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Physiological Dysfunction in Hypophthalmichthys nobilis (Freshwater carp) after Exposure to **Two Insecticides Mixture**

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ABSTRACT

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Pyriproxyfen and Acetamiprid are insecticides, extensively used to eradicate insects. The current investigation was carried out to ascertain the physiological dysfunctions as consequences of Acetamiprid and Pyriproxyfen exposure in fish Freshwater carp. For this purpose 27 Bighead carp were brought from Bahawalnagar hatchery and divided into three groups A (G0), B (G2) and C (G3), fish were acclimatized for 15 days before experiment. Acetamiprid and Pyriproxyfen were applied together with different concentrations, 0, 2 and 3 mg/L in group G0, G1 and G2 respectively. After administration of chemical, different clinical signs such as; bottom running, surface running, gasping, operculum movement, reversed tilting of pectoral fin, convulsions, jerking, surface breathing, unbalanced body movements, static position, and faintness were observed in fish at day 7 and 14. Dissection of fish was performed at equal intervals of day 7 and 14. Additionally, physiological dysfunctions such as reduction in body weight, reduced growth, and malfunctioning of the visceral organs were detected during study. In group G2 in which high concentration was applied, fish showed perceptible physiological dysfunctions and other clinical signs. While the group in which low concentration was provided physiological dysfunctions comparatively was minor, but other clinical signs were recognizable in group G1. Results revealed that the toxicity of Pyriproxyfen and Acetamiprid disrupted the body weight of fish.

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INTRODUCTION

Chemical substances known as pesticides are largely used to protect crops, prevent vector-borne diseases, control, eradicate, or alleviate pests (Richardson, J.R., 2019). With current agricultural techniques, the usage of pesticides is inevitable. Because pesticides suppress harmful weeds, fungus, and insects, agricultural production has been stabilized and altered. But only 1 percent of the pesticide used on crops really reaches the insect; the other 99 percent ends up in the environment, where it contaminates the water, soil, and biota (Mohiddin et al., 2015a; Mohiddin et al., 2015b). Pesticide use benefits include increased yield, labour savings, risk reduction in fertilizer use and crop output (Dasgupta et al., 2001). Growing usage of pesticides since the 1960s has enabled farmers worldwide to significantly boost productivity without experiencing excessive crop losses due to insects, as reported by Oerke (2006). In order to increase effectiveness and lower the cost of crop pest treatment, pesticides are frequently combined in both agricultural and urban settings. The techniques used to apply pesticides (dusting and spraying) allow them to get into the aquatic environment (Elezovic et al., 1994). Therefore, aquatic species certainly interact with a large variety of pesticides that are both structurally and toxicologically varied. Despite the fact that the toxicity of individual pesticides has been well investigated in fish (Joseph and Raj, 2011), nothing is known about the toxicological effects of their mixtures. One of the primary causes of freshwater diversity degradation worldwide is agrochemicals, which has led to growing worries about aquatic hazards from commonly used pesticides, such as neonicotinoids (Schulz, R et al., 2021). Pesticides are

among the most harmful types of pollutants that penetrate the aquatic environment because of their high lipid solubility, resistance to metabolism, and high chemical stability. Pesticides discharged by the agriculture industry have physiological effects on a variety of aquatic creatures, including fish, animals, algae, and shellfish.

Acetamiprid affects the synapses in the insect central nervous system by acting as an agonist on the nicotinic acetylcholine receptor (Wolf, J. C., and Wheeler, J. R. 2018) to produce excitation, paralysis, and death. After being used on crops, this pesticide contaminates a variety of matrices, including soil, water, plants, and aquatic life (Rai and Baby 2015; Morrissey et al. 2015; Hoyle and Code 2016). Acetamiprid has cytotoxic and genotoxic properties in mammals and aquatic organisms. According to reports, it results in chromosomal abnormalities, micronuclei forming in blood cells, and sister chromatid exchanges in cultures (Hladik et al., 2018; Ma et al., 2019). After being taken orally, acetamiprid travels via the circulation, crosses the blood-brain and blood-testes barriers, and is distributed to various bodily organs before being metabolized by the liver and eventually eliminated by the kidney (Ford and Casida, 2006a, b). As a target organ for detoxification, the liver is vulnerable to a number of illnesses brought on by exposure to environmental contaminants. Phase I enzymes, including cytochrome P450 (CYP) and aldehyde oxidase (AO) in mammals, quickly break down neonicotinoids like acetamiprid in the liver (Shi et al., 2009).

