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Biological investigations on the freshwater snail *Pirenella conica* (Blainville, 1829) infected with the developmental stages of *Heterophyes* sp.

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Abstract

Background: Heterophyiasis is an intestinal sickness promoted by infection with the heterophyid digenetic worms. It is one of the major infectious diseases of public health in the developing countries.

Method: Single-cell gel eletrophoresis, or comet assay, was carried out for detecting DNA damage in the digestive gland cells of *Pirenella conica* infected with *Heterophyes* larvae. Besides, apoptosis, some isoenzymes, and two biogenic amines (neurotransmitters) were investigated using the flow cytometric analysis, the starch gel electrophoresis, and the HPLC techniques respectively. Snails were collected from brackish water area around Port Said province during the spring–summer periods of 2012–2013.

Results: The results showed that infection with the larval trematodes increased tail length (length of DNA migration) in the digestive gland cells of infected snails. Meanwhile, the percentage of apoptosis was significantly elevated (58.80%) in the snails infected with the larval trematodes as compared to that of uninfected snails (39. 59%). Apparent polymorphism was detected in the four enzymes obtained from the digestive gland extracts.

Conclusion: DNA damage and increase of apoptosis in the digestive glands of infected snails may end up with a decrease of 5-HT (serotonin) and DA (dopamine) concentrations in all tissues through the course of infection.

Keywords: Comet assay, Isoenzymes, Neurotransmitters, *Pirenella conica*

Background

Heterophyiasis is an intestinal illness initiated by infection with the heterophyid digenetic flukes. It is one of the main infectious diseases of public health and socio-economic position in the developing world (Pica, Castellano, Cilia, & Errico, 2003; Massoud, El-Shazly, & Morsy, 2007 and Youssef & Uga, 2014). The freshwater mollusk *Pirenella conica* represents the intermediate hosts for many species of trematodes, including representatives of the heterophiidae family–Heterophyes. Freshwater snails are not inert hosts for trematodes, as they have an interior system that can identify, control, and remove pathogens (Ataev & Coustau, 1999; Coustau, Gourbal, Mitta, & Adema, 2009). A better understanding of the physiology of vector snails may offer an effective tool in biological control (Abou-El-Naga & Radwan, 2012). The interior defense system is a reason that influences the vulnerability form of the snails. Of all the aspects of snail biology affected by parasitic infection, reproductive success has undoubtedly been of great importance within the field. The biogenic monoamines is one class of molecules that characterizes a probable object for parasitic castration. These molecules show a wide variety of controlling functions in mollusks affecting on almost all physiological systems, comprising reproduction. Serotonin (5-hydroxytryptamine or 5-HT) and dopamine (DA) are amongst the furthermost extensively investigated monoamines (Bai, Johnston, Watson, & Yoshino, 1997; Manger, Li, Christensen, & Yoshino, 1996; Boyle & Yoshino, 2002). Apoptosis is vital for the performance of the immune

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system of mollusks as shown by the apoptosis image detected in circulating digestive gland cells in infected cluster. Accordingly, the antiapoptotic factors show a part in host protection against parasites by restraining the pathogen feast while preventing inflammatory cell injury. Barcinski and DosReis (1999) revealed that control of host cell apoptosis is a serious factor in host–parasite interactions. Unfortunately, studies using the single-cell gel electrophoresis (the comet assay) to measure the special influences of parasitic infection on the DNA damage are relatively lacking. Starch gel electrophoresis of isozymes is a well-recognized technique for detecting genetic polymorphism (Tanksley & Orton, 1983; Soltis & Soltis, 1989; Wang, 1994).

Methods

Collection and maintenance of snails

A total of 1250 freshwater *Pirenella conica* snails, the intermediate host of *Heterophyes* spp., were collected from brackish water area around Port Said province using a hand wire mesh scoop (Araschewski, 1985 and Abou El-Azm, 1991) during spring–summer periods of 2012–2013. They were brought to the laboratory and maintained in separate aquaria containing freshwater and aquatic plants. The snails were successively examined for natural trematode infection by exposure to artificial light at 28 °C for cercarial shedding. Shells of the snails were removed, and the soft tissues were separated into the cephalopedal mass and digestive gland. The fresh tissues were weighed, divided into two groups, and maintained at − 10 °C until use.

