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Manipulated Oviposition in Fresh Water Snail *Lymnaea acuminata* Through Hydrogen Peroxide (H_2O_2)

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Abstract: Eyestalk ablation in *Lymnaea acuminata* causes a burst of oviposition, which gradually tapers off. The present study demonstrated restoration of oviposition in ablated individuals and increased oviposition in their non-ablated counterparts, following Hydrogen Peroxide (H_2O_2) injection. Both processes were accompanied by an increase in protein synthesis (evidenced by higher level of DNA, RNA and protein) and decrease in free amino acids (AA) level in ovotestis. It was hypothesized that while eyestalk ablation acting through the caudo darsal cells (CDC), H_2O_2 might stimulate oviposition through prostaglandins (PGs) via PG endoperoxide synthatase.

Key words: Oviposition · *Lymnaea acuminata* · H_2O_2, Ablation

INTRODUCTION

A masculinezing factor from the tentacles that stimulates spermatogenesis while inhibiting oogenesis has been reported in various studies [1]. Runham *et al.* [2] observed optic tentacles as a source of androgenic factor which helps in differentiation of male sex cells in stylommatophorans. In these studies it was argued that ablation might trigger off neurohormonal system which further controls reproduction. In the slugs *Deroceras retczrlatus* and *L. flmus*, injection of estrogens stimulated egg-laying, but with a lower rate of embryonic development, while androgens increased the rate of embryo development without affecting egg-laying [1]. Further, studies on endocrine control of reproduction confirmed the role of specific neurohormones in preparation of egg mass and ovulation in snails [3, 4].

Increased rate of spawning after eyestalk ablation in *Lymnaea acuminata* have been demonstrated [5, 6], which gradually tapers off within six days. However, how to overcome this tapering was not investigated. Here restoration of spawning in ablated *Lymnaea acuminata* following H_2O_2 injection have been tried. Role of H_2O_2 alone or in combination with PGs has been a subject of study in snails. Morse *et al.* [7] demonstrated H_2O_2 induced spawning in *Haliotis rufeccence*, stimulates the synthesis of prostaglandin endoperoxide synthetase, which in turns raises the endogenous level of prostaglandin. Similarly, Singh and Agarwal [8] observed increased rate of ovulation in *Lymnaea acuminata* with PGE_1 and $PGE_2\propto$, which was as effective as H_2O_2 [9]. In other system also H_2O_2 has been implicated in several reproductive events [10]. It is because of constant production of H_2O_2 by the cells, which exist free in system [11]. H_2O_2 is known to have effect on lipolysis [12], inflammation and collagenase activation [13], protein phosphorylation [14], carbohydrate metabolism [15], steroid hormone production [16], DNA repair [17], Opening of calcium channel [18] and membrane potential coupled with PGs production [19]. Since, more or less all these events are related to the process of ovulation [10, 20, 21], therefore, H_2O_2 could directly or indirectly participate in ovulation by engaging in these processes. In this study, effect of H_2O_2 on ovulation in ablated and non-ablated *Lymnaea acuminata* was investigated on the profiles of DNA, RNA, protein and free amino acids.

MATERIALS AND METHODS

Adult snail (*L. acuminata;* 2.60±0.30 cm. in length) collected from local fresh water pond were first kept in glass aquarium (10 L) for 72h acclimatization and later, divided into four groups (A, B, C and D) in different aquarium, each containing 20 individuals.

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A solution of H_2O_2 of desired strength was prepared in distilled water. All injection (0.05ml) was given in foot of the snails with a micrometer syringe. Control received an equal amount of distilled water.

Group A (non-ablated) remained untreated through out the experiment and served as control. Group B (non-ablated) also left untreated for the first 3 days, but later injected with H_2O_2 (9µg/snail/day) for 3 subsequent days. Group C (ablated) remained untreated for six days. Group D (ablated) was treated similar to group B. Result were expressed in mean±SE. Analysis of variance, Newman-Knaul's test and student t-test [22] were used for locating significant differences.

FERTILITY AND DEVELOPMENT

Spawn of *L. acuminata* are laid in the form of gelatinous ribbon, each consisting of 5-120 eggs. Spawns were collected every 24h from each group, counted and transferred to petri dishes containing dechlorinated tap water. Later, petri dishes were incubated at 30°C for 10 days. At every24h spawns were observed for hatching and survival of embryos. Dead embryos (opaque, lack movement) were discarded.

Ablation of Eye-Stalk: Each individual snail was gently picked out of the aquarium and both eye stalk were quickly snipped off with a pair of iris scissors and were placed back to aquarium.

