The role of Caselio (plant fertilizer) exposure on digestive gland histology and heavy metals accumulation in the freshwater snail, *Lanistes carinatus*

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Abstract

The adverse effects of Caselio, the plant fertilizer (PMR) on the freshwater snail, *Lanistes carinatus* were studied. Snails were collected from Bahr Shebeen canal, Menoufia Province, Egypt. Snails were exposed to 200 and 600 μl/l of the fertilizer (PMR) for 0, 1, 3 and 7 days. Shell, operculum, foot and soft tissue metals concentrations (Cd, Pb, Al, Cu, Mg, Ti and Na), lipid peroxidase and histopatholgy of digestive gland were investigated. Tested metals concentrations accumulated differently in the shell, operculum, foot and soft tissue under the effect of the fertilizer where soft tissue accumulated essential metals and most of the non-essential metals. Exposure to PMR caused significant increase in lipid peroxidase of snails` digestive gland ranging from 52-82 % increase than control snails ($P \leq 0.03$). The fertilizer caused tissue damage in the digestive tubules as necrosis, fusion and increased lipofuscin pigment significantly of exposed snails` (200 and 600 μl/l) when compared to the control group (134.33 ± 2.85, 80.0 ± 8.18 and 69.33 ± 3.18, respectively, $P = 0.0003$) after 7 days of exposure. In conclusion, different organs accumulated different amounts of essential and non-essential metals under the effect of PMR. The fertilizer caused lipid peroxidation and lipofuscin pigments increase (aging) to the digestive gland of *L. carinatus*.

Keywords: *Fertilizer, Lanistes carinatus, Metals, Lipid peroxidase, Digestive gland.*

1. Introduction

For biomonitoring the aquatic systems, there are many criteria for choosing an organism. The organism should be relatively sedentary, widespread and have a moderate life span, accumulate metals from food, water to meet the criteria as good bioindicators (Huang et al. 2007). Metal accumulation in the environment can be a result of both natural and anthropogenic sources. Different Metal concentrations in aquatic ecosystems affect vertebrates and invertebrates of the environment, by modulating their biological activities in several pathways (Rockwood et al., 1990; Kar et al., 2015). Accumulation of metals depends on many parameters. Ibrahim and Mat (1995) showed that the total metal (Mg, Cl, Mn, K, Se, Br, Zn, Co, Fe, Rb and Cd) content is directly proportional to the body weight. Dodd (1965) recorded a relationship between shell size in *Mytilus* sp. and magnesium concentration in the prismatic layer of the shell.

Plant fertilizers are good for plants and increase vegetative production as it supply the plant with nutrients. There are many types of fertilizers; single, binary and multinutrients. Nitrogen fertilizers are made of ammonia (NH_3). Compound fertilizers contain N and P. liquid fertilizer has more rapid effect and easier coverage and added to the irrigation water. Agricultural run-off , where fertilizers are present is the main cause of eutrophication and consume oxygen (Werner, 2009). Nitrogen fertilizers cause soil acidification and in turn inhibit microbial activity and affect soil invertebrates. A negative relationship was found between high rates of fertilizer and populations of earth worms and beneficial invertebrates (Leslie,1994). Agriculture products will make global environmental changes (Tilman et al., 2001).

Magnesium (Mg) is important essential metal in the basic nucleic acid chemistry of cells. Over 300 enzymes require magnesium ions for their catalytic action, such as enzymes utilizing or synthesizing ATP, or those that use other nucleotides to synthesize DNA and RNA. Loss of Mg^{2+} of some tissues caused defective transport, hormonal stimulation, or metabolic impairment (Romani, 2013). Copper (Cu) is an essential element; however its deficiency/toxicity could cause health problems of the organisms. It is essential in metabolic functions as it is a part of proteins, enzymes, bone and connective tissue (Osredkar and Sustar, 2011) and haemolymph of molluscs (Rainbow and White, 1985). Exposure to heavy metals such as Cu SO_4, can cause disturbance in osmoregulation

of the animals tissues (Hu et al., 2015). Secretion of mucous is a sign of Cu toxicity as recorded by Gupta et al., (1981).

Aluminium (Al) is non-essential metal for biological systems. The amount of Al in solution coming from the normal weathering is a small part of the total Al in the environment from other sources. However, surface water contains a large part of Al. Inorganic Al increase with acidic water and organic Al increase with increase of dissolved organic matters. Al bioavailability increases with increase of acidity (under pH 5.0) and decrease of temperature and coupled with drainage lakes (Driscoll and Postek, 1996). Al toxicity could be increased by coupling with phosphorous as $AlPO_4$ (Driscoll and Postek, 1996) or could be reduced with addition to Ca (*Daphnia magna*, Havas, 1985). In addition, Al at low pH affected respiration of the dragonfly *Libellula julia* Uhler (Rockwood et al, 1990). Cadmium (Cd) uptake depended on the external metal concentration (the shore crab, *Carcinus maenas*, Martin and Rainbow, 1998). Rainbow and Black (2005) studied the effects of salinity and osmolality differences on the uptake rates of dissolved cadmium in 3 crabs of different ecologies. Lead (Pb) is a highly poisonous metal affecting almost every organ and system in the body. Long exposure to dietary Pb caused growth deficiency in the snail, *Theba pisana* (El-Gendy et al., 2011). Titanium (Ti) is a biocompatible metals (it is non-toxic and is not rejected by the body). Ti introduced in many medical uses, like surgical implants (joint replacement) and it is not harmful nor plays any biological role in the human body (Emsley, 2001). Pyatt et al., (1997) recorded Ti in the tissues of the freshwater snails, *Lymnaea stagnalis* and not detected in water samples collected from unpolluted site.