Flow cytometric analysis

Preparation and staining of cells

Digestive gland sample was washed through the gauze with Tris/EDTA buffer (pH 7.5) [3.029 g of 0.1 M Tris-(hydroxymethyl-aminomethane), 1.022 g of 0.07 M sodium chloride, and 0.47 g of 0.005 M EDTA]. They were dissolved in 250 ml of distilled water, and then, the pH was adjusted at 7.5 by using 1 N HCl. The cell suspension was centrifuged at 108.67*g* for 10 min whereupon the suspension was aspirated. After centrifugation and aspiration of the supernatant, the cell was fixed in ice-cold 96–100% ethanol approximately 1 ml for each sample. The fixed cells can be stored indefinitely in a refrigerator and can also be mailed without running the sample. Preparation of propidium iodide stain, 0.1 g sodium citrate, 0.1 ml Triton X-100, and 5 mg propidium iodide were dissolved in 100 ml distilled water. The cells were stained according to Nicoletti et al. (2001). Briefly, the tubes containing digestive gland cells were centrifuged at 105.67*g* for 10 min and the supernatant was discarded. The sample was washed with 2 ml phosphate-buffered solution (PBS) and again centrifuged at 105.67*g* for 5 min, and the supernatant was discarded. The cells were re-suspended with PBS, and 200–500 μl was taken from the sample into the polystyrene tube. 1.5 ml of propidium iodide staining solution was added, and the tube was incubated for 1 h at 4 °C in dark place until acquisition. Then, the sample was run in the flow cytometer within overnight after the addition of propidium iodide to analyze the sub-G_1 of apoptosis.

The *flow cytometry* used at Mansoura University Hospital for children is FACSCalibar flow cytometer (Becton Dickinson, Sunnyvale, CA, USA) equipped with a compact air-cooled low-power 15 mW Argon-ion laser beam (488 nm). The average number of evaluated nuclei per specimen was 20,000, and the number of nuclei scanned was 120 per second. DNA histogram derived from flow cytometry was obtained with a computer program for Dean and Jett mathematical analysis (Dean & Jett, 1974). Data analysis was conducted using DNA analysis program MODFIT (verity software house, Inc., version: 2.0 power Mac with 131,072 KB registration no. 42000960827-16193213). This software calculated the CV around the G_0/G_1 peak and the percentage of cells in each phase (G_0/G_1, S, and G_2/M) of the DNA cell cycle for each sample.

Apoptosis was measured by using the sub-G_1 peak staining with iodide (Cohen and Al-Rubeai, 1995). An aneuploid cell population was considered to be present if a distinct peak, in addition to the G_1 diploid peak, was found in position deviating more than 10% from the diploid.

The single-cell gel electrophoresis (comet assay)

The removed digestive glands were prepared according to the technique used by Sasaki, Nishidate, Izumiyama, Matsusaka, and Tsuda (1997).

Starch gel electrophoresis for isozymes

The investigated tissues were macerated in their specific extraction solution. The extract was then filtered. The electrophoresis was performed in approximately 4 h; during this time, the front migrated 8 to 8.5 cm from the origin. The staining reaction was usually stopped by rinsing the gels with tap water several times. Gels were then fixed in 50% glycerol overnight, wrapped in plastic film, and stored in the refrigerator until photographed, or dried and stored afterwards.

