Behavioural and ultrastructural activity relationship as early warning signs in fish species

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Abstract

Larvicides when applied repeatedly to effectively kill mosquito species often accumulate in water bodies affecting non-target aquatic organisms including fish species. It is hypothesised that the behavioural responses from exposure of *Poecilia reticulata* to tolerable concentration of larvicides is unconnected to the ultrastructural alterations found in the exposed fish species. Heterogeneous sexes of fish consisting of 18 females (gravid and non-gravid), and 9 males, in three replicates, were separately exposed to spinosad (49 and 110 μgL^{-1}) and chlorpyrifos (0.4 and 0.8 μgL^{-1}) at dosages that did not cause physical death for 28-days under static renewal bioassay with control. Ultrastructural analysis was performed for control and treatment in 3 replicates each, on randomly selected pre-treated fish species with evidence of behavioural changes or deformities including reduced feeding, loss of equilibrium, hypoactvity and pectoral fin forward. Behavioural changes were consistent with the ultrastructural damage observed in the fish, and demonstrated the strength of each larvicide as fish toxicant. At higher spinosad concentration, reduced feeding in fish manifested as ruptured lysosomonal cells. Fish in lower spinosad concentration behaved similar to control with minimal cellular damage characterised by increased secretory vesicles and mucin. Opercular haemorrhage and skeletal deformities, more likely to be responsible for loss of equilibrium and pectoral fin forward, were mostly found in the chlorpyrifos treated fish. These were as evidenced by mitochondria rupture, gross dead cells and loss of grey area in cytosol. Behavioural changes are reliable diagnostic warning signs of early, and ongoing cellular damage in exposed fish species necessary for rapid detection and prompt intervention against non-target larvicidal effects on aquatic organisms.

Keywords: *Poecilia reticulata;* chlorpyrifos; spinosad; ultrastructural; behavioural.

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Introduction

Spinosad is a naturally derived insecticide with insecticidal potential in mosquito control. It is derived from a bacterium known as *Saccharopolyspora spinosa* Mertz and Yao (Thompson *et al* 2000). Chlorpyrifos is a synthetic organophosphorus compound, and just like spinosad, it has been successfully used against mosquito larvae species (Lawal and Samuel, 2010; Stevens *et al* 2005; Hertlein *et al* 2010). These compounds when applied repeatedly to effectively control mosquito larvae often accumulate in water bodies resulting in increased exposure of non-target aquatic organisms including fish species sharing mosquito larvae habitat. The synergistic/ antagonistic effects of the mixture are hardly interpreted and predicted exclusively from chemical analysis thus, the use of various histological parameters to adequately assess their non-target impacts. *Poecilia reticulata* (Pisces: Poeciliidae), commonly called guppy are natural mosquito regulating-agents. Previous studies have shown non-target effects of larvicides on guppy at low concentrations using various histological biomarkers (Anogwih *et al* 2013a; 2013b).

Histological data are generated from biomarkers which are indicators of the impact of xenobiotic on different levels of biological organizations (Paolini *et al* 2005). They are used to assess damage to cell and tissue structures that are not always visible to the naked-eye. Behavioural changes in organisms due to exposure to chemicals usually indicate the effect of chemicals at organismal level, and therefore used to describe trends over time (Padmini and Rani, 2011). Extensive studies have been carried out on the use of abnormal behavioural responses as diagnostic endpoints for determining the sub-lethal effects of various chemicals. Drummond *et al* (1986) evaluated the use of behavioural and morphological changes in fish as diagnostic endpoints

for screening, and differentiating some chemicals according to their mode of action after exposing 30-day-old fathead minnows *Pimephales promelas*. Spinal deformities in fat head minnows, *P. promelas* and rainbow trout *Oncorhynchus mykiss* exposed to sub-lethal concentrations of chlorpyrifos as well as loss of equilibrium, hanging vertically, rapid gill movement, gulping for air and prolonged motionless behaviour in guppies exposed to various concentrations of deltamethrin have been reported (Halcombe *et al* 1982; Viran *et al* 2003). These behavioural changes were not linked to potential cellular damage in the organisms despite that damage to tissues usually begins as molecular malfunction within specific organelles (Wayne *et al* 2009), hence the current research.

Behavioural symptoms could be attributed to unobservable cellular or tissue damage which when left unattended could degenerate to disease, chronic disorder and eventual death of the organism. More often than not, mortality occurs in exposed fish before the onset of intervention simply because the initial cellular damage was ignored and, had progressed to advanced stage. Behavioural symptoms may be helpful in averting this problem by providing early diagnosis of new and ongoing cellular damage in aquatic organisms necessary for prompt intervention, survival and conservation of non-target species.

