Histopathological Changes Induced by Chronic, Sub-Lethal Diazinon Exposure in Alligator Gar (Atractosteus Spatula) Tissues

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Abstract

Diazinon is a widely used household and agricultural pesticide that accumulates in the aquatic environment and adversely affects non-target organisms such as mammals, birds, and fish. Sub-lethal pesticide levels occur in natural waters, and can impact the health, physiology and fitness of fish populations. This study was conducted to assess the effects of chronic, sub-lethal diazinon exposure to skin, liver, kidney, spleen, heart, gut, intestine, and gas bladder tissues of alligator gar, Atractosteus spatula. In two studies, gar were exposed to sub-lethal concentrations of 0.01, and 0.1 mg/L diazinon for 15 and 30 day periods. Skin, gills, liver, and kidney of exposed fish demonstrated remarkable microscopic lesions. These changes included skin lesions in the head and body, which started as white spots and progressed into deep ulcerations, hepatic vacuolation, swollen hepatocytes, steatosis, aggregation of macrophages, necrosis, and hepatic fibrosis. Gill tissues demonstrated epithelial hyperplasia in the secondary lamellae. Vacuolar degeneration was also present in the hematopoietic tissues of the kidney. Lesion occurrence and severity were correlated to increased diazinon dose and exposure time. Our findings revealed the harmful effects of chronic, sub-lethal diazinon exposure on alligator gar, and suggest other aquatic organisms may also be affected by sub-lethal concentrations of pollutants in their environment.

Introduction

Alligator gar, Atractosteus spatula, are large, air-breathing fish found in fresh water tributaries, brackish estuaries, and coastal waters in the southern U.S., Central America and Cuba. The infraclass Holostei includes the order Lepisosteiformes, the gars, and the genera Atractosteus and Lespisosteus. Alligator gar are the largest gar and can reach 3 meters in length [1]. They are top-level predatory feeders critical to maintain the balance of aquatic ecosystems, and are a native biological control for non-native invasive fish. Recently, they have been proposed as a biological control against Asian carp. Efforts are now focused on the production and reintroduction of alligator gar into areas invaded with Asian carp [2]. In the U.S., most of the waters that alligator gar inhabit run through agricultural areas and receive agrichemical run off. Agrichemical run off is a main source of organophosphate contamination in waterways, and urban run off is another contributing source. More than 500 million kgsof pesticides are used every year in the U.S, and large amounts are washed into watersheds and affect aquatic biota [3, 4]. Only 0.1% of applied pesticides reach their target pests, and the remaining amounts contaminate the environment [5]. Fish play a key role in monitoring the aquatic environment. Gar are one of the best fish to monitor, because they are exposed to pollutants

directly, and indirectly, when they consume contaminated fish. Gar bioaccumulate pollutants, and when multiple species are sampled in a common site, pollutant levels are higher in gar than other fish [6].Due to their large size, gar can be captured, non-lethally sampled and released. Their blood plasma can be used to measure ChE activity as a biomarker for pesticide contamination in aquatic ecosystems [7]. Since the Macondo 252 petroleum oil spill, alligator gar have been the focus of studies in our lab. We documented the effects of the oil spill on wild alligator gar, then documented the effects of crude oil exposure and recovery in a laboratory setting [8, 9]. Diazinon is a common agrichemical pollutant, and we examined the effects of chronic sub-lethal diazinon exposure on the blood parameters and ChE activity in alligator gar [7, 10]. In the current report, we correlated tissue changes following diazinon exposure and evaluated the effects of long-term, sub-lethal diazinon exposures on alligator gar skin, liver, kidney, spleen, heart, gut, intestine, and gas bladder tissues by using 15 and 30 day exposure periods and two diazinon doses.