Determination of the tested monoamines

The dissected frozen soft parts were put in the Eppendorf tubes and weighed. Then, each tissue was homogenized with an ultrasonic cell disrupter in 150 μl 0.1 M perchloric acid inclosing 0.4 mM sodium metabisulfite. The homogenates were later centrifuged at 10,000*g* for 25 min at 4 °C, and the supernatants were filtered via a

Table 1 Digestive gland P53%, BCl2%, and apoptosis % (sub-G_1) in uninfected (control) and infected snails

Flow	Groups	Uninfected (control)	Infected	ANOVA
Mean ± SE	P53%	39.82 ± 1.4	74.49 ± 3	$P \leq 0.05$
Mean ± SE	BCl_2%	65.35 ± 2.6	39.31 ± 2.4	
Mean ± SE	Apoptosis %	39.59 ± 1.5	58.80 ± 2.1	

0.2-μm filter and frozen at − 70 °C until analysis. After that, 5 μl of filtrates was injected into the HPLC system. The mobile phase contained 0.15 M sodium dihydrogen phosphate, 0.1 mM EDTA, 0.5 mM sodium octanesulfonic acid, 10–12% methanol (*v*/*v*), and 5 mM lithium chloride. The mobile phase was accustomed to pH 3.4 with phosphoric acid, filtered over 0.22-μm filter, and degassed with helium. A column temperature of 32 °C and a flow rate of 1.4 ml/min were used.

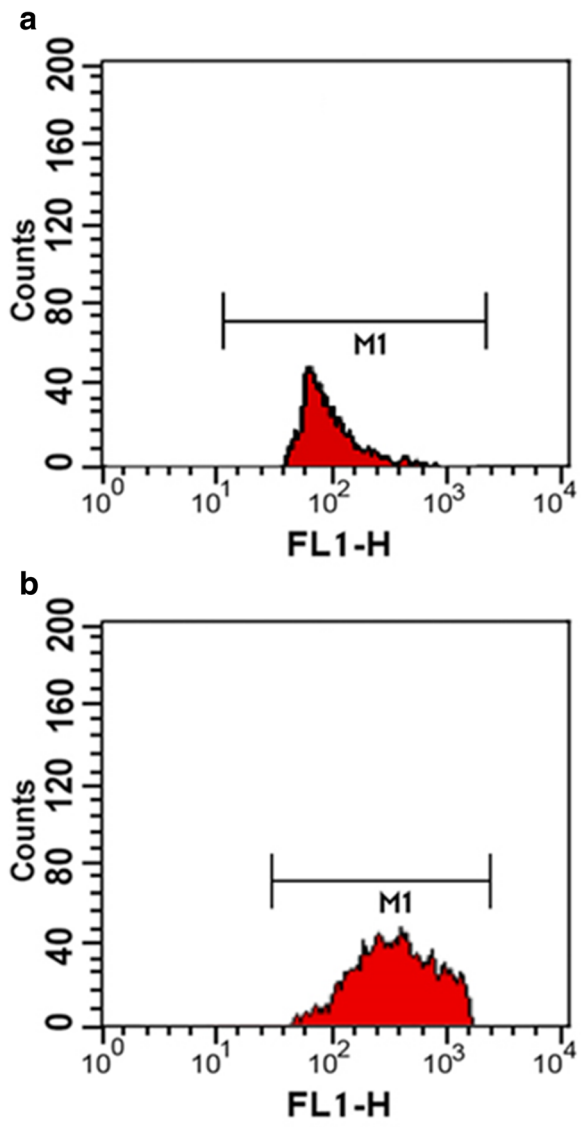

Fig. 1 Digestive gland P53% in uninfected (**a**) and infected (**b**) snails. M1, percent of gated cells stained with +ve P^{53}

Statistical analysis

The results obtained in the current study were assessed by one-way ANOVA test, and post comparison was supported by a Duncan test. The data were stated as means ± standard error ($X \pm SE$). The values of $p \leq 0.05$ were reflected statistically significant (Snedecor & Cochran, 1982).

Results

Flow cytometric analysis of cell cycle

Table 1 and Figs. 1, 2, and 3 illustrated digestive gland P53%, BCl2%, and apoptosis % (sub-G_1) in uninfected and infected snails. Results illustrated that the levels of apoptosis % were significantly elevated (58.80%) in the snails infested with the trematode larvae as compared to that of uninfected snails (39.59%). On the other hand, the data indicated pronounced elevation in the P53% (74.49%) and decrease in the BCl2% (39.31%) in the infected snails compared with those of the control (uninfected) snails which were 39.82% and 65.35% respectively.