Cd, Cu and Pb were analysed in different part of the gastropod *Nerita lineata* collected from different sites in the west intertidal area of Peninsular Malaysia, in soft tissues, shell and operculum (Yap and Cheng, 2009). Cd, Co, Cu, Fe, Mg, Mn, Pb and Zn were anaylzed in the sediment, shell and tissue of different molluscs at south coast of India. The authors found tissue specific accumulation of the investigated metals (Kesavan et al., 2013).

Biomarkers are defined as a biological response to chemical(s) that give a measure of exposure or effect (Peakall, 1994). Lipid peroxidation refers to the oxidative degradation of lipids where free radicals take electrons from the lipids in cell membranes, resulting in cell damage. One of the end products of lipid peroxidation are reactive aldehydes, such as malondialdehyde (MDA, Buege and Aust, 1978). Siwela et al. (2010) studied the effect of polluted water and sediments on the freshwater snails, *Lymnaea natalensis* lipid peroxidase. They recorded significant increase in levels of MDA in the snails tissues exposed to polluted water and sediments. Heavy metals (Zn, Cu, As, Cd, Pb and Hg) were analyzed in the bivalve molluscs, mussels, clams, oysters from different sites in China and compared to each other for biomonitoring purposes (Huang et al. 2007).

Histopathological studies showed variety of organs damage in molluscs exposed to anthropogenic agents. The contamination may be direct by water or indirect by food. Clearly, food route exposure is a concern, because it could be circulated with body circulation. Histological alterations of the liver and vital organs were recorded in the animals reared near the industrial areas (Kar et al., 2015). Exposure to Cd caused morphological and histological malformations of the siphones and gills of the marine clam *Venerupisa urea* (Mohamadein and Desouky, 2002). Many researchers investigated digestive gland as target organ for pollution (the freshwater snail, *Lymnaea stagnalis*; Lance et al., 2010, the Land Snail *Helicella vestalis;* Sharaf et al., 2015).

The objectives of this study were to evaluate the role of Caselio, the plant fertilizer on the digestive gland and metal accumulation in the adult freshwater snail, *Lanistes carinatus* by examining digestive gland histology and lipid peroxidase activity and detecting the pattern of metals accumulation in different tissues.

2. Materials and methods
2.1. Animals
The freshwater snails, *L. carinatus* were collected from Bahr Shebeen Canal at Winter (2014), (body weight, 6.47 ± 0.21 gm) and transported in field water to the laboratory. Snails were held after collection for three weeks for acclimation in filtered declorinated tap water in tanks (5 L tanks, length: 25, width: 16, height: 15.5 cm) filled with 1 litre/tank. The snails were maintained under laboratory conditions with photoperiod 12 h light: 12 h dark and water pH was 7.3 ± 0.2.

2.2. Experimental material
Caselio (fertilizer) is a plant metabolism regulator (PMR) in solution form [contains 7% N, 20% P_2O_3, 4% Zn, 1.8% Fe, 1.3% Mn and 4.5% free amino acids (w/v)]. Caselio is a product of international Egypt Chem, under registry licence No. 2593.

2.3. Experimental design
After 2-weeks of acclimatization to the laboratory conditions, adult *L. carinatus* snails were divided into three groups (one control and two concentrations of the fertilizer [PMR]). Each group consisted of three replicates (10 individual/replicate). The chosen sublethal concentrations was determined according to Sheir, (2015). The 1st group (control) was kept in filtered declorinated tap water, the 2nd group treated with the fertilizer, PMR (200 µl/l, LC_{10}), and the 3rd group treated with the fertilizer, PMR (600 µl/l, LC_{30}). Tanks water were re-dosed every other day after a 100 % water change, and snails were fed on fresh green lettuces. Snails were sampled on day 0, 1, 3, and 7. The assessed parameters were metals analysis (Cu, Al, Mg and Ti) in shell, (Cu, Al, Pb and Cd) in operculum, (Cu, Al, Cd, Pb and Na) in foot, and soft tissue, and lipid peroxidase and histology in digestive gland.

2.4. Metal analysis in tissue

Tissue samples were prepared according to Federici et al. (2007) with minor modifications. Briefly, about 0.05 g of fresh tissue (shell, operculum, foot and soft tissue) was oven dried, digested in 1 ml of concentrated HNO_3 at 70 °C, and then diluted to a final volume of 5 ml with distilled water. Total Cd, Pb, Al, Mg, Ti and Cu were measured using Inductively Coupled Argon Plasma, iCAP 6500 Duo (Thermo Scientific, England) against 1000 mg/l multi-element certified standard solution (Merck, Germany) as stock solution for instrument standardization. Na was measured by Flame Photometer, PFP 7, Jenway, UK.

2.5. Lipid peroxidase

Lipid peroxidase (LPO) forms malondialdehyde (MDA) as one of the products of oxidative stress. The concentrations of malondialdehyde (MDA) as a marker of LPO were determined according to Nair and Turner (1984) based on the reaction with thiobarbituric acid (TBA) with minor modifications. Briefly, 10 mg of digestive gland homogenate was mixed with 300 µl of MDA lysing buffer, and then centrifuged at 13,000 *g* for 10 min. MDA level was measured colorimetrically at 532 nm against MDA standard curve. MDA were expressed as nM/mg of MDA of wet tissue.