A pyridine-based larvicide called pyriprofen is applied directly to water sources to prevent mosquito larvae from multiplying (Caixeta et al., 2016; Dzieciolowska et al., 2017; Peterson et al., 2017; Maharajan et al., 2018). This larvicide is an equivalent of a juvenile hormone that acts metamorphosis the mosquito's inhibit and to embryogenesis (Ohba et al., 2013). Pyripoxyfen exhibits the potential to be ecologically persistent because to its low solubility, hydrophobicity and high partition coefficients. As a result, caution should be taken while using this pesticide on or near water bodies to prevent water contamination. Additionally, homeowners employed pyriproxyfen as a pesticide to control red fire ants, California red scale, and silver leaf whiteflies in citrus, cotton, vegetable, and peanut crops Maharajan et al. (2018) and Legrand et al. (2017). Pyriproxyfen has harmful deleterious effects on non-target species across the food chain and is extremely stable in a variety of habitats. Because pyriproxyfen is applied continuously and persistently, exposed animals have shown a number of negative consequences, such as neurodevelopmental toxicity, decreased reproduction, and disturbance of endocrine homeostasis (Maharajan et al., 2018). Previous research has shown that PPF may cause fish and other aquatic creatures to perish while controlling mosquitoes (Caixeta et al., 2016). Fish in particular may be negatively impacted by these pesticides' adverse effects on non-target creatures.

Aquatic ecosystems accumulate pesticides, which are then passed on to higher-ranking predators. Since fish are at the top of the aquatic food chain, bio-magnification of pesticides is a common occurrence among aquatic creatures (Henny *et al.*, 2003). Furthermore, fish exposure to pesticides is alarming and concerning since they are a representative group of aquatic animals that humans directly consume. Additionally, fish are indicators of environmental contaminants, with approximately half of pesticides remaining in fish for longer than 30 days (Guo et al., 2008a; Guo et al., 2008b). Chronic fish poisoning in an aquatic environment causes physiological and biochemical alterations in cells as well as morphological defects (Fernandes s, 2013). The most often used neonicotinoid in China is acetamiprid (Formula: C10H11ClN4), a first-generation neonicotinoid that produces 8,000 tons a year Shao, X et al., (2013). The toxicity of pyriproxyfen to vertebrates, including fish, is not well documented. There have been reports of embryo abnormalities at very high concentrations of pyriproxyfen (Truong et al., 2016) and erratic swimming (Caixeta et al., 2016).

MATERIALS AND METHODS

This study was carried out in the Aquaculture, Genetic Toxicity, and Molecular Biology Laboratory of the Department of Zoology at the Islamia University of Bahawalpur in Pakistan. The institutional Bioethics Committee (IBC) of The Islamia University of Bahawalpur, Pakistan, mandated that animal handling ethics be strictly followed.

Fish Collection and Chemical

Healthy Bighead carp individuals weighing 100–120 grams were purchased from a nearby fisheries farm in Bahawalnagar, Pakistan. Fish were transported to the testing site in oxygen-filled polythene containers to guarantee their survival. Pyriproxyfen (19.80% w/w) and Acetamiprid (18.35% w/w) issued by orange protection pesticide company in dual formulation were acquired from commercial scientific store at Bahawalpur, Pakistan.