The single-cell gel electrophoresis (comet assay)

From Table 2 and Fig. 4 (1, 2), infection with the larval trematodes increased tail length (length of DNA migration) in the digestive gland cells which exhibited numerical increase of apoptotic cell death.

Starch gel electrophoresis for isozymes

Using the starch gel electrophoresis, characteristic isozyme fingerprints of four enzymes were obtained from the digestive gland extracts of uninfected and infected *Pirenella* snails, and a number of differentially expressed fragments of the digestive gland were identified. All bands migrated anodally. They were labeled in descending order toward the cathode. Apparent polymorphism was detected in the four enzymes used (Fig. 5).

Isoenzymes in acid phosphatase (Ac Ph) have great possibilities of not being significantly different from one another. Three isozymes of glucose-6-phosphatase (G-6-ph) were recognized with difference in the third band. Differences between alkaline phosphatase (Alk Ph) and lactate dehydrogenase (LDH) isozymes could be found with a loss of the first and the fourth isoenzymes, respectively.

Differential expression was verified using UPGMA analysis, which was shown to be a valuable method for this purpose. The dendrogram calculated the similarity between the tested isozymes. The results indicated a similarity of 77.78, 70.00, 58.82, and 80.00% between the isozymes of G-6-Ph, Ac Ph, Alk Ph, and LDH, respectively (Fig. 6).

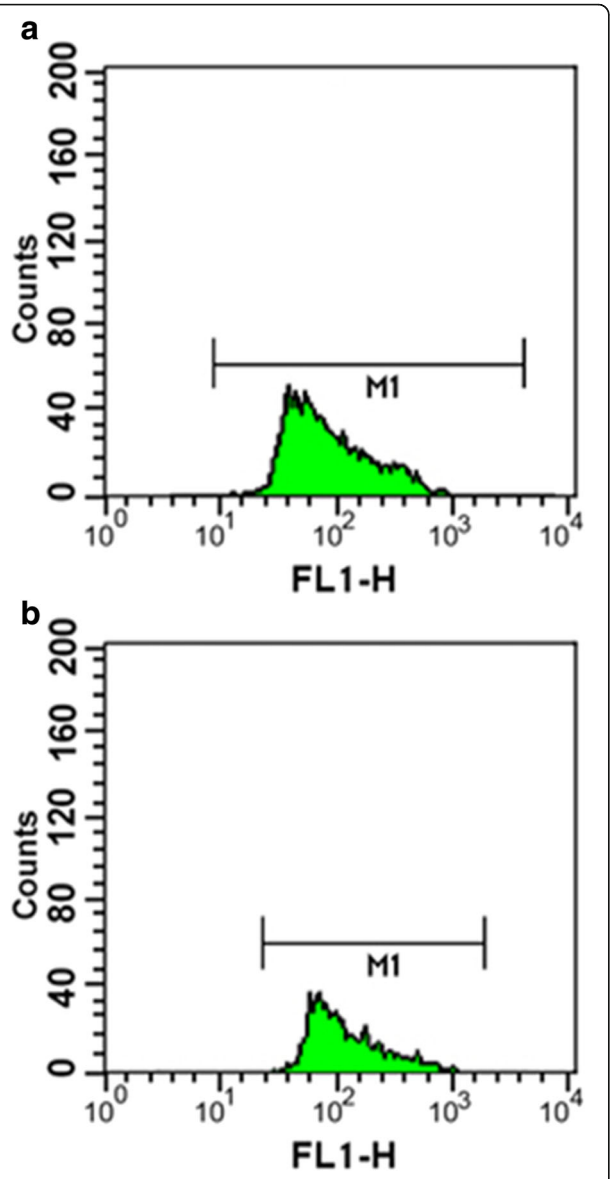

Fig. 2 Digestive gland BCl_2% in uninfected (**a**) and infected (**b**) snails. M1, percent of gated cells stained with +ve BCl_2