2.6. Histology of digestive gland

For histological examination, three snails were selected randomly from each experimental groups including the control group. The most proximal part of the digestive gland was collected after 7 days of the experiment. Digestive gland was dissected and immediately fixed in Bouin`s fluid. After fixation for 24 h, specimens were dehydrated in an ascending series of alcohol. The specimens were cleared in xylene and embedded in melted paraplast at 60 °C. Serial sections were cut at 6 µm thickness and stained with Ehrlich`s Haematoxylin and counterstained by Eosin (Romeis, 1989). The sections were then mounted and covered with glass cover. Histological sections were photographed using Olympus b x. 41, Japans microscope, photo-automated camera.   The number of tubules, total number of lipofuscin pigment and number of lipofuscin pigment/tubule were measured on collected images (20 X), and a mean for each section was derived by randomly counting three replicates from a randomly selected area on a section from each snail (Weibel et al. 1966).

2.7. Statistical analysis

All data were analysed using Statgraphics (v5.1 software). Data were expressed as mean ± SE. Statistical analysis was carried by One-way ANOVA to set the difference between the control and treated groups, setting the probability level to $P \leq 0.05$, where ANOVA could not be applied, Kruskal Wallis test was used.

3. Results
3.1. Effect of the fertilizer (PMR) on some metals accumulation in *Lanistes carinatus* tissues

Metals chosen for representation in the results were different from one organ to another because it depended on its accumulation capacity and others were under detection limits of the chemical analysis.

3.1.1. The shell

Cu, Al, Mg and Ti accumulation were analysed in the shell of the snails by day 7. The fertilizer, PMR exposure caused large increases (1.4, 1.3 and 1.1 fold) in Cu concentration of the shell in the exposed snails to 200 µl/l for 1, 3 and 7 days, respectively (ANOVA, $P > 0.05$). However, 600 µl/l caused decrease in the content of Cu in the exposed snails than the control (Table 1). Al concentration followed the same pattern as Cu in accumulation. 200 µl/l exposed snails shell accumulated Al more than the control and 600 µl/l snails. Meanwhile, 600 µl/l exposed snails lost its content of Al more than control and 200 µl/l snails significantly (ANOVA, $P \leq 0.03$) on 3rd and 7th days. Mg increased in 200 µl/l exposed snails than the control and 600 µl/l snails. The fertilizer, PMR exposure caused reduction in content of Mg of 600 µl/l exposed snails` shell significantly (ANOVA, $P \leq 0.04$) than the control and 200 µl/l on 1st and 3th days. Ti content in the shell of control and exposed snails were significantly higher in accumulation at 0 and 1st days (kruskal wallis, $P = 0.03$), then all groups including the control decreased the Ti content by days 3 and 7 to be similar in concentration.

3.1.2. The operculum

The fertilizer, PMR exposure caused disturbance (increase/decrease) in Cu concentration of the operculum of exposed snails to both concentrations than the control. On the 3rd day, PMR caused significant reduction in Cu content in 200 µl/l exposed groups than the control and 600 µl/l groups (ANOVA, $P = 0.04$, Table 2). Al concentration in the operculum of exposed snails recorded significant increase than the control group during the whole experimental period especially on the 1st and 3rd days (ANOVA/ Kruskal wallis, $P \leq 0.04$). The fertilizer, PMR (600 µl/l) caused significant increase in Pb concentration on day 1 (Kruskal wallis, $P = 0.02$) more than the control and 200 µl/l exposed groups. Then, Pb content decreased than the control in the following days of the experiments (7th day, ANOVA, $P = 0.05$). Cd content generally recorded significant increase in all exposed snails` operculum (ANOVA/ Kruskal wallis, $P \leq 0.05$) than control or between treatments.

3.1.3. The foot

The fertilizer, PMR exposure caused increases in Cu concentration of the foot in the exposed snails to both concentrations on the 1st day. However, decreased for the rest of the experimental time especially on the 7th day by 600 µl/l treatment (ANOVA, $P = 0.05$, Table 3). Al content decreased in both treatments for the total experimental time and continued its significant decrease than the control on 3rd and 7th days (Kruskal wallis, $P = 0.02$). The fertilizer, PMR exposure caused significant increase in accumulation of Cd for both concentrations of the complete experimental period

Tab1e 1. Total Cu, Al, Mg and Ti concentrations in the shell of *L. carinatus* for 7 days exposure to the fertilizer (PMR)

Time	Treatments	Cu	Al	Mg	Ti
Zero	Control	6.3 ± 1.9	100.22 ± 9.60	134.01 ± 4.48	$54.29 \pm 6.56^{\$}$
1 day	Control	6.1 ± 1.2	97.30 ± 13.20	144.02 ± 16.41	$47.42 \pm 4.73^{\$}$
	200 µl/l	8.9 ± 2.8	109.80 ± 27.70	125.02 ± 14.62	49.76 ± 3.86
	600 µl/l	5.6 ± 1.3	64.30 ± 6.70	$78.79 \pm 9.59*$	51.29 ± 6.52
3 days	Control	5.5 ± 1.2	94.69 ± 8.17	134.23 ± 12.19	11.59 ± 0.69
	200 µl/l	6.9 ± 2.3	152.87 ± 30.98	130.17 ± 10.48	15.69 ± 5.15
	600 µl/l	2.9 ± 0.9	$24.36 \pm 1.77*^{\#}$	$91.17 \pm 5.34*^{\#}$	18.01 ± 4.97
7 days	Control	7.8 ± 0.9	99.85 ± 8.17	146.07 ± 14.08	11.92 ± 1.30
	200 µl/l	8.5 ± 2.4	110.84 ± 7.52	129.26 ± 14.24	12.22 ± 0.71
	600 µl/l	6.1 ± 1.7	$35.08 \pm 9.96*^{\#}$	125.78 ± 2.05	13.48 ± 1.84