Material and Methods

Experimental Design

This study was designed similarly to previous studies [7, 10]. To determine the diazinon doses to use, the effect of sub lethal doses on plasma ChE inhibition was determined. The target

dosage was selected as the dose required to maintain the level of ChE inhibition below 25% to ensure the fish would survive the duration of the chronic experiment. Based on these results, 0.01 and 0.1 mg/L diazinon were selected for use in chronic exposures. Thirty-six alligator gar (average weight of 773.9 gm and average length of 53.85 cm) were obtained from the Private John Allen National Fish Hatchery in Tupelo, MS. Fish were acclimated for 10 days while exposed to a natural light cycle, and fed pelleted feed (Rangen, Inc.) once a day. Stainless steel tank sheld 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. Water temperature in each tank was maintained at 21±2°Cusing 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. Changes in color, pigmentation pattern and mucus production were noted. The Mississippi State University Institutional Animal Care and Use Committee (MSU-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, three tanks with final concentrations of 0.01mg/L diazinon and three tanks with final concentrations of 0.1 mg/L diazinon. Fish in the control tanks were exposed to the same concentration of ethanol carrier used with the diazinon exposures. Four fish were used per treatment and the experiments were repeated three times. We previously determined the sub-lethal

dosages to use [7]. Fish were exposed to either the ethanol carrier, 0.01. or 0.01 mg/L diazinon for two different duration times: 15 days and 30 days. The water in each tank was exchanged daily by removing 80% of the water and replacing it with clean water followed by 10 min of continuous flow of clean water. Following cessation of flow, fresh diazinon (to final concentrations of 0.01 and 0.1 mg/L) was added daily. The ethanol carrier was added to the control tanks every day after cleaning. Water quality was measured throughout the experiments to monitor metabolic accumulation of wastes, and to confirm diazinon concentrations.

Tissue Sampling

At the end of each experiment, fish were euthanized by placement in 500mg/L tricaine methane sulfonate. Gill, liver, spleen, kidney, gut, intestine, gas bladder, skin, heart, and brain tissues were surgically removed from each fish, rinsed in physiological saline and fixed in phosphate buffered 10% formalin. The head of each fish was fixed in formalin for 48 h and then decalcified before processing. After fixation, tissues were processed, embedded in paraffin, sectioned at 4μm to 6 μm, and stained with Hematoxylin and Eosin (H&E). Slide identifications were coded so the treatment was not known and viewed on an Olympus BX51 microscope and images were captured by an Infinity 3 Lumenera camera.

Lesion Scoring and Statistical Analysis

Tissues were scored 1(normal), 2 (mild), 3 (moderate) or 4 (severe) if < 5%, 6 to 24%, 26 to 50% or > 50%, respectively, of the tissue demonstrated lesions described in Table 1. For each tissue type and diazinon exposure duration, the low and high diazinon concentration scores were compared to the control score using a student's t-test. Hepatic melano macrophage center (MMC) number and size were analyzed by student's t-test.

Table 1: Summary of tissue lesions in alligator gar following chronic exposure to sub-lethal levels of diazinon, n = 12. Average scores marked with an asterisk were significantly different than the control treatment at p< 0.05. 1 = normal, less than 5% of the tissue demonstrated one or more type of lesion; 2 = mild, 6% to 24% of the tissue demonstrated one or more lesions; 3 = moderate, 26% to 50% of the tissue is affected; 4 = severe, more than 50% of the tissue is affected with marked or severe lesions

Tissue	Duration	Exposure	Lesion	Ave score+ se
Skin	15 days	0 mg/L	Normal	1
		0.01 mg/L	Loss of the dermal and epidermal layers, focal hemorrhage, inflammation, necrosis	2.4 ± 0.66[*]
		0.1 mg/L	Loss of the dermal and epidermal layers, focal hemorrhage, inflammation, necrosis	2.8 ± 0.38[*]
	30 days	0 mg/L	Normal	1
		0.01 mg/L	Loss of the dermal and epidermal layers, focal hemorrhage, inflammation, necrosis	3.5 ± 0.51[*]
		0.1 mg/L	Loss of the dermal and epidermal layers, focal hemorrhage, inflammation, necrosis	3.9 ± 0.28[*]

