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Effects of Long Term Exposure to Sub-lethal Concentrations of Diazinon on Blood Parameters of Alligator Gar (*Atractosteus spatula*)

Ahmad Omar-Ali[1], Russell L. Carr[1,2] and Lora Petrie-Hanson[1]*

[1]Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762, USA

[2]Center for Environmental Health Sciences, Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762, USA

***Corresponding author: Lora Petrie-Hanson,** Department of Basic Sciences, College of Veterinary Medicine P.O. Box 6100, Mississippi State, MS 39762, USA

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Abstract

Agrichemical runoff accumulates in waterways and chronically exposes aquatic animals to sub-lethal concentrations of pesticides. Diazinon is a common organophosphate pesticide. Alligator gar, *Atractosteus spatula,* were exposed to 0, 0.01, or 0.1 mg/L diazinon for 15 days and 30 days. Exposure at both of these durations caused significant alterations in hematological indices. Red blood cell counts, leukocyte counts, and hematocrit values significantly decreased, and hemoglobin values increased. Mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration values significantly increased. Peripheral blood lymphocyte numbers significantly decreased in fish exposed to the high concentration for both 15 and 30-day durations. In conclusion, long-term exposure to sub-lethal concentrations of diazinon induced changes in hematological indices and immune cells of alligator gar. These changes could adversely affect alligator gar health which could have negative ramifications for the entire ecosystem given the importance of apex predators in maintenance of trophic levels.

Keywords: Alligator gar; Diazinon; Organophosphate insecticide; Hematological parameters; Blood

Introduction

The use of pesticides to manage pest problems has become a common practice around the world, and they are used in agriculture, homes, parks, schools and on roads. This extensive use has had a significant impact on non-target organisms such as mammals, birds, and fish, by directly affecting immune, endocrine, and nervous systems [1-9]. Pesticides reach the environment by direct application but can be transported to other areas indirectly through such actions as storm runoff and soil erosion [10,11]. Only about 0.1% of applied pesticides actually reach their target pests and the rest spreads throughout the environment [12].

Diazinon is a common organophosphate (OP) insecticide used for agricultural and urban applications [13-16]. Diazinon is one of five pesticides that have the ability to concentrate in droplets [17] which allows distribution over substantial distances as they travel in the air through spray drift and post-application volatilization [18]. In addition, the U.S. Geological Survey reported that diazinon is the most frequently detected insecticide in urban streams and in the Mississippi River basin [19-21]. In addition, previous studies had reported that diazinon residues can rise to over 1.0 mg/L following application in rice fields and levels were still detectable 4 months after application [22,23].

To be toxic, diazinon must be converted to its oxon metabolite diazoxon which is mediated by cytochrome P450 in the liver [24] and other tissues. Diazoxon inhibits the activity of acetylcholinesterase (ChE), an enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine in cholinergic synapses [13,25-27]. If the inhibition level of ChE are significant, acetylcholine will accumulate in the synapses and will overstimulate the postsynaptic neuron [28,29]. This overstimulation leads to behavioral changes in fish, such as decreased feeding, erratic swimming, convulsions, increased superficial respiratory rhythm, and muscular weakness [30-32].

Since blood reflects the pathophysiological status of the body, hematological parameters and indices can be used to monitor the physiological and pathological state of fish following exposure to various environmental conditions but also exposure to toxic chemicals. These parameters, such as erythrocyte numbers (RBC), hemoglobin (Hb) levels, and hematocrit (Hct), and indices, such as mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), can be used as an indicator for anemia frequently caused by dysfunction of erythropoietic tissues such as kidney and spleen. The leukocytes (WBCs) of fish are also produced by the kidney and spleen [33] and are distributed in specific proportions to specific locations in the body. These cells are the first line of defense against pathogens [34]. An abnormally low level of leukocytes in blood, or leukocytopenia, is a condition that increases the susceptibility of fish to diseases [35]. With respect to diazinon, short-term exposure has been reported to lead to significant alterations in blood biochemical and hematological parameters in multiple species of fish including great sturgeon (*Huso huso*) [36], common carp (*Cyprinus carpio*) [37-39], kutum (*Rutilus frisii*) [40], European catfish (*Silurus glanis*) [41], African catfish (*Clarias gariepinus*) [42,43], Persian sturgeon (*Acipenser persicus*) [44], silver carp (*Hypophthalmichthys molitrix*) [45], Indian Carp (*Cirrhinus mrigala*) [46], and rainbow trout (*Oncorhynchus mykiss*) [47]. The vast majority of these studies involved short term exposure to fairly high levels of diazinon. When the levels of diazinon are reduced and the exposure period is extended, effects on hematological parameters have also been observed in many of these same species [9,37,40,43,48-51] in addition to other species including the Levantine scraper (*Capoeta damascina*) [52] and the grass carp (*Ctenopharyngodon idella*) [53].