Note, Data are means ± S.E., in mg/g dry weight (dw), *n* = 3 snails per group. * indicates a significant difference from the control, # indicates a significant difference between treatments and $ indicates a significant difference between controls within the investigated time (Kruskal-Wallis, $P \leq 0.05$).

Tab1e 2. Total Cu, Al, Pb and Cd concentrations in the operculum of *L. carinatus* for 7 days exposure to the fertilizer (PMR)

Time	Treatments	Cu	Al	Pb	Cd
Zero	Control	27.5 ± 2.5	275.69 ± 69.03	4.76 ± 1.39	0.27 ± 0.04
1 day	Control	26.2 ± 3.4	285.98 ± 20.02	4.99 ± 1.08	0.19 ± 0.03
	200 µl/l	34.4 ± 5.5	$399.57 \pm 50.35*$	7.28 ± 0.96	$0.94 \pm 0.12*$
	600 µl/l	27.3 ± 0.9	$427.18 \pm 40.17*$	$9.64 \pm 0.19*$	$0.27 \pm 0.07\#$
3 days	Control	34.9 ± 5.4	302.76 ± 21.59	4.65 ± 1.44	0.25 ± 0.06
	200 µl/l	$18.4 \pm 3.6*^{\#}$	314.45 ± 52.19	3.63 ± 0.18	0.34 ± 0.02
	600 µl/l	37.2 ± 4.3	353.59 ± 64.89	3.34 ± 0.63	$0.58 \pm 0.13*$
7 days	Control	35.7 ± 4.5	304.32 ± 14.76	4.83 ± 0.48	0.19 ± 0.1
	200 µl/l	30.5 ± 4.4	$455.37 \pm 96.30*^{\#}$	$3.48 \pm 0.27*$	$1.03 \pm 0.17*$
	600 µl/l	21.4 ± 0.6	206.12 ± 5.97	$3.67 \pm 0.15*$	$1.25 \pm 0.03*$

Note, Data are means ± S.E., in mg/g dry weight (dw), *n* = 3 snails per group. * indicates a significant difference from the control, # indicates a significant difference between treatments (ANOVA/Kruskal-Wallis, $P \leq 0.05$).

(ANOVA/ Kruskal wallis, $P \leq 0.03$). The increase continued until reached the highest by 7th day. The differences in percentage of the increase at 200 and 600 µl/l treatments than the control were as follows; 1st day were 47, 142 %, 3rd day were 21, 178 % and 7th day were 172, 405 %, respectively. Pb content was high in the control group and PMR caused significant reduction in its concentration in both treatments by 1st day of the experiments (ANOVA, $P \leq 0.04$, Table 3). PMR caused marginal changes in Na, the electrolyte and insignificant increase/decrease in both exposed snails` foot than the control group during the experimental period.

Tab1e 3. Total Cu, Al, Cd, Pb and Na concentrations in the foot of *L. carinatus* for 7 days exposure to the fertilizer (PMR)

Time	Treatments	Cu	Al	Cd	Pb	Na
Zero	Control	220.3 ± 48.9	36.23 ± 9.42	0.16 ± 0.03	5.08 ± 1.09	1948.8 ± 370.0
1 day	Control	263.0 ± 26.1	38.85 ± 9.34	0.19 ± 0.03	5.83 ± 1.41	2275.0 ± 184.3
	200 µl/l	398.7 ± 60.5	30.04 ± 2.21	0.28 ± 0.04	6.79 ± 1.30	1978.7 ± 185.8
	600 µl/l	464.1 ± 104.3	31.51 ± 2.15	0.46 ± 0.06*#	1.98 ± 0.18*	2168.6 ± 235.0
3 days	Control	262.2 ± 23.1	39.27 ± 4.68	0.19 ± 0.02	5.94 ± 1.01	2258.6 ± 49.0
	200 µl/l	163.5 ± 48.8	26.59 ± 2.64*	0.23 ± 0.06	0.86 ± 0.27*	1819.1 ± 182.8
	600 µl/l	189.3 ± 20.9	12.67 ± 1.02*#	0.53 ± 0.06*#	1.10 ± 0.17*	2168.6 ± 235.0
7 days	Control	234.7 ± 36.9	37.67 ± 4.92	0.18 ± 0.06	5.08 ± 1.41	2169.4 ± 120.9
	200 µl/l	164.1 ± 34.2	18.30 ± 1.64*	0.49 ± 0.09	1.26 ± 0.34*	2196.1 ± 359.6
	600 µl/l	108.3 ± 4.3*	6.82 ± 0.39*#	0.91 ± 0.17*#	1.04 ± 0.17*	2032.9 ± 114.9

Note, Data are means ± S.E., in mg/g dry weight (dw), *n* = 3 snails per group. * indicates a significant difference from the control, # indicates a significant difference between treatments (ANOVA/Kruskal-Wallis, $P \leq 0.05$).

