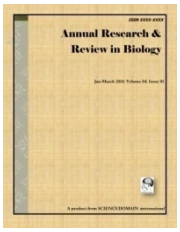

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Influence of Abiotic Factors on Anti-reproductive Activity of Bait-containing Papain in *Lymnaea acuminata*

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Authors' contributions

Author AKS designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors DKS and VKS managed the analyses of the study. Author VKS managed the literature searches.All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To investigate the anti-reproductive action of papain against *Lymnaea acuminata* with respect to abiotic factors. Snail *Lymnaea acuminata* is the intermediate host of *Fasciola* species, which causes endemic fasciolosis in cattle and human population. Fasciolosis is a disease of ruminants worldwide which shows most widespread distribution in comparison to other vector-born parasitic disease.
Study Design: Baits were prepared from starch or serine (20Mm) in 2% agar-agar solution with papain the active component of *Carica papaya* (40% and 80% of 24h LC_{50}). Each regimen of 5 liter water was kept in six aquaria separately, containing 20 snails in each aquarium. Bait containing papain was added in each aquarium except control. Control bait was without papain. After every 24 h, up to 96 h, the total number of eggs laid by the snails was counted in each aquarium. At every 24h spawns were observed for hatching and survival of embryos. Temperature, pH, dissolved oxygen and dissolved free carbon dioxide of different regimen of water were measured, simultaneously. After 96h, the ovotestis and/ or nervous tissue were dissected out and protein [22], free amino acids [23], nucleic acids [24] and enzyme AChE [25,26] activity were then measured.
Place and Duration of Study: Malacology Laboratory, Department of Zoology, DDU Gorakhpur University, Gorakhpur – 273009, U.P., India.The present study was carried

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outinbetween November- 2011 to October- 2012.
Results: Feeding of baits containing papain with starch and serine (40% and 80% of 24h LC_{50}) caused a significant reduction in fecundity, hatchability and survival of young snails. Maximum (1036 eggs/20 snails) and minimum (175 eggs/ 20 snails) fecundity were observed in June-2012 and February-2012, respectively. There was significant (*P* =.05) positive correlation in between the sublethal concentration of baits containing papain (starch/ serine) and variation of temp/pH/CO_2 of water in each month of the year2011-12. Protein, amino acid, DNA and RNA in the ovotestis of *L. acuminata* were significantly decreased when they were fed to bait containing 80% of 24h LC_{50} of papain + serine in different months of 2011 and 2012. Maximum reduction in protein (26.31 % of control), amino acid (28.83% of control), DNA (42.34% of control) and RNA (34.10% of control) level were measured in the ovotestis of *L. acuminata* fed to bait containing 80% of 24h LC_{50} of papain + serine. Feeding of bait containing 80% of 24h LC_{50} of papain + starch caused maximum reduction in the level of protein (28.97% of control), amino acid (56.77% of control), DNA (42.05% of control) and RNA (29.31% of control) in the ovotestis of *L. acuminata*. There was a significant (P=.05) positive correlation between the fecundity in different months and the corresponding AChE activity in the nervous tissue of the snail. Feeding of bait containing 80% of 24h LC_{50} of papain + serine caused maximum inhibition in AChE activity (41.71% of control) was observed in snail exposed to in the nervous tissue of *L. acuminata* in December2011. Feeding of bait containing 80% of 24h LC_{50} of papain + starch caused maximum inhibition in AChE activity (31.92% of control) in the nervous tissue of *L. acuminata* in June 2012.
Conclusion: Papain altered the reproductive capacity of snails. The anti-reproductive action against *L. acuminata* significantly altered with respect to the change in the abiotic factors in different months of the year Nov-2011toOct-2012.

Keywords: Papain;environment; fasciolosis; abiotic factors;reproduction;snail.