Biochemical Estimation: Ovotestis was taken out. Adherent tissue were cleaned and processed for following estimation.

Nucleic Acids: Homogenates of ovotestis (1.0 mg/ml; w/v) in 10% of TCA were prepared and centrifuged at 5000g for 20 min. DNA and RNA were estimated in supernatants by using diphenylamine and orcinol [23].

Protein: Homogenates of ovotestis (1.0 mg/ml; w/v) were prepared as above and centrifuged at 6000g for 20 min. Estimations was made in supernatant by using BSA as standard [24].

Amino Acids: Homogenates of ovotestis (10 mg/ml; w/v) were prepared in 90% of ethanol and centrifuged at 6000g for 20 min. Estimations was made according to the method of Spies [25].

RESULTS

Over a period of six days, a group 20 non-ablated individuals normally laid 150 eggs/day, at an average of 6 spawns/day. This number increased multifold after first 24h of ablation.

In the present investigation non-ablated individuals (Group, A) laid 58% of the total normally laid eggs. Group, B showed a significant increase ($p<0.05$) in number after 72h of injection, which was persisted upto 144h. Group, C did not showed any response. Group, D, on the other hand showed 218% of increase in comparison to Group, A within 24h of injection. However, this trend was not continued on 120 h and 144 h despite every 24h injection and a decline of about 52% of control was observed. Non injected –ablated counterparts on the other hand laid 38 and 19% eggs at 96 h and 120 h, respectively and finally stopped laying at 144h (Table 1). Analysis of variance justifies the effect of treatment which was significantly ($P<0.05$) different on all days.

Endogenous level of DNA was in between 65 to 68 µg/mg of ovotestis which, significantly ($P<0.05$) increased in group-B to 131% compared to controls at four days after ablation. This, however, declined to 126 and 118% at five and six days after ablation with compared to control, respectively (Table 2). Endogenous level of RNA was in between 47 to 50 µg/mg of ovotestis which also significantly ($P<0.05$) increased in group-D to 118% compared to control at four days after ablation. However, it decreased to 89% and 81% on five and six days, respectively (Table 2).

Level of protein increased to 125% compared to control at four days after ablation but went on decreasing continuously five and six days of group-B (H_2O_2 treated snail) (Table 3). Level of free amino acids levels which was between 29 to 30 µg/mg of ovotestis, a continuous increase of 179, 199 and 222% was observed at four, five and six days, respectively after ablation compared to control (Table 3). Two way analysis of variance showed that ablation brought about significant ($P<0.05$) changes in the level of protein and free amino acids during the period of treatment.

H_2O_2 effect was also significant ($P<0.05$) in non-ablated normal individuals where decrease in the level of DNA was 131, 126 and 118%; RNA 127, 112 and 102% and of protein 125, 111 and 108% observed at 96, 120 and 144h of H_2O_2 injection compared to their respective controls, except free amino acid levels which increased to 84, 92 and 96% at same hours and treatment

Table 1: Effect of hydrogen peroxide (9 µg/snail/day) on oviposition in *Lymnaea acuminata* 72 hrs after ablation of eyestalk

	Control (No ablation) Group A		9 µg/snail/day H_2O_2 given to snailsGroup B		Ablated Group C		9 µg/snail/day H_2O_2 72 hrs after ablation Group D	
Hours after ablation	No. of spawns	No. of eggs	No. of spawns	No. of eggs	No. of spawns	No. of eggs	No. of spawns	No. of eggs
24	7.33±0.28 (100)	150.66±5.37 (100)	7.33±0.23 (100)	150.66±5.37 (100)	18.33±0.91(257)	511.16±10.10 (351)	18.33±0.91 (257)	511.16±10.01 (351)
48	5.83±0.18 (100)	148.66±3.32 (100)	5.83±0.18 (100)	148.66±3.32 (100)	6.5±0.54(111)	162.66±8.49 (110)	6.5±0.54(111)	162.66±9.49 (110)
72	7.00±0.28 (100)	131.32±1.25 (100)	7.00±0.28 (100)	131.32±1.25 (100)	3.83±0.33 (55)	87.50±6.90 (66)	3.83±0.33 (55)	87.50±6.90 (66)
96	6.33±0.23 (100)	126.50±4.77 (100)	8.33±0.46* (131)	303.00±7.85* (207)	2.50±0.37* (39)	51.83±5.22+ (38)	9.00±1.26 (142)	319.16±8.27* (218)
120	6.33±0.23 (100)	143.83±9.31 (100)	7.85±0.33* (135)	229.00±5.78* (159)	1.66±0.36* (28)	28.66±3.26* (19)	5.00±0.28 (86)	161.50±4.50 (110)
144	6.16±0.18 (100)	140.00±3.29 (100)	6.66±0.83 (114)	172.33±2.16 (118)	Nil	Nil	2.66±0.23* (46)	76.50±9.24* (52)