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Liver	15 days	0 mg/L	Normal	1
		0.01 mg/L	Steatosis, cytoplasmic vacuolation, hepatocyte hypertrophy, inflammation, fibrosis, necrosis	1.5 ± 0.52*
		0.1 mg/L	Steatosis, cytoplasmic vacuolation, hepatocyte hypertrophy, inflammation, fibrosis, necrosis	2.7 ± 0.45*
	30 days	0 mg/L	Normal	1
		0.01 mg/L	Steatosis, cytoplasmic vacuolation, hepatocyte hypertrophy, inflammation, fibrosis, necrosis	1.5 ± 0.52*
		0.1 mg/L	Steatosis, cytoplasmic vacuolation, hepatocyte hypertrophy, inflammation, fib+rosis, necrosis	3.7 ± 0.45*
Kidney	15 days	0 mg/L	Normal	1
		0.01 mg/L	vacuolar degeneration in the hematopoietic tissues, aggregation of eosinophils	1.5 ± 0.52*
		0.1 mg/L	vacuolar degeneration in the hematopoietic tissues, aggregation of eosinophils	2.6 ± 0.49*
	30 days	0 mg/L	Normal	1
		0.01 mg/L	vacuolar degeneration in the hematopoietic tissues, aggregation of eosinophils	3.5 ± 0.51*
		0.1 mg/L	vacuolar degeneration in the hematopoietic tissues, aggregation of eosinophils	3.6 ± 0.49*
Gill	15 days	0 mg/L	Normal	1
		0.01 mg/L	Normal	1
		0.1 mg/L	Normal	1
	30 days	0 mg/L	Normal	1
		0.01 mg/L	epithelial hyperplasia	1.5 ± 0.52*
		0.1 mg/L	epithelial hyperplasia	3.7 ± 0.45*

Results

Gross lesions observed after 15 days and 30 days exposure.

No exposed fish or control fish died during the experiments. Control fish skin color remained the same throughout the experiment. Fish exposed to low and high diazinon concentrations developed lighter skin color and skin lesions after 15 days. Head and body skin lesions started as small white spots that progressed into skin ulcerations, some of which bled Figure 1A, 1B, 1C. Head skin lesions were observed with both treatment levels and durations. Only body skin lesions were observed on fish exposed to 0.1 mg/L diazinon for 30 days Figure 1D, 1E, 1F. After the 30 day exposure,

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Figure 1: AGross skin and liver lesions of alligator garexposed to diazinon. A-C: arrows denote body skin lesions on fish exposed to 0.1mg/L diazinon for 30 days. D:control fish did not demonstrate gross lesions. E: arrow denotes head skin lesion from fish exposed to 0.01 mg/L for 30 days. F: arrow denotes head skin lesion fromfish exposed to 0.1 mg/L for 30 days. G. liver from control fish. H. liver from fish exposed to 0.01 mg/L, and I. liver from fish exposed to 0.1 mg/L demonstrate changes in color after diazinon exposure.

head skin lesions were more progressed and prevalent in fish exposed to the higher dose. Grossly, control fish tissues were unremarkable. Livers from fish exposed to low and high concentrations for 30 days showed remarkable changes in color Figure 1H, 1I. Livers were spotted with different colors of red, gray, and dark green. Interestingly, livers from fish exposed to the low concentration showed more color changes than livers from fish exposed to the higher concentration. Gross lesions were not observed in any other tissues from exposed fish.

Microscopic Lesions Observed after 15 Days Exposure

After 15 days, tissues from control fish did not demonstrate any lesions, and scored within the normal range. Control gar skin consisted of two layers, the epidermis and the underlying dermis Figure 2A. The epidermis consisted of layered epithelial cells. Mucus cells occurred between the epithelial cells. No lesions were seen in gill, spleen, brain, air bladder, gut, intestine and heart tissues of fish exposed to 0.01 and 0.1 mg/L diazinon, and these tissues scored within the normal range .At 15 days in fish exposed to 0.01 and 0.1 mg/L diazinon, there were skin lesions on the head. The epidermal and dermal layers were absent, and necrosis extended into the underlying muscle. Intact epidermis surrounding these lesions was edematous Figure 2B. These lesions were more severe in fish exposed to the higher diazinon concentration, and the edematous epidermis was congested