Acclimatization

Fish are acclimatized for one week in glass aquaria with a 150-liter water capacity and dimensions of 14" L \times 10" W \times 12" H. International water quality criteria that are appropriate for freshwater fish species were fulfilled by all water qualitywater quality. Aerators were used to control oxygen levels, and frequent fecal waste removal was done to prevent ammonia toxicity. Fish (n=27) were split evenly into three groups (G0-G2) based on their level of acclimation. In each tank, nine fish were kept in a 100-liter water space.

Experimental Setup

We arranged three aquariums and given them names like group G_0 , group G_1 and group G_2 . Group G_0 was control group which receive normal diet daily while the remaining groups G_1 - G_2 were experimental groups treated with different concentration of Acetamiprid and Pyriproxyfen @0, 2 and 3 mg/L respectively. The fish were fed commercial feed that contained 30% protein every day at a rate of 2% of their body weight. Each tank was inspected daily for signs of clinical illness and death. Two samplings were conducted during the experiment: the first took place on day7, and the second took place on day 14.The temperature of the laboratory was kept 28- 29° C. The aerators were established in these aquariums for proper aeration. The feed was provided to the fishes regularly, and the aquariums are cleaned and siphoned regularly.

Physicochemical Parameters

After the distribution of the fish into different groups, physicochemical parameters had been observed at the day first to check the effect of toxicant on the fish and their environment. Following parameters are calculated:

Clinical Observations

Clinical signs which were observed are Bottom running, Surface running, Gasping, Operculum movement, Caudal fin movement, Convulsions, Unbalance body, Surface breathing, Jerking, Faintness and tilting of fin, Rapid gulping due to difficulty in breathing, Loss of appetite, low rate of feeding makes immune system weak and fish become susceptible to disease.

Statistical Analysis

The statistical analysis was conducted using SPSS V.26 software. One Way ANOVA was used for the intergroup comparison. The control and treated groups were compared using the t-test. For various variables, the mean and standard deviation had p values that were Significant 0.05. For creating graphs, the software GraphPad Prism (version 7) was used.

RESULTS

Behavioral Responses

Control group (G_0) fish were found without any mortality and clinical reactions. Fish of treated groups (G₁-G₂) with low to high doses of Acetamiprid and Pyriproxyfen showed moderate to severe clinical indications at day 7 and 14 such as gasping, jerking, convulsion, bottom running, surface running, surface breathing, body unbalancing, operculum movement, tin tilting, and static lurization were observed at higher concentration (2 and 3 mg/L). The maximum intensity of these clinical signs was observed in fish exposed to higher concentration of Acetamiprid and Pyriproxyfen in group G_1 and G_2 throughout the present experiment. Clinical signs were categorized as Absent (-), Mild to Moderate (+), Severe (++), very severe (+++). During first seven days normal clinical signs were observed in groups G_0 and $G_1 G_2$ but group G₂ indicates mild clinical abnormalities (Table 1). During 7-14 days normal clinical signs were observed in group G₀ but groups G₁ and G₂ indicate severe clinical abnormalities (Table 2)

Results at Day-7

At day 7, the gasping nearly remains same in the group G_1 and increased gasping at the surface was seen in group G2 in contrast to group G0, the control group as fish got stress due to lack of oxygen as a result of insecticide application. When compared to the control group A, the jerking in group G1 is marginally higher, but it increases significantly in group G2. Fish lose control of their muscles and nerves, which causes them to begin migrating away from the area of application.

While the convulsions remain generally slight in group G1 in contrast to group A, the control group but increased in the G₂, due to stress, compared to the G0 control group. The surface breathing at day 7 was increased in the G1 and G2 control groups when in contrast to the G0 control group as introduction of insecticide causes loss of oxygen in water. Surface running is higher in group G1 than in control group G0, although it mostly increases in group G2. Groups G1 and G2 experience higher levels of bottom running under stress than group G0, which is the control. The body unbalancing was observed in groups G1 and G2 in contrast to group G0, the control group Normal operculum pumping was seen in control group G0, however rapid operculum movement was observed in groups G1 and G2, which is caused by an accelerated rate of breathing. The pectoral fins of the fishes were reversed. Since pesticides harm the nervous system, the strange behavior of the experimental fishes may be the result of neurotransmitters' ability to operate normally being impeded by the stress of the pesticide. Pectoral fin tilting was seen in groups G1 and G2, but not in group G0, which served as the control. The static position at day 7 is slight or sometimes occurs.