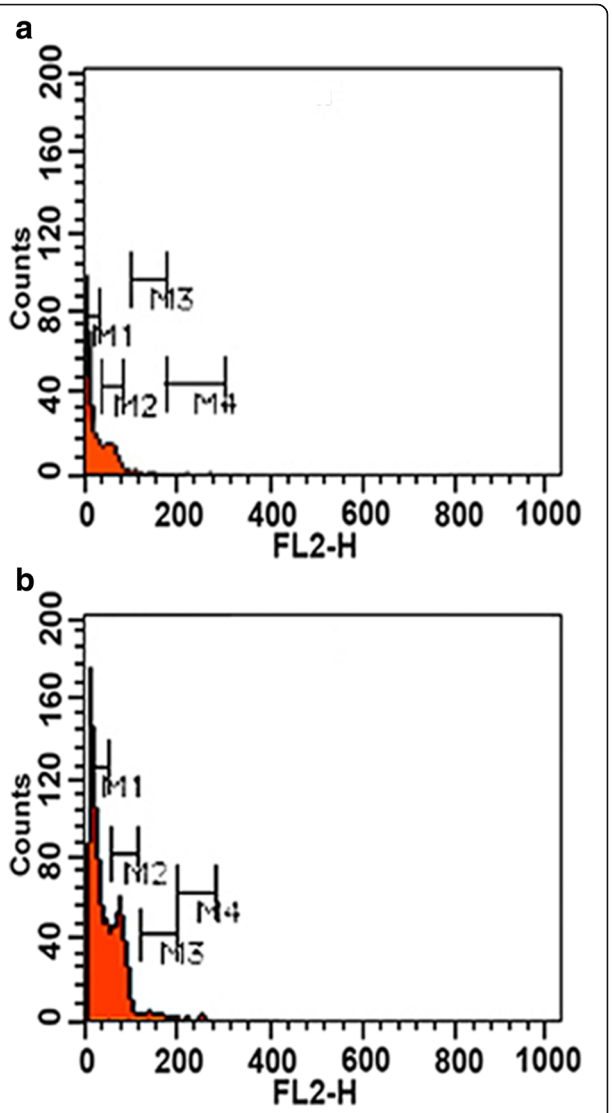

Fig. 3 Digestive gland apoptosis % (sub G1) in uninfected (**a**) and infected (**b**) snails. M1, percent of apoptosis

Determination of the tested monoamines

Two biogenic amines, dopamine (3,4-dihydroxytyramine or DA) and serotonin (5-hydroxytryptamine or 5-HT), were recognized and measured by HPLC in homogenates of snails' soft parts. Chromatograms from HPLC analysis of separate tissue samples were reliable in displaying peaks for these two biogenic amines (Fig. 7). They were recognized by their coalition with reliable amine standards and evaluations of retaining times in standard buffer and electrode potential circumstances.

The levels of DA in the soft parts were comparable between *Heterophyes* infected and uninfected *P. conica* (Table 3). Significant decreases in dopamine relative to control were detected.

The overall effect of infection was a decrease of 5-HT content in all tissues through the infection course.

Discussion

The obtained results showed that the overall number of apoptotic cells in infested snails was significantly increased than that of control ones. This result may be

Table 2 Comet assay of apoptotic digestive gland of both uninfected control (C) and infected (I) snails

Group	% Tailed		% Untailed		Tail length (μm)		% Tail DNA		Tail moment (unit)	
	C	I	C	I	C	I	C	I	C	I
Mean	5	17	95	83	1.49	3.56	1.59	3.20	2.368	11.298
±SE	0.3	0.5	0.3	0.5	0.07	0.37	0.06	0.07	0.04	0.3