1. INTRODUCTION

Fasciolosis is a parasite disease, affecting a wide range of mammalian species [1]. It is caused by *Fasciola hepatica* and *F. gigantica* [1,2]. *Fasciola*species are of great importance in ruminants in which they inflict heavy economic losses [1,2]. Infection of human host was very sporadic for the last two decades [3]. An increasing number of cases of human's fasciolosis are now reported throughout the world [1,4]. Globally, the estimated number of human infections ranges from 2.4 million to 17 million people and approximately 180 million at risk of infections [5]. The fresh water snails of the genus *Lymnaea* act as the intermediate host of these flukes [6]. The *Lymnaea acuminata* reproduce round the year and lays eggs on the lower surface of aquatic vegetation.Earlier studies have shown that the reproductive capacity of snail significantly altered from one season to other [7]. Several mechanisms are involved in the eggs laying process of *Lymnaea* hormone by environmental factors [8,9]. *Lymnaea* breeds round the year and their population in aquatic environment is nearly same throughout the year except extreme condition [10,11] so that availability of intermediate host i. e. snail for completion of life cycle of fluke *F. gigantica* is sufficient round the year [12]. One of the best approaches to control the incidence of fasciolosis is to interrupt the life cycle of the parasitic trematodes by eliminating or keeping the number of intermediate host below threshold level [13-15]. Baits are the best approach for the management of vector snails [16]. Use of a combination of snail attractant and molluscicides in bait formulation is an effective tool for the pest management [16]. Earlier it has been reported that *Carica papaya* has great potential as molluscicides [17]. The active component is papain. The aim of the present

study is to explore the anti-reproductive capacity of papain at sublethal concentration in bait formulations against the snail *Lymnaea acuminata*. Simultaneous changes in different abiotic factors in each months of the year 2011-12 were also measured. A correlation with reproduction of snail and abiotic factors will provide a tool which can be used in effective control of fasciolosis.

2. MATERIALS AND METHODS

2.1 Test Animals

Adult *Lymnaea acuminata* (2.25±0.20 cm in length) were collected from Ramgarh Lake, Gorakhpur, India in each month in between November-2011 to October-2012. Gorakhpur district lies between Lat. 26º13′N and 27º29′N and Long. 83º05′E and 83º56′E.The snails were acclimatized for 72 hours in dechlorinated tap water .The pH of the water was 7.1-7.3 and dissolved oxygen, free carbon dioxide and bicarbonate alkalinity were 6.5-7.2, 5.2-6.3, and 102.0-105.0 mg/l, respectively.

2.2 Pure Compound

Papain (Cysteine protease) was procured from Sigma Chemical Co. (USA). Agar-agar, starch and serine purchased from Qualigens Fine Chemicals and Sisco Research Laboratories Pvt. Ltd. Mumbai, India.

2.3 Preparation of Bait Formulations with Plant Molluscicide

Bait formulations were prepared by the method of Madsen [18] as modified by Tiwari and Singh [19,20]. In brief, 0.02 g of starch or serine (20 mM) added in to 2% agar- agar solution separately. After boiling papain (40% and 80% of 24h LC_{50}) was added to the solution. The mixture was stirred constantly for 30 minute and spread a uniform thickness (5mm). After cooling, the bait pellets were cut out from the layer with a corer (5 mm diameter). Two type of baits one containing papain+serine and papain +starch were used for the toxicity study.

2.4 Experimental

The bioassay was performed by the method of Tiwari and Singh [19,20]. Each regimen of 5 liter water was kept in six aquaria separately, containing 20 snails in each aquarium. The effect of bait prepared papain+starch or papain+serine on oviposition and survivability of the hatched embryo. Snails in control aquaria were exposed to dechlorinated tap water and fed without papain. *Lymnaea acuminata* laid their eggs in the form of elongated gelatinous capsule containing 5-180 eggs on the lower surface of leaves of *Nelumbonucifera*. After every 24 hours up to 96 hours, the total number of eggs laid by the snails was counted in each aquarium. Hatchability of eggs and survivability of embryo were observed on eggs collected from experimental groups of snails exposed to the bait containing papain (40% and 80% of 24h LC_{50}). The collected eggs were incubated at 30ºC for 24h in petridishes. The time of hatched embryo was monitored. Development of the hatched snails was observed at regular intervals using a binocular microscope until all eggs are hatched. Dead embryos (opaque, lack movement) were discarded. Temperature, pH, dissolved oxygen and dissolved free carbon dioxide of different regimen of water were measured simultaneously. Temperature, pH were measured by thermometer and digital pH meter, respectively. Dissolved O_2 and CO_2 were estimated according to methods prescribed by APHA [21]. To