 Table 1: Analysis of physicochemical parameters at the start of trial period

Sr. No	Parameters	Group A	Group B	Group C
1	рН	7.8	7.8	7.8
2	Temperature (°C)	29°C	29.1°C	28.8°C
3	EC (mS/cm)	0.77	0.74	0.73
4	Salinity (ppt)	0.3	0.4	0.3
5	TDS (ppt)	0.41	0.	0.38

Table 2: shows clinical signs at day 7.

Clinical Signs	Treated Groups			
C C	G_0	G ₁	G ₂	
Gasping	+	+	++	
Jerking		++	++	
Convulsions	_	_	+	
Faintness	_	_	+	
Surface breathing	+	+	+	
Surface Running	+	+	++	
Bottom Running	+	+	++	
Body unbalancing	_	_	+	
Operculum movements	+	++	++	
Tilting of Fin	+	+	++	
Static position	+	+	+	

Table 3: Clinical signs at day 14.

Clinical Signs	Treated Groups		
-	G_0	G1	G_2
Gasping	+	+	_
Jerking	+	+	++
Convulsions	_	_	++
Faintness	_	_	+
Surface breathing	+	+	+
Surface Running	+	+	_
Bottom Running	+	+	++
Body unbalancing	_	_	+
Operculum movements	++	++	+++
Tilting of Fin	+	+	++
Static position	+	+	+

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Fig. 1: Various physiological dysfunctions observed in *H.nobilis* (a) Rapid gulping (b) Body unbalancing (c) Surface running (d) Bottom running (e) Static movement (f) Faintness during the 14 days of experimental period.

Results at Day 14

At day 14 following physiological dysfunctions were detected. The gasping at day 14 moderately increases in the group and in group G₂ gasping was not detected, when compared to the control group G_0 as fishes got stress due to lack of oxygen become slightly adapted to it. The jerking mainly increases in the group G₁ largely severe at intervals in G₂ in contrast to the G0 control group. Convulsions indicate that a fish has lost its ability to regulate its body and are symptoms rather than a single illness. It happens when a fish is under extreme stress. The convulsions at the day 14 remain generally slight in the G1 group in contrast to the G1 control group but increased in the G₂, due to stress, in contrast to the G0 control group

The surface breathing was slightly increased in group G1 and group G2 control compared to group G0 control as introduction of insecticide causes loss of oxygen in water with increasing duration. Surface running is more in group G_1 when compared the control group G_0 but in group G_2 it is detected at imperceptible rate. The bottom running with stress increases in groups G1 and G2 in contrast to group A, the control group due to loss of body control by dysfunction neurotransmitter that carries out the effective of movements. Groups G1 and G2 showed noticeable bodily un-balancing in comparison to group G0, which served as the control. While normal operculum pumping was seen in control group GO, rapid operculum movement was observed in groups G1 and G2, which is attributed to an accelerated rate of breathing. The pectoral fins of the fishes were reversed along with non-specified and non-oriented movements. Due to stress of the pesticide, the unusual behavior of the experimental fishes may be due to the obstructed function of neurotransmitters as pesticides cause damage to the nervous system. Tilting of pectoral fin was detectable in group G_1 and G_2 but not in control group G_0 . The static position at day 14 is slight or sometimes occurs in group G_1 but can remain same in G_2 .