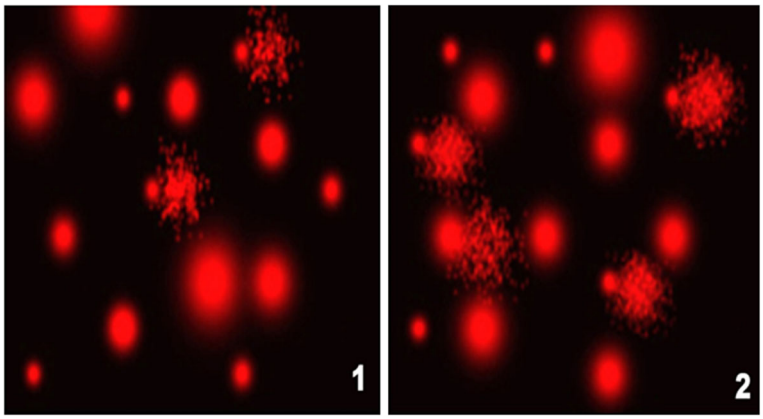

Fig. 4 Comet assay of the digestive gland cells of the uninfected (**1**) and infested snails (**2**). Note DNA migration and increased stretching apoptotic cells in infected individuals

due to the decline in lysosomal stability initiated by reactive oxygen species (ROS). Under conditions affecting oxidative stress, the lysosomal membrane may well rupture leading to the discharge of enzymes into the cytoplasm (Cheng, 1983; Öllinger & Brunk, 1995; Zdolsek, Olson, & Brunk, 1990; Winston, Moore, Kirchin, & Soverchia, 1996; Zhao, Antunes, Eaton, & Brunk, 2000). Thus, lysosomes have been assumed as being responsible for the death of cells: the leak of lytic enzymes into the cytosol might cause degenerative changes and initiate cell death. Apoptotic processes are characterized by many morphological features, which have been stated by Sweet, Passino-Reader, Meier, and Omann (1999). Furthermore, Romero, Estévez-Calvar, Dios, Figueras, and Novoa (2011) stated that the adequate clearance of damaged, senescent, and infested cells without inflammation are supported by apoptosis. It is well known that the molluscan immune system is based on innate mechanisms, whereas humoral and cellular processes approve cytotoxic and antimicrobial roles (Auffret, Rousseau, Boutet, Tanguy, & Baron, 2006).

Outcomes of the present study is in agreement with former studies of biogenic amines in freshwater gastropods (for example, Hetherington, McKenzie, Dean, & Winlow, 1994; Ottaviani, Caselgrandi, Petraglia, & Franceschi, 1992). Chiang, Bourgeois, and Beuding (1974) separated and identified dopamine and 5-HT in numerous tissues of *Balanus glabrata*, plus the ganglia, cephalopedal area, and mantle. To evaluate the role of bioamines in controlling molluscan reproductive activity, the effect of trematode larvae infection on reproduction of host and bioamine concentration was of real interest. Infection of parasite could cause major depressions, as compared to matched controls, of biogenic monoamine contents in the soft parts. Parasitic induction for depressed DA and 5-HT levels can be supported by several mechanisms. A large population of parasites mount

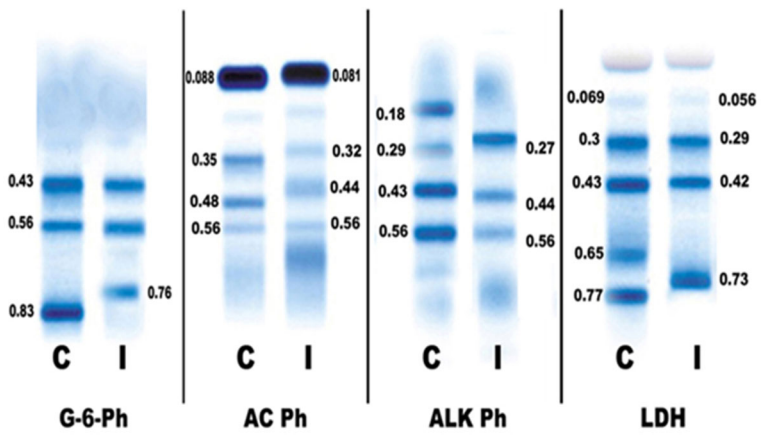

Fig. 5 Mean gels showing positions of bands electrophoresed on both uninfected control (C) and infected (I) groups stained for G-6-Ph, Ac Ph, Alk Ph, and LDH