determine the effect of bait containing papain with starch or serine on *Lymnaea acuminata*ovotestis/ nervous tissue, the experimental snails were fed bait containing (80% of 24h LC_{50}) of papain. Six aquaria were set up for each experiment. Control aquaria contained only dechlorinated tap water without bait. After 96h the treated snail were removed from aquaria and washed with fresh water. The ovotestis /nervous tissue were quickly dissected out and placed on filter paper for remove the adherent water and then weighed. Protein, free amino acids, DNA, RNA and enzyme AChE activity were then measured.

2.5 Biochemical Estimations

Protein (µg/mg) was estimated according to Lowry et al. [22] using bovine serum albumin as a standard. Homogenates of ovotestis were prepared in 10% (w/v) trichloroacetic acid (TCA). Total free amino acids (µg/mg) were determined according to the method of Spies [23]. Estimation of DNA and RNA levels were done according to the method of Schneider [24] using diphenylamine and orcinol reagents, respectively. Homogenates (1.0 mg/ml, w/v) of ovotestis were prepared in 10% TCA at 90°C and centrifuged at 5000 g for 20 minutes. The supernatants were used for estimation of DNA and RNA.

2.6 Enzyme Acetylcholinesterase (AChE)

Acetylcholinesterase activity was measured according to the method of Ellman et al. [25] as modified by Singh et al. [26]. This is a calorimetric method that measures the enzyme activity in terms of ACh breakdown. Fifty milligram of nervous tissue of *L. acuminata* was taken around the buccal mass and homogenized in 1.0 ml of 0.1M phosphate buffer pH 8.0 for 5 minutes in an ice bath then centrifuged at 1000 g for 30 minutes at 4ºC. Enzyme activity was measured in a 10mm path length cuvette using an incubation mixture consisting of 0.1mL of enzyme source, 2.9mL of 0.1M buffer pH 8, 0.1mL of chromogenic agent DTNB (5, 5-dithio-bis-2-nitrobenzoic acid), and 0.02mL of freshly prepared ATChI (acetylthiocholine iodide) solution in distilled water. The change in optical density at 412 nm was recorded for 3 minutes after every 30 second interval at 25ºC. Enzyme activity has been expressed as µmole 'SH' hydrolyzed/ min/ mg protein.

2.7 Statistical Analysis

All experiment was replicated at least six times and values are expressed as Mean±SE of six replicates. LC_{50} value was calculated by using the POLO computer programme software of Russell et al. [27]. Two way of ANOVA was applied to determine significant (P=.05) difference between abiotic factors (temperature, pH, dissolved O_2 and CO_2) and fecundity/ survival of snails. Product moment correlation coefficient was applied to determine significant (P=.05) differences in between abiotic factors such as temperature, pH, dissolved O_2, CO_2 and fecundity of snails treated with bait containing papain. Student's t-test was applied between control and bait fed groups to locate significant (P=.05) variations [28].

3. RESULTS AND DISCUSSION

There was significant (P =.05) variation in the fecundity of *L. acuminata* fed to bait formulations containing 40% and 80% of 24h LC_{50} of papain and attractant serine/starch. In the control group 20 snails laid maximum 1145 eggs in June and minimum 625 egg/20 snails in March (Table 1, 2). Feeding of baits containing 40% of 24h LC_{50} of papain + serine caused maximum fecundity of 965 eggs/ 20 snails and minimum 175 eggs/20 snails in June