Alligator gar inhabit coastal ecosystems and waterways adjacent to agricultural areas in Louisiana and Mississippi. These fish are apex predators and very important to maintain the balance of natural fish populations as well as to control invasive fish species. Their populations have been in decline due to habitat loss and alteration (dams, channel straightening, road construction, flow alteration, pollution, etc.) [54]. With respect to pollution, many types of fish are affected by environmental toxicants but gar may be more susceptible to these effects because of their high trophic position, long life span, and bottom-dwelling habits [55]. All gar are lepisosteids, one of the four members of the “ancient fishes”. They are also non-teleosts and actually differ genomically from teleosts [56,57]. Most of the studies involving the effects of environmental pollutants such as OP insecticides have focused on teleosts with fewer studies on the effect of exposure on primitive fish. This study was designed to simulate chronic sub-lethal environmental exposure to agricultural chemicals in waterways located in agricultural areas and determine the effects of that exposure on blood parameters of alligator gar. Our previous study reported that 30 day chronic exposure to diazinon significantly inhibited plasma ChE activity in alligator gar [58]. In teleost studies, the inhibition of plasma ChE is accompanied by changes in blood parameters [40,50]. In this study, we investigated the effect of exposure to long-term, sub-lethal levels of diazinon on blood parameters in the non-teleost alligator gar.

Materials and Methods

Experimental setup

Seventy-two alligator gar (average weight of 773.9 gm and average length of 53.8 cm) were obtained from the Private John Allen National Fish Hatchery in Tupelo, MS and were acclimated following transport in fresh water at the Mississippi Agriculture and Forestry Experiment Station, South Farm Aquaculture Unit. For experimentation, four fish were placed into exposure tanks and acclimated for 10 days prior to exposure. Fish were fed pelleted feed (Rangen. Inc.) daily throughout the experiment and held under a natural light cycle.

Stainless steel tanks held 350 liters of well water, and an air stone was in each tank at all times during each experiment. Water in each tank was exchanged every day to ensure optimum water quality and maintain diazinon concentrations. Water temperature in each tank was maintained at 21 ± 2°C using heaters; dissolved oxygen was maintained at 7.3 ± 0.2 mg/L, and the pH was 7.5 ± 0.2. Fish were observed two times a day and physical changes noted. Swimming patterns and feeding activity of diazinon exposed fish were compared to control fish, and differences noted. Changes in scale color, pigmentation pattern and mucus production associated with diazinon exposure were also noted. The MSU Institutional Animal Care and Use Committee (IACUC) approved fish holding and experimental protocols.

Diazinon exposures

Diazinon (99% purity) was purchased through Chem Service Inc. (West Chester, PA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The stock dilution of diazinon was dissolved in 3 mL ethanol to concentrations of 1.167 mg/mL and 11.667 mg/mL prior to use. Nine separate tanks were used: three control tanks with alcohol carrier, three tanks with final concentrations of 0.01 mg/L diazinon and three tanks with final concentrations of 0.1 mg/L diazinon (nominal water concentrations). Fish in the control tanks were exposed to the same concentration of ethanol carrier used with the diazinon exposures. A previous study determined that 0, 0.01, or 0.1 mg/L diazinon were the sub-lethal concentrations to use [58]. It is unclear if these are environmentally relevant concentrations. However, based on the literature available, only one previous study investigating diazinon used levels below 0.1 mg/L [49]. Previous studies reported that levels of diazinon in rice fields following application exceed these levels [22,23].