and necrotic Figure 2C. The skin lesion scores for 0.01 mg/L diazinon averaged mild to moderate, and for 0.1 mg/L the scores averaged moderate. Liver tissues from control fish were normal Figure 2D. Liver tissues from fish exposed to 0.01 mg/L diazinon demonstrated steatosis, vacuolation of the hepatic cytoplasm, and focal aggregation of macrophages Figure 2E. The scores averaged normal to mild, and were significantly greater than the control tissue score. Liver tissues from fish exposed to 0.1 mg/L diazinon demonstrated MMCs and individual macrophages, severe steatosis (fatty liver), vacuolated hepatocytes, swollen hepatocytes, fibrosis, and necrosis in liver tissues Figure 2F. The scores averaged mild to moderate, and were significantly greater than the control tissue score. Counting and measuring the size of MMCs revealed a significant increase in the number and size in fish exposed to the high dose when compared to control fish. In fish exposed to the low dose, there was not a significant difference in the number and size of MMCs compared to the control fish Table 2. The bile ducts of control fish and fish exposed to the low dose were unremarkable. In contrast, the bile ducts of fish exposed to the high dose demonstrated rod let cells in the wall of the bile duct, and eosinophilic granules present in the bile duct. Kidney hematopoietic tissues from control fish appeared normal Figure 2G. Kidney hematopoietic tissues from fish exposed to 0.01 mg/L diazinon demonstrated vacuolar degeneration. The scores averaged normal to mild, and were significantly greater

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than the control tissue score. Vacuoles were larger and more prevalent in fish exposed to the higher dose Figure 2H, 2I. Aggregations of eosinophils were present around these vacuoles in fish exposed to the high diazinon dose Figure 2I. The scores averaged mild to moderate, and were significantly greater than the control tissue score.
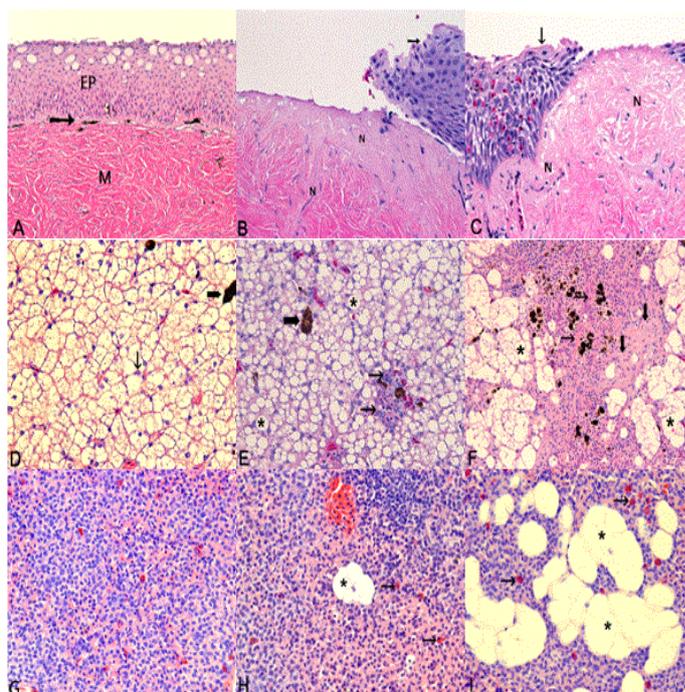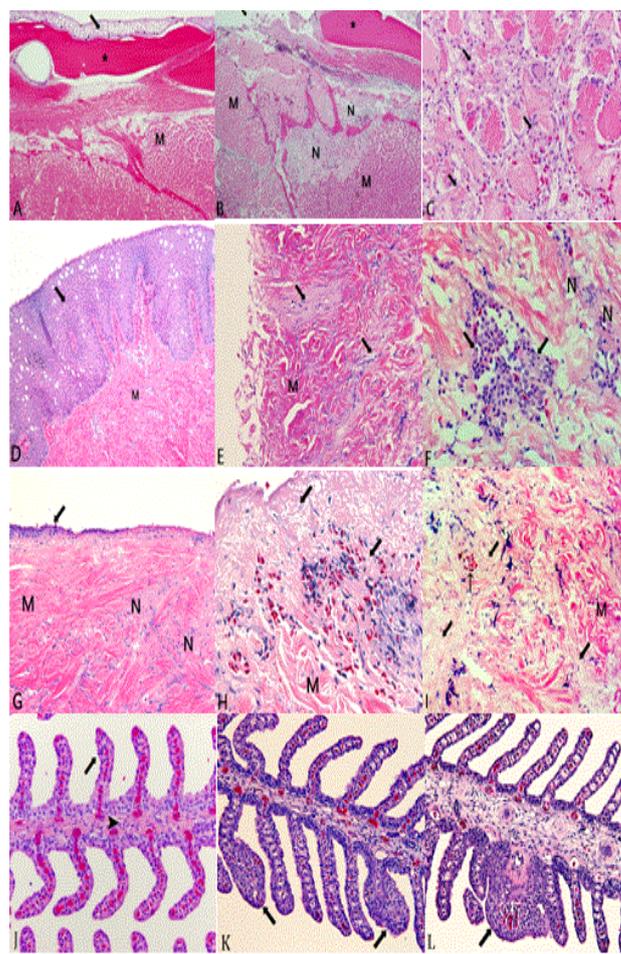