Therefore, the aim of this study is to investigate behavioural changes in exposed guppy linked with ultrastructural damage found in the fish's tissue, necessary for rapid detection and management of non-target pesticide effects on aquatic organisms.

Materials and methods

Ethical approval

The Ethical Committee of the University of Lagos approved the research according to the rules guiding the use of animals for experimental research.

Fish rearing

Poecilia reticulata Peters 1859 (Pisces: Poeciliidae) were collected from open drains in Yaba City in Lagos, Nigeria, (6.533048N, 3.388424E). Fish species were gently released into a holding tank of capacity 200L containing dechlorinated tap water at pH 7. They were reared under laboratory conditions of 28ºC±0.8ºC, 72 ± 2% RH and 12:12 h light: dark regime. The tank was drained, washed and refilled with fresh dechlorinated tap water twice weekly to prevent the accumulation of fish metabolic wastes. After 8-days of acclimatization period, selected brood stocks were transferred into 5L plastic containers to obtain offspring F1 generation. After 3-4 weeks period of completion of a cycle of reproduction, 2-day old juveniles were separated from adults and introduced into container of 2L well-aerated, with a portable aerator (Frabill Aqua-Life™), in dechlorinated tap water where they were allowed to mature into adult sizes of mean length 3.5±0.2 cm (snout to tail) that were used for bioassay.

Test compound

Spinosad, 1.25 g/kg AI consisting of Spinosyns A (CAS: 131929-60-7), and D (CAS: 131929-63-0) was sourced from Nigeria Stored Product Research Institute (NSPRI), Yaba, as spintor dust, Naturalyte® (Dow Agrosciences, South Africa) while chlorpyrifos, Pyrinex® (Adama, UK) CAS: 3383-98-8 containing 480 g/l AI was purchased from Afcott Ltd located in Lagos, Nigeria.

Preparation of test concentrations

Low concentrations of spinosad and chlorpyrifos within the range that killed 30 to 70% population of a more tolerant mosquito species (*Culex quinquefasciatus*) but did not cause physical death of fish at preliminary studies were selected (Anogwih *et al* 2013a).

Ultrastructural study was conducted on pre-treated fish samples that showed signs of one or more behavioural changes.

Behavioural analysis

Fish were not fed 24 hours before testing. Heterogeneous sexes of fish consisting of 18 females (gravid and non-gravid), and 9 males, in three replicates for each concentration of larvicide, and control respectively were exposed for 28 days under static renewal bioassay (Wester and Canton 1992) and then evaluated for behavioural changes. The following concentrations of larvicides were used for spinosad, 49 ìgL^{-1} and 110 ìgL^{-1}; and Chlorpyrifos, 0.4 ìgL^{-1} and 0.8 ìgL^{-1}. Gravid females were included only in the higher larvicides concentrations and in the untreated dechlorinated tap water that served as control.

Behavioural changes in guppy were critically monitored throughout the experimental period, at every 24 hours interval. Responses were recorded if they differed from control, and occurred in $\geq$ 10% of the fish within each test chamber (Rice *et al* 1997). Control mortality was less than 5% and a fish was regarded as dead if it failed to move when gently probed with the edge of a glass rod.

Ultrastructural analysis

Three randomly selected fish from each treatment that showed either deformities or behavioural changes different from control including reduced feeding, loss of equilibrium, hypoactivity, pectoral fin forward, skeletal deformities and opercular heamorhage were dissected with control at the end of the 28-day exposure period. Fish intestinal tissue was prepared for ultrastructural analysis as in Anogwih *et al* (2013b). Briefly, dissected tissues were immediately fixed in 1.25% Glutaraldehyde (EMS, USA) in 0.10M phosphate buffered solution, pH 7.4 at 4°C for 1h in the dark and 2% osmium tetroxide for 2 hours under the hood followed by an ascending

series of dehydration of graded alcohol (25% to 100%). Ultrathin sections (80 nm) were imaged at 80 kV with Philips CM-10 Transmission Electron Microscope following a staining regime of 2% Uranyl acetate and Reynold's lead citrate for 30 and 3 minutes respectively. Intestinal tissues were selected because like the gills, they are prime sites of fluid/nutrient uptake and absorption in fish species. It also protects the fish from external impacts of chemicals and metals (Marijic and Raspor 2007). Therefore, the health of these tissues is pivotal in the proper functioning of *P. reticulata* as a mosquito control agent.