Two separate studies were performed to investigate two durations: 15 days and 30 days. The 15 day study represented a mid-range of time, and the 30 day study represented long-term exposure. Each concentration was assigned 3 replicate tanks with 4 fish in each tank, and resulted in a total of 12 fish per treatment. Tanks were cleaned daily and refilled with fresh water and the assigned rate of alcohol carrier alone or diazinon in alcohol. Fish status was monitored, and any change or abnormal behavior was recorded. In addition, water ammonia and diazinon concentrations were monitored though out the experiments.

Blood sampling

Diagnostic hematology kits and reagents were purchased from Sigma-Aldrich. At the end of each exposure period, fish were individually placed in a mixed solution of anesthetic (500 mg/L tricaine methanesulfonate MS-222) and euthanized. Blood samples were collected through the caudal vein using an evacuated blood collection system. The vacuum blood collection system consists of a 17-gauge double-pointed needle, a plastic holder, and a vacuum tube with rubber stoppers. These tubes contained sodium heparin and 0.9 g/L NaCl to prevent the blood from clotting. Hematological analyses included counting the numbers of erythrocytes (RBC), leukocytes (WBCs), measuring hemoglobin (Hb) using Drabkin's Reagent (Sigma), hematocrit (Hct), the mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH), and the mean corpuscular hemoglobin concentration (MCHC), following the unified methods of hematological examination of fish [59]. Blood smears were prepared from each blood sample, fixed with ethanol for 45 seconds, and stained with Giemsa (Sigma) following the manufacturer's instructions. Differential counts for leukocytes were performed based on morphological appearance, and cells were identified based on previous hematological study on alligator gar [60]. One hundred leukocytes were counted on each slide under light microscope 100x magnification. Peripheral blood differentials of alligator gar from fish exposed to 0.01 and 0.1mg/L diazinon were compared to control fish.

Statistical analysis

Data obtained in this study were tested for normality and then analyzed by two way analysis of variance (ANOVA) with factors of treatment and duration with three tank replicates per treatment. Significant differences were tested among measured variables of the control and exposed fish groups. Data are presented as mean ± SEM. Statistical analyses were performed using SPSS® version 23.0 software program for Windows 10, and. the differences between means were considered significant if $P > 0.05$.

Results

Behavioral observations

Behavioral observations were similar after 15 days and after 30 days of exposure to the low and high concentrations of diazinon. Fish swam actively during the acclimation period and in the first week of exposure. By the second week, diazinon exposed fish were motionless for most of the time. However, there were no changes in opercula movement, and fish gulped air normally. We did not observe a change in appetite. Diazinon exposed fish developed lighter skin color by the end of second week. The behavior, physical appearance and appetite of control fish were the same during the 15 and 30-day experiments, and did not change. No deaths occurred in either diazinon concentration or the control fish in both the 15 day study and the 30 day study (Table 1).

Hematological findings after 15 days exposure to 0.01 and 0.1 mg/L diazinon

There were significant differences between the control fish and the two chronic exposure groups (Table 2). Erythrocyte, leukocyte, and hematocrit values from low and high concentration exposed fish were significantly lower than the values from the control fish. There were no significant changes in hemoglobin and MCV values from low and high concentration exposed fish compared to the control group. Hemoglobin values from exposed fish were statistically higher than those from control fish.

Table 1: Clinical observations of alligator gar chronically exposed to two levels of diazinon.

Indices	Control 15 & 30 days	0.01mg/L diazinon 15 & 30 days	0.1mg/L diazinon 15 & 30 days
Death	No	No	No
Appetite	Normal	Normal	Normal
Opercula movement	Normal	Normal	Normal
Swimming	Active swimmer	Motionless	Motionless
Skin color	Normal	Lighter	Lighter

Table 2: Mean values of hematological analyses after 15 days continuous diazinon exposure.

Blood parameters	Control	0.01 mg/L	0.1 mg/L
RBCs (×10⁶/mm³)	1.1 ± 0.06	0.9 ± 0.05*	0.8 ± 0.05*
WBCs (×10³/mm³)	32.1 ± 2.3	25.9 ± 1.5*	22.5 ± 1.6*
Hb (mg/L)	33.4 ± 8.6	68.9 ± 15.8*	49.4 ± 10.7
Hct (%)	41.2 ± 1.5	36.1 ± 1.3*	33.3 ± 1.1*
MCV (fl)	407.9 ± 28.9	409 ± 21	364.1 ± 26.9
MCH (pg)	330.8 ± 92.3	713.6 ±142.8*	554.8 ± 112.3
MCHC (%)	70.7 ± 18.1	190.7 ± 42.7*	148.5 ± 31.8*

*Indicates significant changes (*P*>0.05), mean ± SEM.