Figure 2: Alligator gar tissues from control fish and fish exposed to diazinon for 15 days.A:head skin from control fishdemonstrated epidermal (EP) and dermal layers (arrow), and underlying muscle (M). B: head skin lesion from fish exposed to 0.01 mg/Ldemonstrated loss of epidermis and edematous epidermis around the affected area (arrow), and necrosis in the muscle (N). C: head skin lesion from fish exposed to 0.1 mg/L diazinon demonstrated loss of epidermis and congested and swollen epidermis around the affected area (arrow), and necrosis in the muscle (N). D: liver tissue from control fish demonstrated MMCs (bold arrow), and hepatocyte nucleus (thin arrow). E: liver tissue from fish exposed to 0.01 mg/Ldemonstrated vacuolation of hepatocytes (asterisk), MMCs (bold arrow), and aggregation of macrophages (thin arrows). F: liver tissue from fish exposed to 0.1 mg/L demonstrated steatosis, fibrosis (bold arrow), necrosis (thin arrow), and vacuolar degeneration of hepatocytes (asterisk). G: kidney hematopoietic tissue from control fish was normal. H: kidney hematopoietic tissue from fish exposed to 0.01 mg/L demonstrated vacuolar degeneration (asterisk) with aggregation of eosinophils (arrows). I: kidney hematopoietic tissue from fish exposed to 0.1 mg/L demonstrated progressed vacuolar degeneration (asterisk), and eosinophilic infiltration (arrows). Hematoxylin and eosin (H&E); (200X).