3.1.4. The soft tissue

Cu concentration of the soft tissue of the exposed snails to both concentrations, increased at the 1st day, and then significantly decreased than the control (ANOVA, $P \leq 0.05$, Table 4). Al content decreased under the effect of exposure to PMR at both treatments, especially at the 3rd and 7th days. Cd concentration in the exposed snails` soft tissue increased significantly (ANOVA/kruskal wallis, $P \leq 0.03$) than the control snails` at all time points of the experiment. The increase in 200 and 600 µl/l treatments was 1.2, 2.4 fold for the 1st day, 2.4, 4.6 fold for the 3rd day and 2.1, 5.3 fold for the 7th day, respectively. Pb concentration decreased after exposure to PMR than the control at all time points of the experiment significantly (ANOVA, $P = 0.04$), except at the 1st day, it increased by 200 µl/l significantly than the control and 600 µl/l (kruskal wallis, $P= 0.03$). Na of the soft tissue followed similar pattern of changes in concentration as in the foot. Insignificant increase/decrease in both exposed snails` soft tissue when compared to the control group was recorded during the experimental period.

Tab1e 4. Total Cu, Al, Cd, Pb and Na concentrations in the soft tissue of *L. carinatus* for 7 days exposure to the fertilizer (PMR)

Time	Treatments	Cu	Al	Cd	Pb	Na
Zero	Control	1068.8 ± 421.9	125.17 ± 18.95	0.71 ± 0.29	8.49 ± 1.19	2017.9 ± 246.0
1 day	Control	1012.5 ± 129.5	117.78 ± 22.45	0.77 ± 0.10	9. 57 ± 0.47	2279.5 ± 71.5
	200 µl/l	1567.6 ± 429.5	101.32 ± 8.24	0.92 ± 0.24	17.11 ± 1.23*	2087.9 ± 183.3
	600 µl/l	1506.8 ± 489.2	121.24 ± 11.62	1.88 ± 0.26*#	7.17 ± 0.02#	2506.4 ± 283.1
3 days	Control	1063.5 ± 230.9	121.89 ± 1.66	0.73 ± 0.26	9.06 ± 1.52	2129.3 ± 231.4
	200 µl/l	264.4 ± 83.9*	77.19 ± 3.33*	1.77 ± 0.50	6.07 ± 0.70*	2539.5 ± 167.5
	600 µl/l	539.0 ± 145.8	47.16 ± 1.65*	3.39 ± 0.28*#	3.59 ± 0.38*	2030.9 ± 125.3
7 days	Control	1149.9 ± 18.9	119.83 ± 20.99	0.80 ± 0.09	8.39 ± 0.18	2375.2 ± 13.6
	200 µl/l	654.3 ± 136.7*	59.12 ± 1.64*	1.65 ± 0.51	5.14 ± 1.30*	1939.0 ± 322.9
	600 µl/l	620.9 ± 182.3*	72.23 ± 13.61	4.25 ± 0.08*#	4.76 ± 0.72*	1852.8 ± 146.2

Note, Data are means ± S.E.M., in mg/g dry weight (dw), *n* = 3 snails per group. * indicates a significant difference from the control, # indicates a significant difference between treatments (ANOVA/Kruskal-Wallis, $P \leq 0.05$).

Generally, the fertilizer, PMR caused increase in the accumulation of Al in the exposed snails` shell and operculum, but caused decrease of Al in foot and soft tissue. However, the treatment with the fertilizer, PMR caused disordered pattern of accumulation in Cu content in operculum, foot and soft tissues.

Cu concentration followed a pattern for accumulation magnitude in soft tissue > foot > operculum > shell. Al content mainly accumulated in operculum > soft
tissue > shell > foot. For Cd, soft tissue recorded the second highest accumulation after the operculum followed by the foot. Pb highly accumulated in the soft tissue followed by the foot then the operculum. Na content was mainly present in soft tissue followed by the foot.

3.4. Effect of the fertilizer (PMR) on adult *L. carinatus* lipid peroxidase

LPO levels were significantly higher in the snails exposed to PMR compared to control group (ANOVA/kruskal wallis, $P \leq 0.03$, Fig. 1). At zero time, LPO concentration in snails recorded 3.37 ± 0.39 μM/mg. At the 1st day, the increase in percentages of 200 and 600 μl/l treatments than the control were 61, 70 %, at the 3rd day were 52, 82 % and at the 7th day were 66, 52 %, respectively.

3.5. Histological alterations under the effect of the fertilizer (PMR) on adult *L. carinatus* digestive gland

The control *L. carinatus* digestive gland is composed of bundles of tubules, digestive tubules. Each tubule lined by a single layer of columnar epithelial cells. The epithelial cells are divided into digestive and secretory (basophilic, calcium-excretory) cells. The two types of cells rest on basement membrane and enclose a lumen in the centre of

the tubule. The tubules connected to each other by intertubular connective tissue. The average number of digestive gland tubules per image (20 X) was 9.7 ± 0.88. The digestive tubules had lipofuscin pigments (69.33 ± 3.18). The number of lipofuscin pigments/tubule was 10.89 ± 0.68 (Figs. 2a, b and 3).