Table 3: Mean values of hematological analyses after 30 days continuous diazinon exposure.

Blood parameters	Control	0.01 mg/L	0.1 mg/L
RBCs (×10⁶/mm³)	1 ± 0.06	0.8 ± 0.04*	0.6 ± 0.0.07*
WBCs (×10³/mm³)	31.4 ± 1.6	23.8 ± 1.8*	22.8 ± 2.1*
Hb (mg/L)	59.6 ± 15.7	94.3 ± 18.7	90.5 ± 14.7
Hct (%)	40.5 ± 0.9	36 ± 1.25*	32.8 ± 0.91*
MCV (fl)	404.4 ± 24.9	442 ± 19.3	405.1 ± 21.4
MCH (pg)	586.6 ± 142.2	1250 ± 245.6*	1142.4 ± 199.8
MCHC (%)	150.5 ± 42.06	280 ± 52.8*	285.3 ± 51.3*

*Indicates significant changes (*P*>0.05), mean ± SEM.

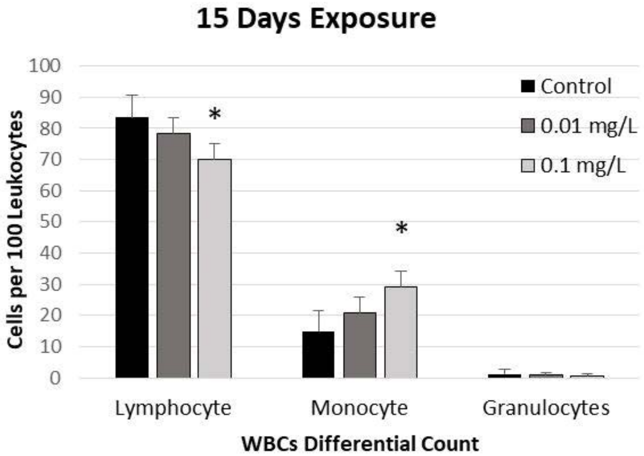

Figure 1: Differential counts of alligator gar peripheral blood leukocytes after diazinon exposure for 15 days. A significant decrease in lymphocyte number and a significant increase in the monocyte number was observed following exposure to 0.1 mg/L diazinon when compared to the control group. No change was observed with granulocyte count following exposure to either concentration of diazinon. There was no significant change in leukocyte counts in fish exposed to 0.01 mg/L diazinon.

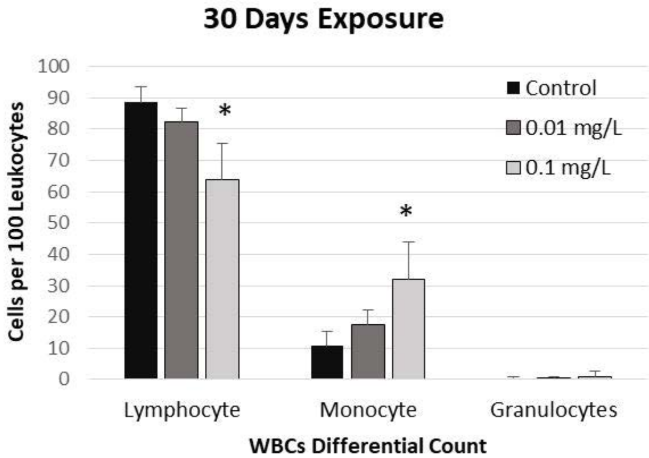

Figure 2: Differential counts of alligator gar peripheral blood leukocytes after diazinon exposure for 30 days. A significant decrease in lymphocyte number and a significant increase in the monocyte number was observed following exposure to 0.1 mg/L diazinon when compared to the control group. No change was observed with granulocyte count following exposure to either concentration of diazinon. There was no significant change in leukocyte counts in fish exposed to 0.01 mg/L diazinon.

MCH values from the low concentration were significantly higher than those from the control group. There was no significant difference between MCH values of high concentration exposed fish compared to the control group. There was a significant increase in MCHC values from low and high concentration exposed fish compared to the values from control fish.