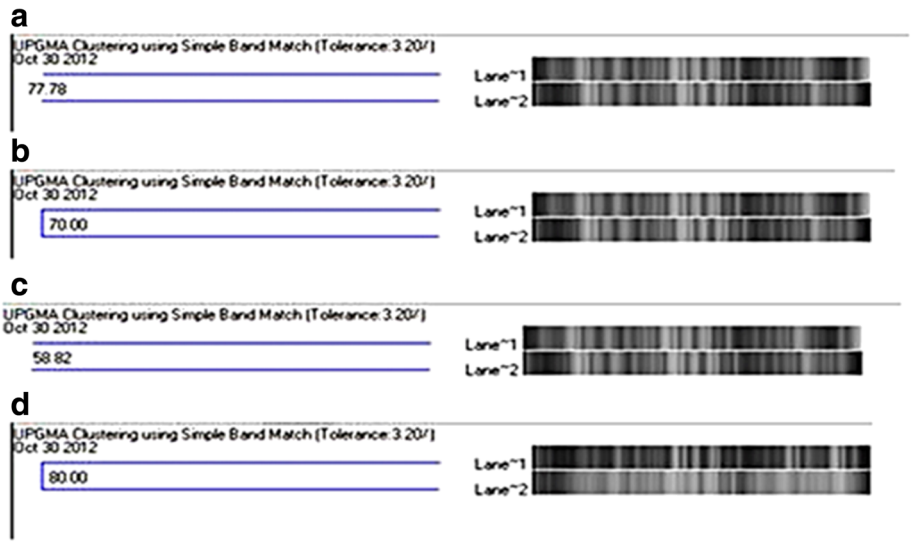

Fig. 6 a–d Similarity dendrogram resulting from analysis of isozymes bands of both uninfected and infected groups

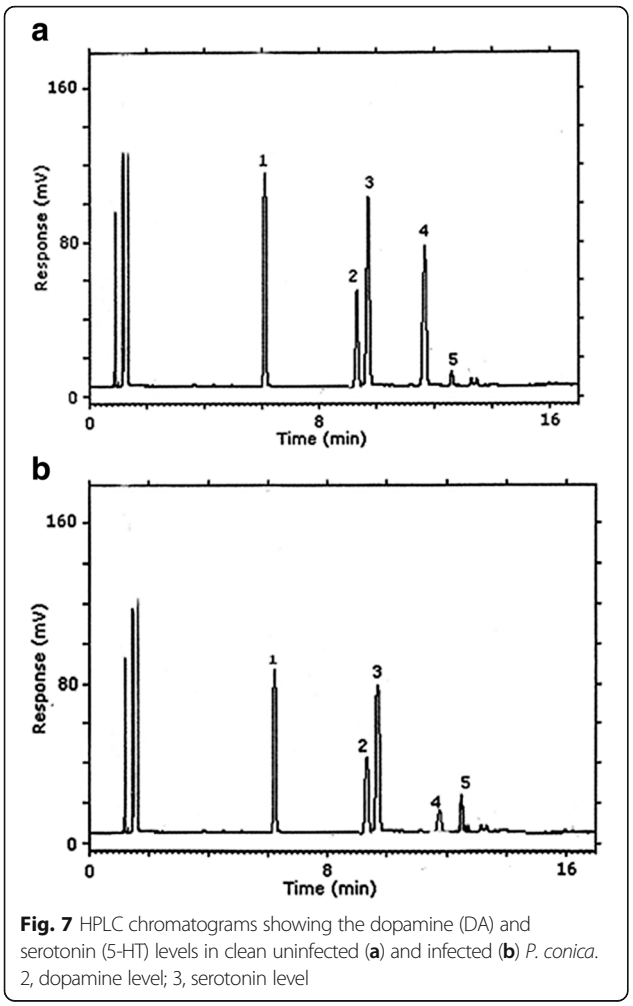

Fig. 7 HPLC chromatograms showing the dopamine (DA) and serotonin (5-HT) levels in clean uninfected (**a**) and infected (**b**) *P. conica*. 2, dopamine level; 3, serotonin level