Microscopic Lesions Observed after 30 Days Exposure

Gill, skin, liver, brain, spleen, gut, intestine, kidney, heart and air bladder tissues from control fish scored within the normal range. Brain, spleen, gut, intestine, heart and air bladder tissues from fish exposed to 0.01 mg/L and 0.1 mg/L diazinon scored within the normal range. Epidermal lesions were observed on the heads and bodies of fish exposed to 0.01 mg/L and 0.1 mg/L diazinon for 30 days. These lesions demonstrated loss of epidermal and dermal layers with aggregations of macrophages and necrosis of the underlying musculature Figure 3A- 3I. The average lesion scores were moderate to severe in fish exposed to both doses. These scores were significantly greater than the control. Gill tissues from control fish were normal and the secondary lamellar diameter averaged 14 μm Figure 3J. Gill tissues from fish exposed to 0.01 mg/L diazinon demonstrated moderate lamellar epithelial hyperplasia and the secondary lamellar diameter was significantly greater than the control, and averaged 18 μm, with one fish measuring 45 μm. Figure 3K.The average lesion score for the low dose exposure was normal to mild, and was significantly greater than the control. In fish exposed to 0.1 mg/L diazinon, gill tissues demonstrated severe epithelial hyperplasia and fusion of lamellae Figure 3L. The secondary lamellar diameters were significantly greater than the control, and averaged 19 μm, with the most
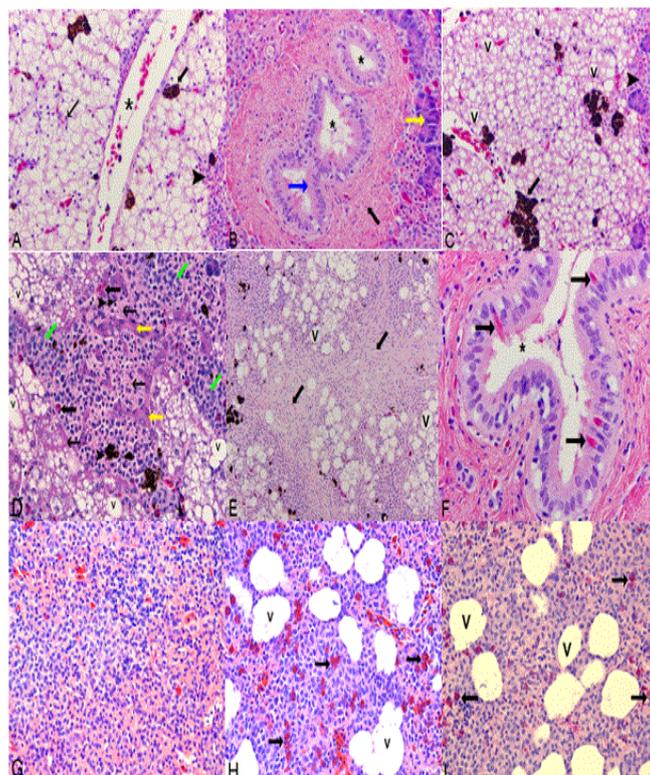
affected lamellar diametermeasuring65 μm. The average lesion score for the high dose was moderate to severe, and was significantly greater than the control. Liver and bile duct tissues from control fish scored in the normal range Figure 4A, 4B. Liver tissues from fish exposed to 0.01 mg/L diazinon demonstrated lipidosis and hepatocyte vacuolation Figure 4C. The average lesion score was normal to mild and was significantly greater than the control. Liver tissues from fish exposed to 0.1 mg/L demonstrated severe steatosis with marked hepatocyte vacuolation, diffuse fibrosis, hepatocyte nuclear hypertrophy and hepatic necrosis with macrophage aggregations Figure 4D, 4E. The average tissue score was moderate to severe and was significantly greater than the control. Rodlet cells were also observed in the bile duct wall and eosinophilic granules were within the bile duct lumen of fish exposed to the high dose Figure 4F. Melano macrophage centers were scattered throughout the liver tissues in control and exposed fish, but the numbers of MMCs were significantly higher in diazinon exposed fish Table 2. Kidney tissues from control fish scored within the normal range Figure 4G. Kidney tissues from fish exposed to 0.01 mg/L and 0.1 mg/L diazinon demonstrated vacuolar degeneration and eosinophilic infiltration and aggregation Figure 4H, 4I. The average tissue scores for both exposure levels were moderate to severe, and were significantly greater than the control.

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Figure 3: Alligator gartissues from control fish and fish exposed to diazinon for 30 days. A: control skin showed epidermis (bold arrow),a scale (*) and muscle (M). B: a body skin lesion from fish exposed to 0.1 mg/L demonstrated loss of epidermal and dermal layers (arrow), necrosis (N) in muscle (M), a scale (*). C: shows the necrotized area (arrows) in B in higher magnification. D: head skin from a control fish demonstrated epidermis (arrows), and muscle (M). E: head skin lesion from a fish exposed to 0.01 mg/Ldemonstrated loss of epidermal and dermal layers and necrosis (arrows) in the muscle (M). F: higher magnification of the area shown in E, demonstrated macrophage aggregations(arrows) and necrotic muscle (N). G: head skin lesion from a fish exposed to 0.1 mg/Lshowed loss of epidermal and dermal layers (arrow), necrosis (N), muscle (M). H: shows the lesions in G in higher magnification,demonstrating necrotized muscle and hemorrhage (arrows) within muscle (M). I: demonstrates necrosis (bold arrows) in muscle tissue in higher magnification, muscle (M), blood vessel (thin arrow). J: gill tissue from a control fish demonstrated primary (arrow head) and secondary lamellae (arrow). K: gill tissue from a fish exposed to 0.01 mg/L showed epithelial hyperplasia in secondary lamella (arrows). L: gill tissue from a fish exposed to 0.1 mg/L demonstrated severe epithelial hyperplasia in secondary lamella (arrow). Hematoxylin and eosin (H&E); (200X).