Differential leukocyte counts showed significant decreases in lymphocyte counts in fish exposed to the high concentration compared to control group (Figure 1). There was no significant difference in lymphocyte count between control and low concentration exposed fish. Monocyte counts from high concentration exposed fish were significantly higher than in the control fish. There were no significant differences between monocyte counts from low concentration exposed fish compared to control fish. Also, there was no change in granulocyte counts from either low or high concentrations compared to the counts from control fish (Figure 1).

Hematological findings after 30 days exposure to 0.01 and 0.1 mg/L diazinon

Erythrocyte, leukocyte and hematocrit values from low and high concentration exposed fish were significantly lower than the values from control fish (Table 3). There were no significant changes in hemoglobin and MCV values from fish exposed to low and high concentrations compared to the control group.

Mean corpuscular hemoglobin values from low concentration exposed fish were significantly higher compared to control fish. There were significant increases in MCHC values from low and high concentration exposed fish compared to the control fish. Lymphocyte counts of fish exposed to 0.1 mg/L diazinon were significantly lower than the control group (Figure 2). Lymphocyte counts from fish exposed to 0.01mg/L diazinon were not statistically different than control fish. Lymphocyte counts from high concentration exposed fish were significantly lower than fish exposed to the low concentration and control fish. Monocytes significantly increased in fish exposed to 0.1 mg/L while there was no change in fish exposed to 0.01 mg/L diazinon when compared to the control group. Granulocyte counts did not show any significant changes with either treatment in comparison to the control fish (Figure 2).

At the end of 30 days continuous exposure of 0.1 mg/L diazinon, erythrocyte counts were significantly lower than after 15 days continuous exposure of the same concentration. After 30 days, Hb, MCH and MCHC were significantly higher than after 15 days exposure of 0.1 mg/L diazinon.

Discussion

The decreased swimming activity observed after 7 days of exposure could negatively impact reproduction given the propensity for alligator gar to move into shallow areas for spawning [61,62]. In addition, since OP exposure can increased predation in other fish species [63], decreased swimming due to diazinon exposure could decrease the ability of small gar to evade predators. The appetite of diazinon exposed alligator gar was normal during the exposure suggesting that the alligator gar would engage in normal predatory activities. However, eating easily available pelleted feed is not the same as the normal active feeding predatory behavior that the alligator gar would be involved in in a natural setting. Thus, it is possible that the predatory behavior of alligator gar could be negatively affected. While this would certainly be detrimental to the individual fish, decreasing the numbers or activity of an apex predator could have negative ramifications throughout multiple trophic levels thereby changing ecosystem structure [64,65].

Normal gar skin color is grayish to dark green dorsally [66]. In this study, the color of fish exposed to both concentrations and at both duration times became lighter. Similar studies in teleosts have reported changes in skin color after exposure to chronic sub-lethal concentrations of diazinon [38,67,68]. El-Sherif et al. [69] reported that the color of skin is controlled by the sympathetic nervous system and the change in color was due to, at least in part, the effects of the OP insecticide on nervous system signaling. Given that alligator gar are ambush predators that commonly utilize shading and structure as camouflage [62,70], lighter skin color could negatively impact the ability of the gar to elude visual detection of their prospective prey and decrease feeding success.

Our study revealed that significant changes in blood cell parameters were affected by sub-lethal chronic exposure of alligator gar to low and high concentrations of diazinon for 15 days and 30 days. The observed reduction in the number of erythrocytes and hematocrit percentages suggest that the exposed fish may be suffering from hemolytic anemia or some other destructive effect of diazinon on the blood forming organs. In addition, the reduction in erythrocyte count was statistically much lower in fish exposed to the higher concentration for 30 days when compared to fish exposed to either the lower concentration for 30 days or the low or high concentration for 15 days. Similar findings were reported in other sub-chronic diazinon exposure studies in teleosts using concentrations, albeit slightly higher, that were in the same range as those we used in this study [40,48-51,53]. In contrast to what observed in these teleost studies, total Hb in diazinon exposed alligator gar was numerically increased at both 15 and 30 days following both low and high diazinon exposure. Unfortunately, these data were highly variable with only one statistically significant time point (15 days with the low concentration of diazinon). Regardless, an increase in total Hb combined with a decrease in RBC numbers suggests that there were increased levels of Hb protein inside the RBCs. This could be a compensatory mechanism to maintain healthy oxygen and carbon dioxide levels in blood and tissues in response to the reduced number of RBCs resulting from the diazinon exposure. The significant increases in MCH and MCHC also indicate that the Hb content in the RBC is elevated. This increase in Hb occurs without any changes in RBC size as evidenced by the lack of an effect of diazinon treatment on MCV. No previous study investigating sub-chronic exposure to diazinon in teleosts observed similar changes with respect to MCV, MCH, and MCHC changes that we observed in the non-teleost alligator gar.