up in the snail after a fairly short period time of the asexual reproduction of the parasite. So, a progressive decrease of neurotransmitters in the plasma and later in infection and in amine-producing tissues may be expected. Then, an extensive physical or pathophysiological disorder of the snails' aminergic system or secretion by parasite-derived molecules or specific interference of host bioamine production can be caused by parasites. On the other hand, Chiang et al. (1974) indicated that they were unable to identify any effects of infection of schistosome larvae on snail biogenic amine contents, although no records were offered to support this statement. This identified difference may reflect the relative sensitivities of the applied techniques, though other explanations including variances in sample analysis, age of the host at the time of infection, preliminary infection dose, and the accurate timing of castration might have been causative factors.

In summary, a prominent class of biomolecules that role in many physiological processes of mollusks, including reproduction, is characterized by biogenic monoamines. Hence, these molecules represent a probable objective for parasite manipulation of host reproductive activity. The present investigation verifies a damage of 5-HT and dopamine in the tissues of *Pirenella* through

Table 3 Effect of *Heterophyes* larvae infection on dopamine (DA) and serotonin (5-HT) in clean uninfected (C) and infected (I) *P. conica*

Group	DA		5-HT	
	C	I	C	I
Mean	55.68	42.45	105.06	81.51
±SE	0.7	0.7	0.7	0.8

infection by *Heterophyes* larvae that is linked with the start of parasitic castration. As endogenous serotonin is inhibited in *Pirenella* via infection by trematode, it has a stimulatory effect on reproductive processes in these snails. Finally, parasitic castration may be intermediated in the snail by the parasitic manipulation of host serotonin concentrations.

The performed study has revealed that invasion with cercariae of trematodes *Heterophyes* lead to the statistically significant increase of DNA fragmentation and migration in the molluscan tissues as compared with control. This was in agreement with Mohamed (2011) who found that flow cytometric analysis of cell cycle and comet assay caused DNA damage in *Biomphalaria alexandrina* hemocytes exposed to *Schistosoma mansoni* infection when compared to control group.

There is an unlucky inclination in the study of Johnson (1973) to propose that multiple electrophoretic bands establish straight indication of heterozygosity at a protein locus. Breeding investigates identical to those carried out by Oxford (1973) and Brussard and McCracken (1974) are best confirmed by genetic relationships. Several cases of isoenzyme variation were documented to be due to molecular instability (Beardmore, 1970).

There are still many aspects to be investigated in host–trematode interactions. To our knowledge, this is the first description of the changes and modification of metabolic interactions in the *Pirenella–Heterophyes* model.

Conclusions

In conclusion, DNA damage and increase of apoptosis in the digestive glands of infected snails may end up with a decrease of 5-HT (serotonin) and DA (dopamine) concentrations in all tissues through the course of infection.

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Availability of data and materials
The datasets generated and analyzed during the current study is available from the corresponding author on a reasonable request.

Authors' contributions
ESR wrote the manuscript, contributed to the design of the study, and carried out the data analysis. SH coordinated the design of the study and was responsible for the execution of the experiment. SAE helped to write the manuscript. FAR contributed to the design of the study, contributed to the literature review, and critically revised the manuscript. Finally, AR provided the idea for the study, coordinated the study, and critically revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The authors were asked to participate in a satisfaction survey on a voluntary basis. Participants were informed that this survey was part of a research project. This study was approved by the Social Science Ethical Committee of Faculty of Science, Tanta University and Research, and complied with the Egyptian Code of Conduct for Scientific Practice, National institute of research, Egypt.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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