Table 2: Hepatic melanomacrophage center number and size (expressed as mean+se) in control and diazinon exposed fish. Asterisk indicates significant difference at p<0.05

Treatment	15 days exposure		30 days exposure	
	Number	Size	Number	Size
Control	29.7 ± 9.5	6.5 ± 1.2	35 ± 6.4	8 ± 1.3
0.01 mg/L diazinon	37.5 ± 8.3	7 ± 2.2	64.5 ± 16.3*	10 ± 2.1
0.1 mg/L diazinon	62 ± 6.7*	12 ± 1.4*	78.2 ± 11.1*	18 ± 1.7*

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Figure 4: Alligator gartissues from control fish and fish exposed to diazinon for 30 days. A:liver tissue from control fish; hepatocyte nucleus (arrow), MMCs (bold arrow), hepatic portal vein (*). B: bile duct from control fish shows hepatic pancreatic tissue (yellow arrow), columnar epithelium (blue arrow), bile duct lumen (*), and smooth muscle (arrow). C: liver tissue from fish exposed to 0.01 mg/L demonstrated steatosis with vacuolar degeneration (V), MMCs (bold arrow) adjacent to portal vein, and hepatic pancreatic tissue (arrowhead). D: swollen hepatocytes (yellow arrows) and necrotic hepatocytes (thin arrows), macrophage aggregates (green arrows) and eosinophils (bold arrows), vacuoles (V), and MMCs (arrowhead). E: liver tissue demonstrated fibrosis (bold arrows), vacuoles (V). F: liver bile duct; arrows denote rodlet cells in bile duct wall, bile duct canal (*). G: healthy kidney hematopoietic tissues from control fish. H: kidney hematopoietic tissue from a fish exposed to 0.01 mg/L showed vacuolar degenerations (V), and eosinophilic infiltration (arrow). I: kidney hematopoietic tissue from fish exposed to 0.1 mg/L demonstrated vacuolar degeneration (V), and eosinophilic infiltration (arrow). Hematoxylin and eosin (H&E); (200X, 400X).

Discussion

Exposure to sub-lethal diazinon concentrations for 15 and 30 days induced gross and microscopic lesions in alligator gar tissues. Changes in fish skin color following diazinon exposure have been reported [10 - 13]. Liver color changes were also observed in alligator gar exposed to water accommodated fractions of crude oil for 48 hours and in other fish species following toxin exposure [14, reviewed in 15]. In the crude oil study, gar liver color returned to normal after a seven day recovery period. Exposure to chronic sub lethal diazinon concentrations also caused changes in gar behavior and blood parameters [10]. In skin tissues, lesions cores were normal for control fish, and mild to moderate for the 15 day low and high dose exposures. Lesion scores were mild to moderate for the 30 day low dose and moderate to severe for the 30 day high dose exposures. Skin lesions occurred only on the head after 15 days of diazinon exposure. The lateral line canal system is concentrated in the head of the gar [16, 17]. The epidermal pits and neuro mast cells are probably the most sensitive epidermal structures in the gar, and are the first to be affected by diazinon exposure. Lesions at the end of the nose could also results from mechanical injury if the fish contacts the side of the tank repeatedly. Skin lesions on the head were more progressed after a longer and higher diazinon exposure. Skin lesions occurred on the gar body after 30 days of exposure. The histopathological alterations in the skin ranged from loss of epidermal and dermal layers to severe inflammation, congestion, and myonecrosis. In addition, the epidermis above the muscle lesions was edematous in some fish and edematous and congested in other fish. Mild epidermal vacuolar degeneration was seen in Nile tilapia after exposure to edifenphos pesticide [18]. This is the first study to report severe skin lesions in alligator gar following diazinon exposure. In a previous study, chronic diazinon exposure resulted in increased epidermal mucus production, and this increases skin protection [7]. However, epidermal lesions are an entry for secondary pathogens. Chronic exposure to chemical toxicants was suggested to lead to necrotizing dermatitis in fish [19].