Each value represents Mean ±SE of six replicates. Non-ablated (B) and ablated snails (D) were injected with H_2O_2 (9 µg/snail/day for 3 days) three days after the day of ablation, Control (A) and ablated (C) did not receive H_2O_2

Values in parentheses indicate percent change with controls taken as 100%

*- Significantly different (P<0.05) from controls when t-test was applied

+- Analysis of variance (P<0.05) demonstrated that the effect of treatment was significant on all days

Table 2: Changes in levels of DNA and RNA (µg/mg ovotestis) after eyestalk ablation and H_2O_2 injection in the snail *Lymnaea acuminata*

	Control (A) No ablation		9 µg/snail/day H_2O_2 injected to un-ablated snails (B)		Ablated (C)		9 µg/snail/day H_2O_2, 3 days after ablation (D)	
Days after ablation	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA
Four	67.59±2.13^a (100)	47.77±1.61^{ab} (100)	88.85±2.21* (131)	60.58±2.28* (127)	24.65±2.13* (36)	18.19±0.92* (38)	83.88±3.03* (124)	56.18±2.15* (118)
Five	65.64±1.02^a (100)	49.75±1.82^{ab} (100)	82.90±1.66* (126)	55.61±0.35* (112)	16.41±2.11* (25)	12.31±1.57* (25)	79.95±2.44* (122)	44.09±1.40* (89)
Six	66.22±1.73^a (100)	48.50±1.87^{ab} (100)	78.06±2.54* (118)	49.68±1.72* (102)	14.46±2.20* (22)	7.03±0.74* (14)	49.11±2.54* (74)	39.31±1.44* (81)

Each value represents Mean ±SE of six replicates

Values in parentheses indicate percent change with controls taken as 100%. Experimental details and dose are given as in Table 1

*- Significantly different (P<0.05) from controls when t-test was applied

Effect of Treatment (a) and time (b) significant (P<0.01) when two way analysis of variance was applied

Table 3: Changes in levels of protein and amino acids (µg/mg ovotestis) after eyestalk ablation and H_2O_2 injection in the snail *Lymnaea acuminata*

	Control A No ablation		H_2O_2 treated snails (B)		Ablated (C)		9 µg/snail/day H_2O_2, 3 days after ablation (D)	
Days after ablation	Protein	Amino Acids	Protein	Amino Acids	Protein	Amino Acids	Protein	Amino Acids
Four	82.51±1.46^a (100)	29.88±3.014^a (100)	103.08±1.68* (125)	25.16±5.97 (84)	30.92±1.66* (37)	53.44±1.97* (179)	95.25±1.05* (115)	27.13±4.60 (91)
Five	85.21±2.94^a (100)	29.42±2.59^a (100)	94.38±2.58* (111)	27.04±3.10 (92)	26.49±0.72* (31)	59.44±2.31* (199)	88.76±2.20 (104)	29.82±2.00(101)
Six	82.58±1.76^a (100)	30.78±2.22^a (100)	89.51±2.81* (108)	29.60±2.98 (96)	24.35±0.58* (29)	66.52±2.19* (222)	42.63±0.96* (52)	53.28±1.98* (173)

Each value represents Mean ±SE of six replicates

Values in parentheses indicate percent change with controls taken as 100%

Untreated snails and ablated snails were injected with H_2O_2 (9µg/snail/day) after 72h

Experimental details and dose are given as in Table 1

*- Significantly different (P<0.05) from controls when student t-test was applied

Effect of Treatment (a) and time (b) significant (P<0.01) when two way analysis of variance was applied

(Table 2, 3). In ablated individuals (Group D) H_2O_2 also produced significant (P<0.05) changes in DNA level which increased to 124 and 122% at four and five days, respectively compared to control. However, this increase was not continued as the level decreased to 74% at six days compared to control.

RNA level, on the other hand increased to 118% at four days but decreased to 89 and 81% at five and six days, respectively compared to control (Table 2). It should be noted that in spite of lower level of DNA and RNA at six days, these level were 3 and 6 times higher in Group-C during same period where ablated individuals were not injected H_2O_2. Two way analysis of variance showed that H_2O_2 treatment brought significant (P<0.05) time dependent changes in the level of DNA, RNA, protein and free amino acids.