Physiological dysfunction in organ's weight

The results on the body weight of fish were presented in in Fig.2 (graph A). Before application of chemicals, during acclimatization period, the body weight of fish remain same in all the groups from G₀.G₂. After application of chemicals in experimental groups G1 and G₂ body weight significantly reduced at day 14, when compared to control group G_0 . The results on absolute and relative weight of intestine are presented in graph B. When comparing experimental groups G1 and G2 to control group G0, the absolute weight of the fish's intestines stayed rather constant. Comparing groups G1 and G2 to control group G0, the relative weight of the intestines dropped considerably. The results on absolute and relative weight of liver are presented in graph C. In comparison to control group G0, the fish in experimental groups G1 and G2 had nearly identical liver absolute weights. Relative weight of the liver was substantially lower in groups G1 and G2 than in control group G0. The results on absolute and relative weight of Heart are presented in graph D. Heart absolute weight of fish remained almost same in experimental group G_1 and G_2 , when compared with control group A. Heart relative weight significantly decreased in group G₁ and G₂, when compared to control group G₀. The results on absolute and relative weight of Spleen are presented in graph E. Spleen absolute weight of the fish remained almost same in experimental group G₁ and G₂, in contrast to the G0 control group. When comparing groups G1 and G2 to control group G0, the relative weight of the spleens dropped dramatically. The results on absolute and relative weight of brain are presented in graph F. Fish in experimental groups G1 and G2 had nearly identical brain absolute weights when compared to control group G0. When comparing groups G1 and G2 to control group G0, the relative weight of the brains dropped considerably.















Fig. 2 (A) Body weight of fish (*Hypophathalmicthys nobilis*) before and after the experiment. (B) Relative and absolute weight of intestine (C) Relative and absolute weight of liver (D) Relative and absolute weight of heart (E) Relative and absolute weight of spleen (F) Relative and absolute weight of brain.

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DISCUSSION

The purpose of this study was to evaluate the toxicity of two active insecticides on Bighead carp fish Acetamiprid and Pyriproxyfen under the controlled conditions. The juveniles of *Hypophathlmicthys niloticus*, after 15 days of acclimatization, were exposed for 14 days to low (2mg/L) and high (3mg/L) concentrations of (pyriproxyfen and acetamiprid). The tests were conducted in aquariums under carefully monitored circumstances. Fish may exhibit changed behavioral patterns in polluted environments, such as aggressiveness, avoidance, and movement activity. These behaviors may be an attempt by the fish to cope with the stressor or to flee it (Morgan, J. D., 2002).

Clinical signs that correspond with behavior include the series of measurable actions that are controlled by the central and peripheral nervous systems (Baatrup, E. 2009) as well as the cumulative expressions of physiological and biochemical processes that are vital to life, like feeding, reproduction, and avoiding predators. It differs greatly among fish species, exposure circumstances, and toxicant types and enables an organism to adapt to both internal and external stimuli in order to best face the challenge of surviving in a changing environment. The current study's findings show that the only medication that altered skin color was acetamiprid. Similar color changes in guppy fish (Poecilia reticulate) have been documented (Ylmaz, M., 2004). Adewoye (Adewoye, S. O. 2010) explained this discoloration of the skin observed with the fingerlings and the adults of African catfish Clarias gariepinus exposed to soap and detergent effluents to the weakening or inhibition of melanin production, due to the extent of the depletion of the oxygen content of the main melanin components. The discoloration that fish exposed to toxicants suffer, however, may be caused by the dispersion of melanin pigment in the chromatophores, according to Mishra and Shukla (Mishra, R., and Shukla, S. P.94).

In toxicity testing, pesticides often enter through the gills, which means that the respiratory system may be negatively impacted (Velmurugan, B., 2009). Our findings show that the combination of acetamiprid and pyriproxen in this research increased opercular activity via lowering dissolved oxygen. According to Kind (Kind, P. K., 2002), alterations in fish behavior may have been made to compensate for the chemicals' effects on aquatic hypoxia. In situations where escape from hypoxic stress is impossible, hyperventilation may be triggered in order to make up for the inadequate oxygen supply.