Gill lesions cores were normal for control fish, normal to mild for the 15 day low and high dose exposures, mild to moderate for the 30 day low dose and moderate to severe for the 30 day high dose exposures. Similar findings were reported in studies of fish exposed to diazinon, quinalphos, edifenphos pesticides, and crude oil [20, 21, 18, 13, 22, 23]. Liver lesions cores were normal for control fish, mild for the 15 day low dose exposures, mild to moderate for the 15 day high dose, mild to moderate for the 30 day low dose and moderate to severe for the 30 day high dose exposures. Documented liver lesions following exposure to pesticides or petroleum compounds ranged from fatty degeneration to necrosis and fibrosis [24, 25, 21, 27, 28, 18, 22, 9]. In the current study, liver tissues from exposed fish demonstrated mild to severe steatosis, hepatic fibrosis, cytoplasmic vacuoles and necrosis. These lesions were dose and duration correlated. Liver fibrosis and accumulation of extracellular matrix and collagen fibers commonly results from chronic chemical exposure [29, 30]. With prolonged chronic exposure, fibrosis can develop into cirrhosis and impaired liver function [29]. Liver lesions can impair liver functions, and multiple organ damages can follow [31]. Liver lesions resulting from diazinon exposure could have contributed to lesions observed in other tissues. In alligator gar exposed to crude oil for 48 hours, liver tissue had the most severe lesions demonstrating similar lesions as observed in the current diazinon study [9]. In the current study, chronic diazinon exposure caused rodlet cell accumulation in the bile duct walls. Rodlet cells are unique to some fish species, and little is known about their nature and function [32]. It is suggested they play a role in intracellular non-specific response or are a response to sub-lethal toxin exposure [32-34, 38].Significant increases in the number of hepatic MMCs occurred in fish exposed to low and high treatments for 30 days and high dose for 15 days. Additionally, the size of hepatic MMCs significantly increased in fish exposed to the high dose for 15 and 30 days. Melano macrophage centers in fish increase in size and number after exposure to environmental pollutants [36, 37, 38, 39, 8].When alligator gar were exposed to crude oil for 48 hours, hepatic MMCs decreased, but returned to normal after a 7 day recovery period [9]. Kidney hematopoietic tissue lesions cores were normal for control fish, normal to mild for the 15 day low dose exposure, mild to moderate for the 15 day high dose, mild to moderate for the 30 day low dose and moderate to severe for the 30 day high dose exposures. Severity of kidney hematopoietic tissue vacuolar degeneration was correlated to increased diazinon dose and exposure duration. This degeneration resulted in tissue dysfunction and decreased red blood cell and leukocyte numbers [10]. Exposure to other pollutants induced kidney interstitial tissue lesions, including eosinophilic infiltration and vacuolar degeneration [40, 41, 9, 25, 42].Chronic, sub-lethal exposure of chlorpyrifos caused anemia in tilapia [43]. Since the renal hematopoietic tissue is the bone marrow equivalent in fish, chronic diazinon exposure can impact multiple aspects of fish health. Diazinon exposure inhibited plasma cholinesterase activity in alligator gar negatively impacting their physiology [7]. Hepatic biomarkers ⍰-naphthoflavone and

17-⍰-estradiol of alligator gar juveniles were also adversely affected by diazinon exposure [44].Chronic exposure to diazinon at low and high concentrations caused skin, liver and kidney lesions; severities were correlated to dose level and exposure duration. Studies in other fish species reported similar findings [24, 45, 46, 13, 47, 23]. Histological changes following exposure to sub-lethal concentrations of pesticides can lead to further physiological changes [48, 38, 49, 40, 9]. In conclusion, the present study shows that long-term exposure to diazinon, even at a low concentration, led to dose and duration correlated histological lesions in skin, gill, liver, and kidney tissues of alligator gar. Fish kills directly attributable to agrichemicals are rare. But long term exposure to low levels of these compounds can be insidious, causing physiological, hematological and histological changes. The question is, can the fish adapt to these changes, or will the population decline? Sub-lethal effects can become lethal effects if behavior, reproduction or disease susceptibility are negatively impacted. Our findings, viewed with other alligator gar studies, strongly suggest that populations of this high-trophic level predatory fish are being adversely impacted by chronic exposure to sub-lethal levels of environmental pollutants. Alligator gar are unique fish that are an integral part of aquatic ecosystem balance and health monitoring. Alligator gar habitat needs to be preserved, and natural populations enhanced.

Acknowledgments

Declarations

The authors declare there are no conflicts of interest.

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