DISCUSSION

The result of this study clearly showed that ovulation can be significantly enhanced by H_2O_2 at very low concentration in ablated and non-ablated freshwater *Lymnaea acuminata*. H_2O_2 has been shown to stimulate ovulation in prosobranch snail *Haliotis rufescence* [7]

and other aquatic systems [26]. However, the mechanism (s) involved in H_2O_2 induced ovulation is not clear. Some theories have been postulated that H_2O_2 stimulated synthesis of PG endoperoxide synthatase which in turn increases level of PGs [7]. There are some reports whereas H_2O_2 alone or in combination with PGs (synergistically) act on the system. Hsu and Goetz [26] observed that ovulation activator increases *In vitro* ovarian PGs production, through, the PGs thus produced may not mediate the ovulation induced by these agents. Although the function of these PGs is not known, the data indicate second messenger pathway may be involved in the regulation of PGs production during ovulation and spermiation [27].

Singh and Agarwal [5] suggested that increased oviposition in ablated and H_2O_2 injected snail may be governed by caudo-darsal cells (CDC) but whether the CDC themselves are involved, with the synthesis of prostaglandins is also not known [4]. However, it appears that the eyestalks either through the nervous system or a neuroendocrine material inhibit the activity of the caudo-dorsal cells. When the eyestalk is removed the inhibitory effect is abolished and the caudo-dorsal cells through their secretion accelerate ovoposition. It is proposed that the secretion of the caudo-dorsal cells do not act on the gonads directly but through a prostaglandin like substance because it is clear that exogenously administered PGs induces ovulation [7, 8] and H_2O_2 also brought changes similar to PGs [9].

The process of ovulation has been suggested to be type of an inflammatory reaction [20] and a number of compounds directly or indirectly take part in the process without interfering, the system. Ecosanoids compounds (including PGs) play role in inflammation [13] and H_2O_2 induces ecosanoids synthesis by triggering the release of arachidonic (AA) acid a precursor of PGs [28]. The released AA could be subsequently converted to various ecosanoids by cyclooxygenase and lipooxygenase. H_2O_2 has also selective effect on the activities of various lipooxygenase [29]. Thus, it is hypothesized that H_2O_2 may not require PGs but elevated level of PGs any how, in the present investigation also.

Here, increased ovulation in ablated and H_2O_2 injected snails was accompanied by marked changes in the biochemical composition of ovotestis. Thus, DNA, RNA and protein level in ovotestis rose sharply at 24h after ablation. It seems that initial ablation due to some neurohormonal changes [2, 3] speed up the process of rate of spawning [5] in just ablated snails. But after certain hrs or beyond that secretion of nurohormones stopped and spawning gradually tapers off. And H_2O_2 at this juncture might speed up several processes like DNA repair [17], protein metabolism [14], hormone [16] and PGs [19] production, therefore, a rise in the level of DNA, RNA and protein was observed. Because, all these events are directly/ indirectly related to reproduction.

The reason of reduced level of amino acids at the same hour is a matter of justification. This reduction may due to utilization of newly produced amino acids because of increased protein synthesis confirmed at 48 and 72 hrs, when level of protein along with DNA and RNA were lower and amino acids concentration was higher. Injection of H_2O_2 thus increased the level of DNA, RNA and protein and caused a significant reduction in amino acid levels.

On the basis of the above it is proposed that (1) during profuse ovulation level of DNA, RNA and protein was higher, but level of amino acid is lower and (II) during decreased ovulation (tapering off) the level of DNA, RNA and protein is lower and level of Amino Acid is higher. Both processes are the result of increased and decreased protein synthesis, respectively.

Now, how H_2O_2 and endogenous PGs in the above process are associated is explained. It is possible that first, H_2O_2 stimulate PG endoperoxide synthetase to maintain a desired level of PGs and, secondly this newly produced PGs by acting directly on ovotestis or indirectly on related target organs [30] provoke release of eggs, similar to other aquatic systems [26].

It was concluded that ovulation in ablated and non-ablated *Lymnaea acuminata* is stimulated by H_2O_2. This may be at least in part also stimulating PGs production. H_2O_2 in this way be used as cheaper substitute of costly ovulating agents like HCG, Steroids, PGs and Ovaprim [31] and the most favorable thing is that it could be used round the year without special storage (at -20°C) at the own will of fish farmers/aqua culturists for artificial culture/ induced breeding.

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