After the exposure to 200 μl/l of the plant fertilizer (PMR), some of the connective tissue became necrotic. The nuclei of epithelial cells from the digestive tubules were unclear in some cells. The most obvious symptoms of pathology were necrotic, degenerated and fused digestive tubules and cellular disintegration with almost normal connective tissue. Exposure to 600 μl/l of the plant fertilizer (PMR) caused similar pathological alterations in the digestive gland as in 200 μl/l but worse with necrotic basement membrane of some tubules. The average number of digestive gland tubules per image (20 X) was significantly higher than 600 μl/l exposed snails (13.33 ± 1.85 and 7.33 ± 1.33, respectively, kruskal wallis, $P = 0.05$). The digestive tubules had significantly higher lipofuscin pigments than 600 μl/l exposed and control snails (134.33 ± 2.85 and 80.0 ± 8.18, respectively, ANOVA, $P = 0.0003$). The number of lipofuscin pigments/tubule was significantly higher than 600 μl/l exposed and control snails (16.78 ± 1.64 and 12.22 ± 0.96, respectively, ANOVA, $P = 0.03$, Figs. 2a, b, c, d and 3).
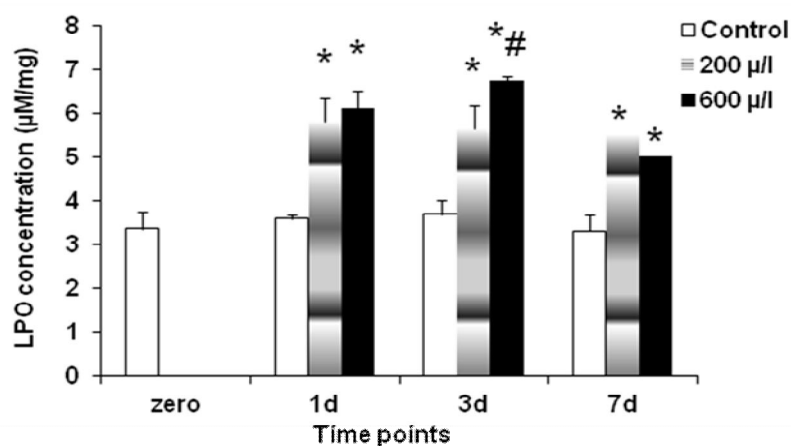
Fig. 1. Effect of the fertilizer (PMR) on adult *L. carinatus* lipid peroxidase activity for 7 days of exposure. * indicates a significant difference from the control and # indicates a significant difference between treatments (ANOVA/Kruskal-Wallis, $P \leq 0.05$).
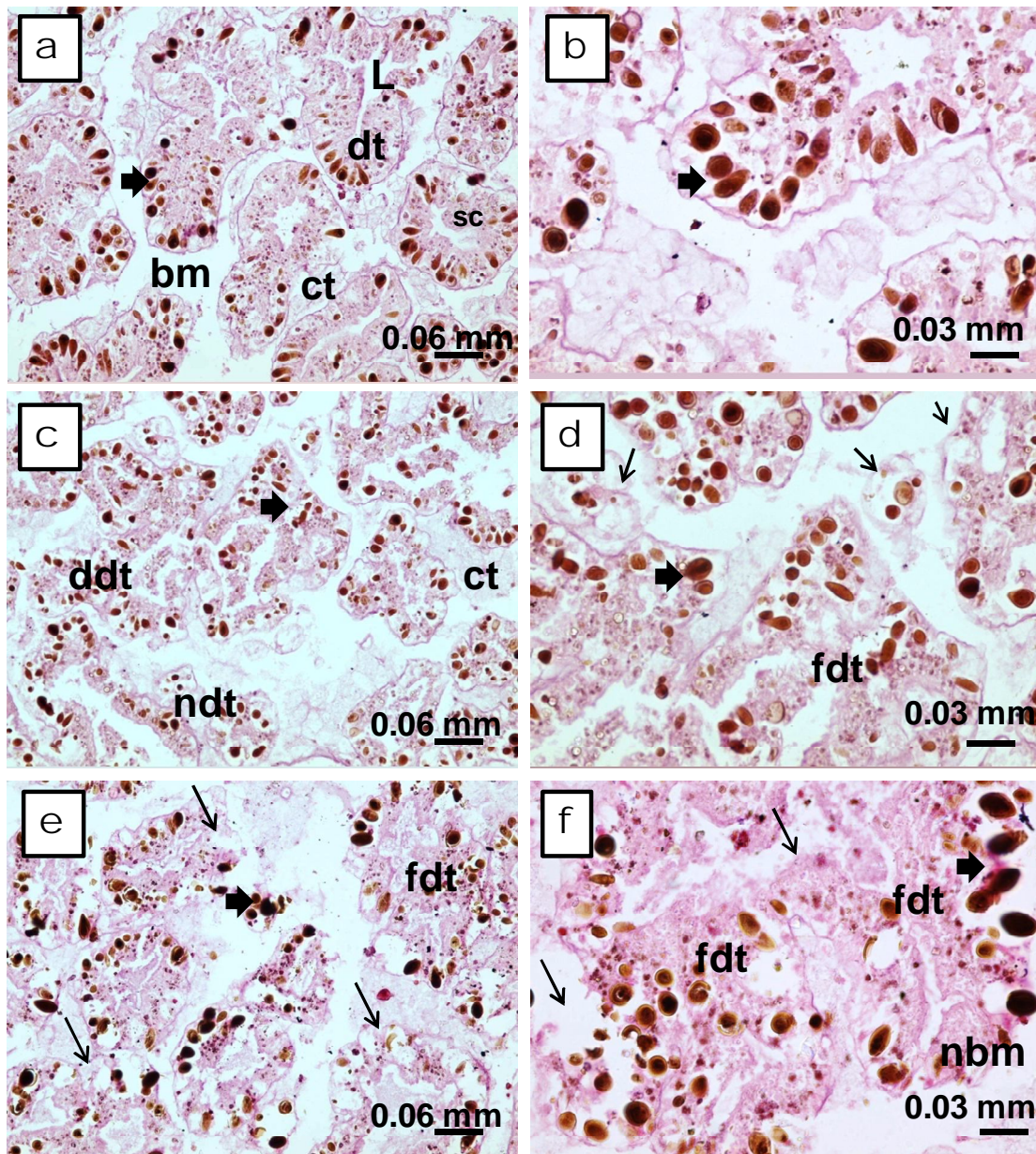
Fig. 2. Light micrographs of sections through digestive gland of adult *L. carinatus* stained with Haematoxylin and Eosin at 6 µm thickness after 7 days of exposure to the fertilizer (PMR). a and b, control snails; c and d, 200 µl/l exposed snails; e and f, 600 µl/l exposed snails. dt; digestive tubule, L, lumen, bm; basement membrane, sc; secretory cell, ct; connective tissue, ➡; lipofuscin inclusions, ndt; necrotic digestive tubule, ddt; degenerated digestive tubule, fdt; fused digestive tubule, ↘ ; degeneration, nbm; necrotic basement membrane.
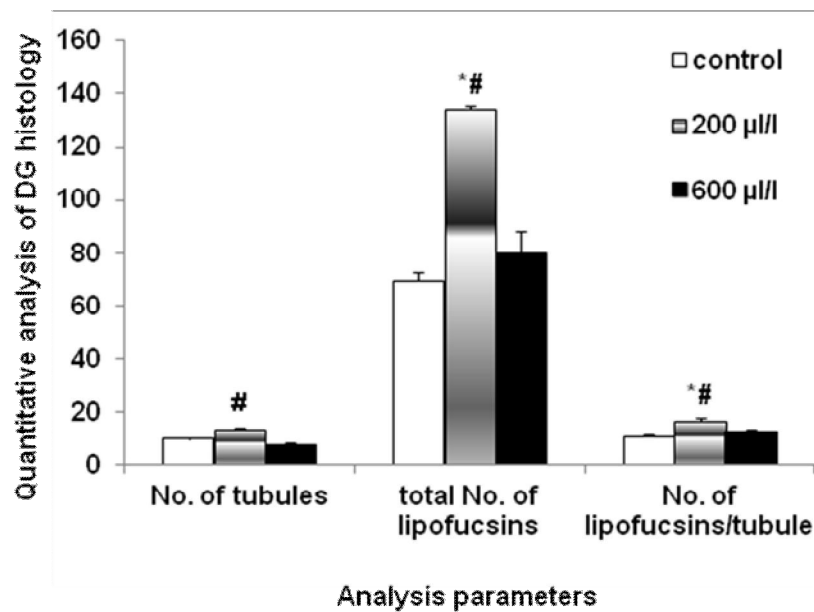
Fig. 3. Quantitative histological analysis of adult *L. carinatus* digestive gland after 7 days of exposure to the fertilizer (PMR). * indicates a significant difference from the control and # indicates a significant difference between treatments (ANOVA/Kruskal-Wallis, $P \leq 0.05$).

