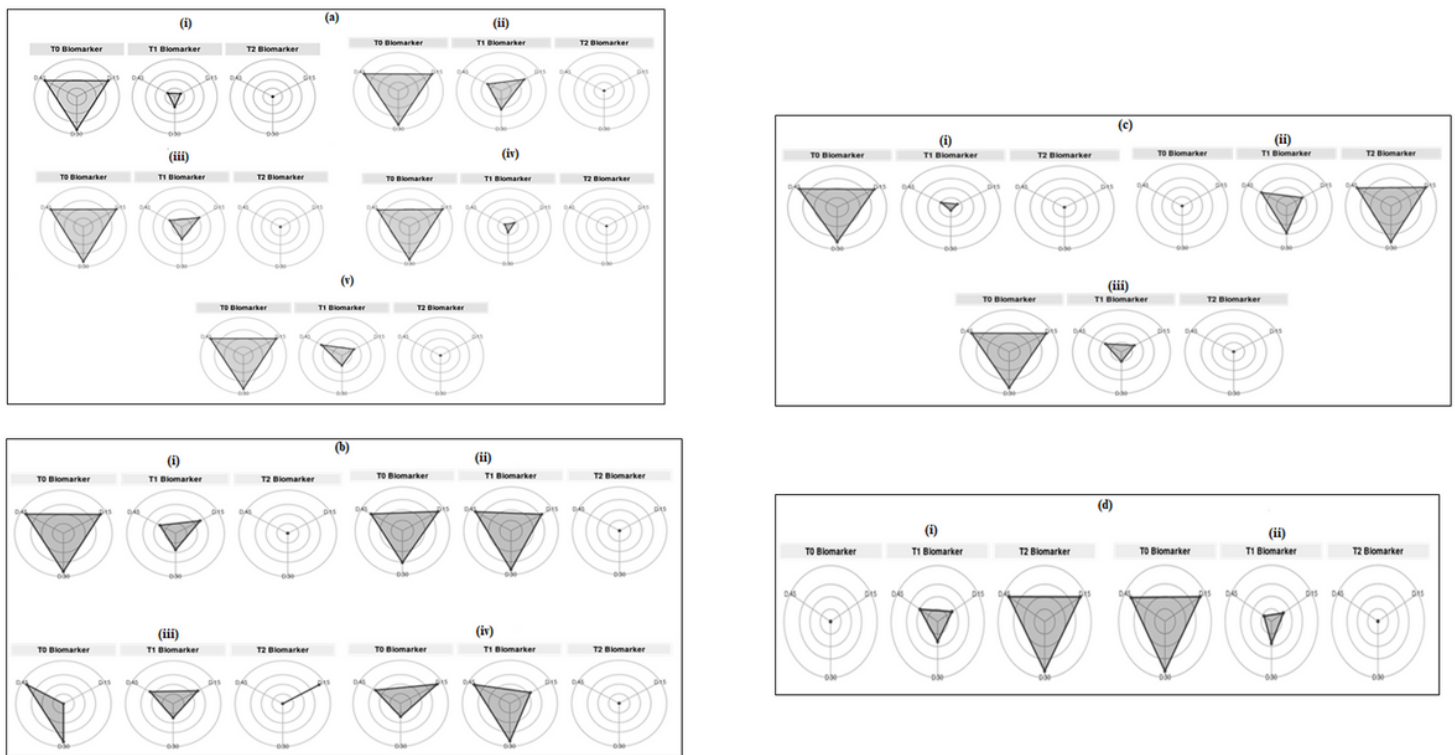


Figure 6

(a-d): IBR star plots for evaluating (a) haematological (i- Hb; ii- HCT; iii- TWBCC; iv- TRBCC; v- MCH) (b) growth (i- K; ii- HSI; iii- SGR) (c) endocrine (i- GH; ii- T3; iii- T4; iv- T3: T4) (d) biochemical (i- GCSS; ii-

PCSS) biomarker responses in *C. batrachus* exposed to different Captan® concentration (0.53 and 1.06 mg/l).