Table 1. Behavioural changes in *Poecilia reticulata* after 28 days exposure to larvicides.

Days	Behavioural symptoms	Control	Chlorpyrifos (µg/L)		Spinosad (µg/L)	
			0.4	0.8	49	110
1	Hyperactivity					
	Abnormal lateral flexure					
3	Hatching					[c]X
	Hypoactivity				X	X
7	Haemorrhage			[b]X		
14	Abnormal lateral flexure					
	Loss of equilibrium			X		
	Mortality			X		
	Pectoral fin forward			X		
	Haemorrhage		[a,b]X	[a,b]X		
	Hypoactivity			X		
	Reduced feeding		X	X		X
	Scoliosis			X		
15	Loss of equilibrium			X		
	Reduced feeding			X		
	Pectoral fin forward			X		
	Hypoactivity			X		
16	Scoliosis					
	Loss of equilibrium			X		
	Haemorrhage			[a,b]X		
	Pectoral fin forward			X		
	Hypoactivity			X		
	Reduced feeding			X		X
	Lordosis			X		
	Mortality			X		
17	Mortality		X	X		
	Hatching			[c]X		
	Lordosis			X		
20	Mortality			[d]X		
	Lordosis			X		
21	Reduced feeding			X		
	Hatching	[c]X				
22	Mortality			X		
	Reduced feeding			X		
23	Reduced feeding			X		
	Mortality					
24	Reduced feeding			X		
	Mortality			X		
27	Reduced feeding			X		
	Mortality			X		

[1]Responses were recorded if they differed from control and occurred in e"10% of the fish within each test chamber. [a]Male fish; [b]Opercular region; [c]Gravid Female; [d]Fries.

Results

Behavioural changes in Poecilia reticulata

Chlorpyrifos was more toxic to guppy than spinosad with several behavioural symptoms ranging from fish hypoactivity to reduced feeding, opercular heamorrhage and death (Table 1). Fish in spinosad treatment were seen to behave not too differently from control especially in the lower concentration where fish became hypoactive on day 3 thereafter, swam normal as in control (Table 1). Gravid fish placed in higher concentrations were seen to hatch their fries earlier than in control indicating premature birth (Table 1).

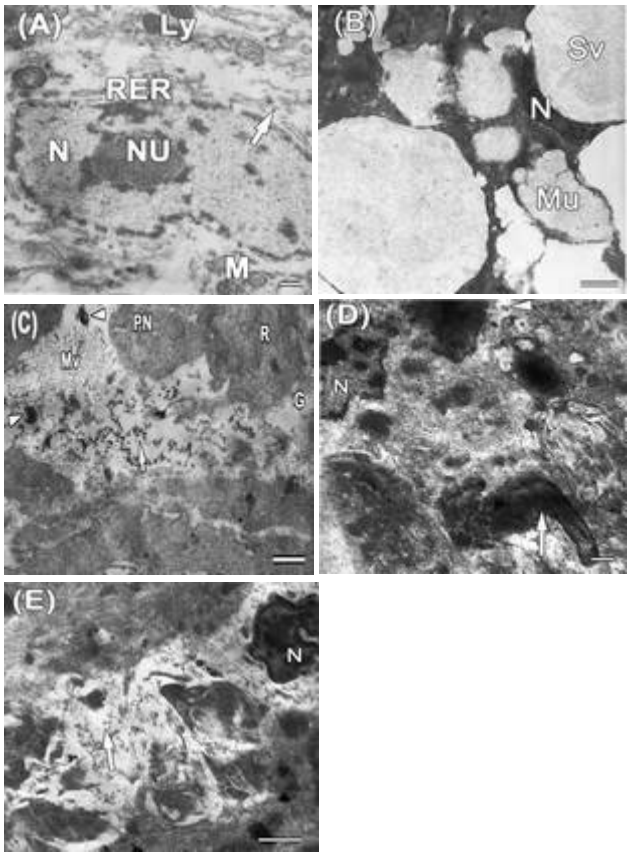
Ultrastructural alterations in Poecilia reticulata

The severity in ultra-cellular damage increased at increasing concentration with fish placed in spinosad treatments presenting milder alteration than chlorpyrifos treated ones (Figures A to J). Control fish were with intact cytoplasmic and nuclear membrane, well-defined nucleus, and nucleolus. Other organelles were intact as in mitochondria with distinct cristae, and well defined matrices (Figures A and F).