4.Discussion

Caselio, the plant fertilizer caused different patterns of metal (essential and non-essential) accumulation in the exposed snails. Oxidative stress (peroxidation) was confirmed in the exposed snails digestive gland histological alterations and malondialdehyde (MDA) production. The findings of this investigation recorded the possible effect of PMR exposure to cause cell aging.

In the present investigation, several metals were picked and accumulated in the tissues of the snails from the experimental declorinated tap water with/without the effect of the fertilizer (PMR). Many studies on the tap water metals were done from different Governorates of Egypt. Drinking water samples from the Great Cairo, Egypt were 2.0, 0.15 and 2.78 µg/l for lead, cadmium and copper, respectively, a study done by Lasheen et al. (2008). They identified pH is a key factor for Pb release from pipes. Fe, Mg, Pb, Ni, Cr, Zn, Cu, Co, Al, and Cd concentrations were analyzed in surface drinking water at Dakahlia Governorate, Egypt (Mandour and Azab, 2011). They mentioned that most of the analyzed metals (Al, Cd, Ni and Pb) exceeded the permissible limits. Heavy metals such as Cadmium, lead, chromium and iron were detected at Menoufia Governorate, Egypt in drinking tap water samples. The presence of those metals was correlated to alteration of the systemic physiology of the residents (Badr et al., 2011).