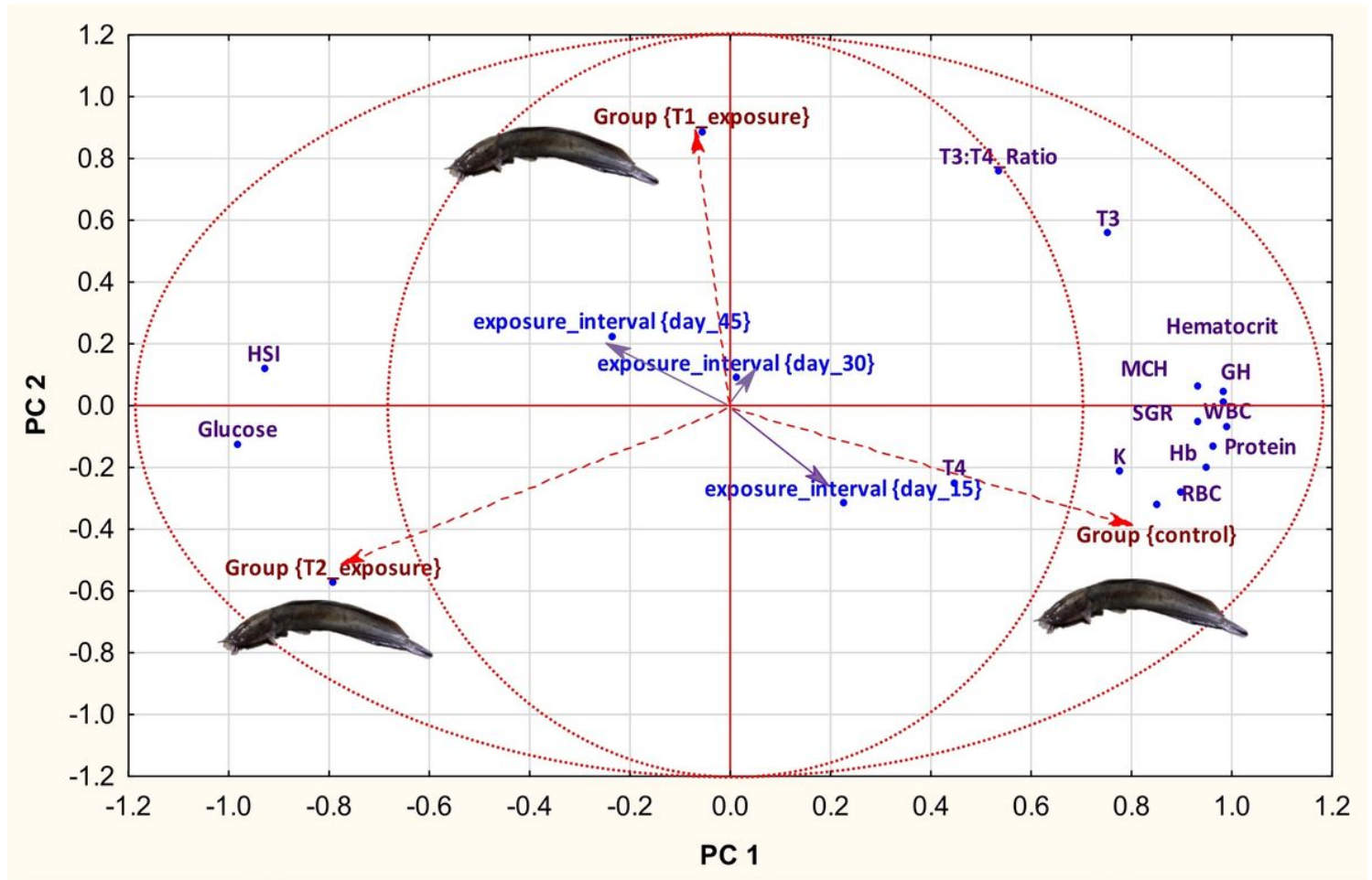


Figure 7

Principal component biplot of dependent variables (haematological, endocrine, growth and biochemical parameters) and independent /effect variables (exposure interval and exposure group).

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