In our previous study, we observed that 30 day chronic exposure to the two concentrations of diazinon used in this study (0.01 and 0.1 mg/L) significantly inhibited plasma ChE activity (62% and 72%, respectively) in alligator gar [58]. When comparing the plasma ChE data to the current data, the concentration related changes are very similar. For almost all of the hematological parameters measured at 30 days, the values differ less than 10% between the low and high concentrations which matches very well with the plasma ChE data. The exception was MHC and where the difference between the low and high concentrations was 18% but they were not statistically different. Thus, it is very likely that the altered hematological parameters are related to the inhibition of ChE.

Total leukocyte counts were significantly reduced in alligator gar exposed to both concentrations of diazinon at both times indicating immune suppression possibly due to the malfunction of hematopoietic tissues. Many studies reported significant WBC decreases in various teleosts exposed to diazinon [38,41,71] and these findings in teleosts are similar to what we observed in this study. Specifically, alligator gar exposed to the high diazinon concentration for 15 and 30 days demonstrated lymphopenia. Other studies have documented that lymphopenia occurs in teleosts after exposure to pollutants, and increases the susceptibility of fish to infectious diseases [35,38,60,72,73]. In contrast to the lymphocyte numbers, exposure to the highest diazinon concentration resulted in an elevation in the number of monocytes. This elevation agrees with previous work performed on Nile tilapia (*Oreochromis niloticus*) [74] but is in contrast with work in other teleosts who observed either no effect or a decrease in monocyte numbers [38,40,48,49,51,53,75].

While not investigated in our experiments, short term exposure to high concentrations of diazinon altered mitogen-induced lymphocyte proliferation in Nile tilapia suggesting direct effects of diazinon on immune function [76]. However, this altered response may not be due to the direct action of diazinon or its active metabolite on the lymphocytes but indirectly due to the increased levels of acetylcholine that occurred as a result of the inhibition of ChE [77,78]. It is thought that the activation of the cholinergic receptor leads to calcium elevation which, in turn, produces reactive oxygen species [79]. The production of oxidative stress in various tissues have been observed following *in vivo* exposure of teleosts to diazinon [80-84]. Many of these studies suggest that oxidative stress plays a role in the toxicity of diazinon in fish species. Diazinon may also alter immune function as well. *In vitro* exposure of a macrophage cell line to diazinon increased oxidative stress levels but also enhanced pro-inflammatory cytokine levels [85]. Exposure of Nile tilapia to diazinon increased oxidative stress levels in leukocytes and reduced the phagocytic ability of these cells [79]. While reactive oxygen species production were not measured in this study, it is possible that this production played a role in the changes that were observed in alligator gar. Regardless, the reduction in the number of lymphocytes suggests that long-term exposure to diazinon would ultimately increase the susceptibility of alligator gar to pathogens and parasites.

Conclusion

Alligator gar normally inhabit backwaters and swampy lowlands which is a common destination for agricultural runoff and increases the chances of exposure of the inhabitants to agricultural chemicals such as the insecticide diazinon. In this study, long-term exposure to diazinon, even at low concentrations, significantly affects the hematological parameters in alligator gar. While not overtly toxic, these changes may indicate potential alteration of respiration and a compromised immune function. The alligator gar is an apex predator that relies on stealth and quickness for feeding success. The physiological parameters altered by diazinon exposure could decrease the predation and feeding efficiency of the gar which would also affect the populations of prey species, including endogenous and invasive species. Reduced feeding efficiency could decrease the alligator gar population as would an increased susceptibility to disease. This reduction could have negative ramifications throughout multiple trophic levels thereby changing ecosystem structure [64,65].

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Competing Interests

The authors declare that there are no conflicts of interest in the publication of this paper.

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