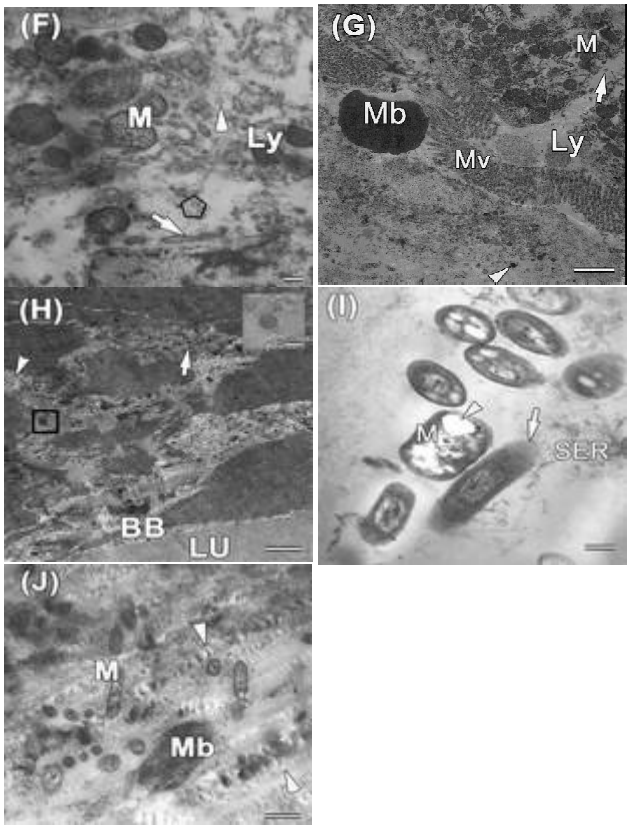
Fish placed in lower spinosad concentration were not too different from control. Nuclei and mitochondria alterations differing from control that were found in this group included large secretory vesicles, elongated nucleus without nucleolus, rearranged chromatin, mucin and electron dense cytoplasm (Figures B and G). In the higher concentration of spinosad, the following were

evident: severe cellular alterations of the electron dense cytoplasm namely pycnotic nucleus, ruptured lysosome, degraded cell membrane, fewer mitochondria and cristae (Figures C and H).

Chlorpyrifos-treated fish were mostly with loss of grey area in cytosol, several ruptured mitochondria and dead cells that increased over concentration increase essentially the nuclei, mitochondria and lysosomonal cells (Figures D, E, I and J).



Figures A-E: TEM of Intestinal Nuclei for control and exposed fish. **(Figure A)**: Control fish with intact Cytoplasm, Nuclear membrane, well-defined Nucleus (N) and one Nucleolus (NU). Cell organelles are intact as in Mitochondria (M); Lysosomes (Ly); Rough endoplasmic reticulum (RER); Smooth endoplasmic reticulum (arrow) **scale bar 1 ìm**. (**Figure B)**: At 49 μgL^{-1} of spinosad, features included Electron dense cytoplasm and nucleus compared to control, elongated Nucleus (N) with rearranged chromatin, presence of large Secretory vesicles (Sv) and Mucin (Mu), **scale bar 1 ìm. (Figure C):** Severe distortion of electron dense cytoplasm at spinosad 110 μgL^{-1} (arrow) characterised by Pycnotic nucleus (PN) with ruptured Lysosome (arrow heads). Golgi body (G), Microvilli (Mv), **scale bar 0.5 ìm. (Figure D):** At 0.4 μgL^{-1} of chlorpyrifos there was severe damage to Cytoplasm (arrow), few dead cells including Nuclei (N), Smooth endoplasmic reticulum (arrow head) **scale bar 1 ìm. (Figure E):** Chlorpyrifos at 0.8 μgL^{-1} was characterised by lots of dead cells including Nucleus (N) and loss of grey area in cytosol (arrow), **scale bar 0.5 ìm**.