The hyperventilation seen in this study may potentially represent a respiratory distress, which is one of the first signs of pesticide poisoning and may result in modified gill histology. However, fish gills are essential organs for their respiratory and osmoregulatory processes and are the main entrance point for pesticides (Val, A. L., 2000). Indeed, research by Velmurugan et al. (Velmurugan, B., 2007) on the gill tissue of mrigal carp exposed to sublethal concentrations of lambda-cyhalothrin revealed changes in histopathology, including edema, epithelial lifting, aneurism, epithelial necrosis, desquamation, epithelial hyperplasia, lamellar fusion, and shortening of secondary lamellae. The effects of the active ingredients on the central nervous system could account for additional behavioral changes seen in this study, including jerking, depth activity, erratic swimming, loss of balance, lethargy, operculum movement, bottom running, surface running, static movement, loss of appetite, and convulsions.

There were other indications that the body had been exposed to contaminant besides behavioral а abnormalities. All of these behavioral alterations were only seen following the assessment of certain markers and are generally the consequence of modifications in physiological and biochemical processes at the individual level. It appeared that acetamiprid was not very harmful to fish. The key factors influencing fish mortality from pesticide exposure are their susceptibility to the toxicants, as well as the exposure's concentration and duration (Kamble, S. M., 2011). Prior to doing more research on animal physiological alterations, it is crucial to assess the LC50 concentration of contaminants.

Bighead carp juveniles exposed to the water/solvent control did not perish or have any less severe side effects, according to all confirmed acute toxicity investigations. A relationship between fish direct mortality and concentration was shown by the fact that fish mortality rose as the toxicant concentration rose. The first mortalities were recorded at the same time (2 h postexposure). Our results show that Pyriproxyfen and Acetamiprid are extremely toxic to Bighead carp as they can cause disruption in its normal behavior and physiological dysfunctions to visceral organs should be avoided. Acetamiprid affects the synapses in the insect central nervous system by acting as an agonist on the nicotinic acetylcholine receptor (Wolf, J. C., and Wheeler, J. R. 2018) to produce excitation, paralysis, and death. Many matrices, including water, soil, plants, and aquatic animals, are contaminated by this pesticide once it is sprayed to crops (Raj and Baby 2015; Morrissey et al. 2015; Hoyle and Code 2016).

Conclusion

The purpose of this research was to examine the physiological dysfunctions in Bighead carp by applying the mixture of Acetamiprid and Pyriproxyfen. For this purpose 27 freshwater fishes were split into three groups at random (9 fishes in each group) named as G0, G1 and G2. Fish exposed to Acetamiprid and Pyriproxyfen in group G1 and group G2 were dissected after 7 days, their sampling and analysis indicated that fishes exhibited surface breathing, jerking, body tilting, erected fins, and lethargy. sluggishness, static movement, bottom breathing, faintness and irregular operculum movements. Other physiological dysfunctions are reduced body weight, decrease in relative weight of liver, kidney, brain, intestine, heart etc. conditions become severe when sampling was done on 14th day. However, no mortality was detected during the period of experimental analysis. Hence, we concluded that Acetamiprid and Pyriproxyfen induce physiological dysfunctions fish in Hypophathalmicthys nobilis. This research unequivocally showed the negative physiological dysfunctions by pyriproxyfen and Acetamiprid pesticides on freshwater fish Hypophthalmicthys nobilis. It is concluded that in the realm of environmental bio-monitoring, the clinical and physiological characteristics examined in the current

studies can be employed as possible biomarkers of pesticide toxicity to fish and other aquatic creatures.

Author Contribution

Adeeba Naseer: Conceptualization, Investigation; Formal Analysis, Writing -original draft, Data Curation, Methodology, Visualization. Taskeen Shakir, Bushra Tariq and Sanya Naz, Review and editing. Nusheen Ahmad, Saud Iqbal Azhar Ullah and Muhammad Usman: Review final draft and editing.

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Ethical Statement

This research was executed according to the guidelines of the ethical committee of the institution.

Availability of Data and Material

All the data is available and can be obtained on reasonable request from the corresponding author

Consent to Participate

All the authors equally participated in this research.

Consent for Publication

All the authors agreed and gave their consent for publication.

Competing Interest

Authors declare no conflict of interests.

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