Al, Ti, Pb and Cd are representative toxic metals, which are biologically non-essential. However, Cu, Mg and Na are essential elements for good biology of the cell, but their excess can cause oxidative stress then toxicity to the organism. In the present work, different accumulation patterns were taken place in the different analysed organs. The pattern for internalisation of different metals were different to each other. Some metals mainly accumulated in fatty tissue (Cu, Pb, Na; digestive gland), muscular tissue (Cu, Pb, Na; foot) or mineralized structures (Ti; shell, Al, Cd; operculum). As discussed by Tunca et al. (2013) who found correlations between metals accumulation and different tissues plus groups of metals in specific tissues in the freshwater crayfish, *Astacus leptodactylus*. In addition, accumulation of Hg was mainly in gills (Sheir et al., 2010) and Cd was in digestive gland (Sheir and Handy, 2010) of the marine mussel, *Mytilus edulis* exposed to Hg and Cd, respectively. This could be explained that some metals accumulate through food and others through dissolved phase in water. Fisher et al. (1996) reported that dissolved phase of metals were predominantly bound to the shell, however, those ingested (food) predominantly associated with the soft parts. Cd, Cu and Pb were analysed in different part of the gastropod *Nerita lineata* collected from different sites in soft tissues, shell and operculum, which showed different accumulation patterns for the essential and non-essential metals (Yap and Cheng, 2009). They added that soft tissues accumulate most for essential metals and least for non-essential metals. Pyatt et al. (1997) detected Mn, Ti and Cu in tissues and shell of the freshwater snail *Lymnaea stagnalis* even they were not detectable in the collection site. Cairns et al. (1975) discussed the interactions between more than one stressor. They suggested that the effect may be synergistic, additive

or antagonistic, and the combined stressors have less effect on the organism than the individual stressor does alone. As Al became more soluble and more bioavailable and toxic to aquatic biota at low/acidic pH (Muniz and Leivestad, 1980). Decreased essential metals and increased non-essential metals in tissues of the marine mussel, *Mytilus edulis* after pre-exposure history to mixture of pollutants was a sign of synergistic effect of the pollutants (Sheir et al., 2013).

In the present work, Na was not affected in tissues by the exposure to PMR. Beadle, (1969) discussed the possible role of the envelope and capsular membranes of the egg masses which separate the eggs from the external water are freely permeable to water and inorganic ions in *Biompalaria sudanica* and *Lymnaea stagnalis* embryos. Greenaway, (1970) discussed that Na concentration in the adult freshwater snail, *Limnaea stagnalis* followed the concentration of the external medium.

In the present investigation, LPO was increased significantly in the exposed snails than the control group. Increased lipid peroxidation (malonaldehyde) was a sign of exposure to a mixture of Cu and thiram (fungicide) in the gills and the digestive gland of the freshwater bivalve, *Unio tumidus* (Doyotte et al., 1997). Barata et al. (2005) recorded increase in lipid peroxidation and lipofuscin pigment in *Daphnia magna* juveniles after exposure to sublethal levels of menadione, paraquat, endosulfan, cadmium and copper for 48 h. Sheehy, (1992) reported that peroxidation of the cell components can cause increase of lipofuscin pigments in the crayfish, *Cherax quadricarinatus* brain. In addition, Oxidative stress increase accumulation of lipofuscins as mentioned by Brunk and Terman (2002). In the present work, lipofuscin pigments were detected in the control and exposed snails` digestive gland. Many studies were done on the lysosomal activity in the digestive glands of molluscs. Lipofuscin deposition was used as indicative of organic contaminant exposure revealing a general response to pollution in molluscs (*Mytilus galloprovincialis*, Marigomez and Baybay-Villacorta, 2003). The lysosomal system activity was limited altogether with raised lipofuscin inclusions and reduced amount of lipids in early winter of the mussel, *M. galloprovincialis* (Koukouzika et al., 2009). The presence of lipofuscins in the control digestive gland could be attributed to the unfavourable winter season that animals might be subjected to where they used their lipids contents as speculated by Koukouzika et al. (2009). Necrosis and degeneration of the digestive tubules were the major lesions of the exposed snails in the current study. Sharaf et al. (2015) recorded alteration in the histology and total lipids and protein of the digestive gland of the Land Snail after exposure to Methiocarb and Chlorpyrifos. Oxidative stress leads to lysosomal instability and causing apoptosis due to release of lysosomal contents into the cell, and might cause cell aging (crayfish, Sheehy, 1992; cell culture, Brunk and Terman, 2002). Koukouzika et al. (2009) suggested that digestive gland was the site of lipid storage. Marzabadi and Jones (1992) discussed the cause of lipofuscin pigments increase in metal exposed rat myocytes

as (1) inhibition of lysosomal activity, (2) increased damage due peroxidative reactions, or (3) general toxicity with increased autophagy or transformation of lysosomal lipids to lipofuscin via lipid peroxidation (Dimitriadi et al., 2004). In the present study, the number of digestive tubules (20 x image) in the exposed snails to 200 μl/l was significantly higher than 600 μl/l exposed snails. That can be attributed to the fusion between the tubules which made it bigger in volume and lower in number. However, the number of the exposed snails to 200 μl/l was higher and in 600 μl/l exposed snails was lower than the control group. That could be due to the smaller tubules in the former, fused and large tubules in the later.

In conclusion, different investigated organs of *L. carinatus* accumulated metals from declorinated tap water of the experiment with/without the effect of Caselio, the plant fertilizer and depurated others. Therefore, *L. carinatus* is a good organism for detection of metals in drinking tap water. Caselio, (PMR) caused oxidative stress (lipid peroxidation) and increased induction of lipofuscins (aging-pigment) in the digestive gland of *L. carinatus* which is a biohazard for organisms health.

5. References

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