Figures F-J: TEM of Intestinal Mitochondria for control and exposed fish. **(Figure F):** Control fish with intact organelles including Mitochondria (M); Smooth endoplasmic reticulum (arrow head); Rough endoplasmic reticulum (arrow); free Ribosomes (Polygon shape); Lysosomes (Ly); **scale bar 200 nm**. **(Figure G):** At 49 μgL^{-1} no marked difference from control. Intact cristae in Mitochondria (M), Microbodies (Mb), Microvilli (Mv), Smooth endoplasmic reticulum (arrow), Lysosome (Ly), Lipid droplet (arrow head) **scale bar 1ìm**. **(Figure H):** At 110 μgL^{-1} of spinosad there was marked degradation of electron dense Cytoplasm (arrow) including Mitochondria with indistinct cristae (inset), Brush border (BB), Lumen (LU), Microvilli (arrow head), **scale bar 1ìm**, **Inset 0.25 ìm**. **(Figure I):** At 0.4 μgL^{-1}, there was marked Mitochondria rupture (arrow head) with degenerated cytoplasmic Membrane (arrow), Smooth endoplasmic reticulum (SER) **scale bar 0.25 ìm**. **(Figure J):** At 0.8 μgL^{-1} of chlorpyrifos, Cell membrane was shrunken (arrow head), presence of numerous dead cells including Mitochondria (M) and Microbody (Mb) **scale bar 1 µm.**

Discussion

Results of both biomarkers were similar and demonstrated the strength of each larvicide as toxicant to the bio-control fish agent. The presence of large secretary vesicles and mucin in guppy exposed at the lower spinosad concentration probably indicates that the fish were beginning to get affected by the toxin hence the initial hypoactive behaviour that seized after day-3. These alterations were not found at higher concentrations suggesting a threshold level of occurrence above

which internal cells would begin to die, resulting in the physical death of fish over time and if not diagnosed early enough. The production of mucin and secretory cells in fish species after exposures to low concentration of deltamethrin and temephos have been reported, though with no emphasis on behavioural biomarker (Al-Ghanbousi *et al* 2012; Ba-Omar *et al* 2011). Mucus secretions by gills and intestines play a major role in the protection of the tissues from environmental impacts of xenobiotics, and have been presumed to be an initial protective response by fish to environmental effects of larvicides (Sorensen 1991; Pawert *et al* 1998; Matey *et al* 2008).

Hatched fries from treated gravid females placed in higher concentrations were recorded as in the control. Although hatching occurred prematurely in the treated-fish which suggests that both larvicides at these concentrations lacked the potential to inhibit fish growth and development but are likely to cause premature birth of fish species. More studies at higher concentrations than currently utilised in this study should however be tested to validate this assumption**.** Premature fries were more unlikely to make it to adults or may grow into unfit adults thus jeopardising their ability to perform effectively as mosquito control agents.

Reduced feeding was found to characterise the higher concentrations of spinosad and chlorpyrifos, manifesting as ruptured lysosome in cells contained in higher spinosad treatment. The higher toxic effect of chlorpyrifos compared to spinosad must have caused total destruction of lysosome beyond recognition in the picture explaining why fish exposed at this concentration also showed reduction in feeding just as spinosad-treated ones but with no evidence of lysosomonal alterations. There are over 40 heritable lysosomonal storage diseases known, each being characterised by harmful accumulation of a specific substance or class of substances commonly polysaccharides or lipids that would normally be catabolised by the hydrolytic enzymes present within the lysosome or transported out of the lysosome therefore, lysosomonal defects may impair digestion and recycling of cellular components that are no longer needed in the fish (Wayne *et al* 2009).

The pattern of mitochondria rupture observed in chlorpyrifos treatments was similar to those reported for pirimiphos methyl (Anogwih *et al* 2013b), and could be diagnostic of organophosphorus toxicity in fish species. Cell ruptures are signs of hypoxia and respiratory failures in organisms (Richmonds and Dutta 1989) explaining why pectoral fin forward was mostly observed in this group of fish. Hypoactivity, heamorrhage and increased fish mortality in chlorpyrifos treatment may be attributed to the fewer mitochondria cristae, matrix damage, and the gross death of mitochondria and nuclei characterising the group. Cristae are sites of oxidative phosphorylation and electron transport while the matrices are sites of Kreb cycle enzymes (Taylor *et al* 1997). Any damage to these components would definitely lead to oxidative stress in the fish affecting their respiratory behaviour and eventual physical death.

Conclusion

This study has demonstrated that behavioural changes in addition to being a promising diagnostic tool for the screening of larvicides, can serve as indicators of new and ongoing cellular damage invisible to the naked eye necessary for rapid diagnosis of non-target chemical impacts on aquatic species. Usually, cellular damage begins as molecular malfunction within specific organelles, and may gradually progress into a disease or chronic disorder hence the need for early diagnosis and intervention through constant behavioural monitoring. Behavioural monitoring should be made to complement other biomarkers of stress in any toxicological study to achieve a reliable and more meaningful result.

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