and February 2012 (Table 1). Whereas, snails fed with 40% of 24h LC_{50} of papain + starch caused maximum fecundity of 1036 eggs/20 snails and minimum 254 eggs/20 snails in June 2012 and October 2011 (Table 2). There was significant (*P* =.05) positive variation in between the 40%/or 80% of 24h LC_{50} and variation of temperature/ pH/ CO_2 of water in each month of the year 2011-12. Positive (*P* =.05) correlation was observed only 80% of 24h LC_{50} of concentration and dissolved O_2 concentration of water. There was a negative (*P* =.05) correlation only in between 40% of 24h LC_{50} bait containing papain with serine and dissolved O_2 concentration in each month of the year 2011-12. There was significant positive correlation was observed in between the different abiotic factors in each month of the year 2011-12. A significant (*P* =.05) variation in fecundity / hatchability/ survivability of young snails was noted in each month of the year 2011-12 (Table1, 2). Complete embryonic development was lacking in the eggs of snail fed with bait formulation containing 80% of 24h LC_{50} of papain. Per cent hatchability of eggs laid by snails fed with papain + serine or papain + starch was 94.3-42.2% or 94.3-57.6% of control, respectively (Table 1, 2). In control group usually eggs hatched in 7-9 days. The hatching period of eggs in treated snail was prolonged from 9-19 days. Maximum hatching period of eggs (15-19 days) was noticed in January 2012, whereas minimum (8-11 days) in July 2012 (Table 1, 2).

There was a significant (*P* =.05) change in the endogenous level of protein, amino acid, DNA and RNA in ovotestis of bait fed snails. Feeding of papain + serine bait formulations caused maximum reduction in level of DNA (42.34% of control) was noted in July and RNA (34.10% of control) in the April. Maximum reduction in ovotestis protein (26.31% of control) and amino acid level (28.83% of control) were observed in April and June (Table 3). Feeding of papain + starch bait formulations caused maximum reduction in the level of amino acid (56.77% of control) in May and protein (28.97% of control) was observed in October. Highest reduction in the level of DNA (42.05% of control) was observed in June and RNA (29.31% of control) in May (Table 4).

The acetylcholinesterase activity in nervous tissue of snail fed to 80% of 24h LC_{50} of bait containing papain was significantly inhibited in different months of the year 2011- 2012. There was a significant (P=.05) positive correlation between the fecundity in different months and the corresponding AChE activity in the nervous tissue of the snail. Feeding of bait containing 80% of 24h LC_{50} papain + serine caused maximum inhibition in AChE activity (41.71 % of control) in the nervous tissue of *L. acuminata* in December (Table 3). Feeding of bait containing 80% of 24h LC_{50} papain + starch caused inhibition (31.92% of control) in AChE activity in the nervous tissue of snails in June (Table 4).

Present studies clearly indicate that use of papain in bait formulations has great potential in reducing the reproductive capacity of snail *L. acuminata*. Although, molluscicidal activity of papain is reported by Jaiswal and Singh [17] when, papain is directly released in water. In the present study papain was used in bait formulations with attractant serine/starch. Toxicity (24h LC_{50}) of papain in bait formulations in different month was in between 8.58% to 16.85%. It indicates a great variation in LC_{50} about two times in 12 months.

Table 1.Effect of sub-lethal concentration (40% and 80% of 24 h LC_{50}) of papain + serine bait formulation on the reproduction of snail *L. acuminata*.

Months	Treatments	24h LC_{50}	Sub-lethal dose of 24h LC_{50}	Abiotic Factors				Fecundity/20 snails/ Day				Hatchability (%) (In days)	Survival (%)		
				Temp	pH	DO ppm	CO_2 ppm	24h	48h	72h	96h		24h	48h	72h
	Control	-	-	**24ºC**	**7.3**	**3.8**	**18**	**825±6.3**	**365±6.5**	**142±3.2**	**74±5.6**	**100 (7-9)**	**100**	**100**	**100**
November	Pa+Se+Ag	11.14	40% (4.45)	24 ºC*	7.4*	2.7*	14*	608±6.5*	265±9.6*	54±4.5*	36±6.3*	84.7±6.3* (10-13)	94.3±6.5*	63.4±4.1*	41.3±2.3*
2011			80% (8.90)	24 ºC*	7.6*	2.5*	10*	242±2.5*	89±8.6*	23±3.6*	0	56.4±5.2* (12-15)	76.5±4.8*	42.4±3.6*	23.6±8.5*
	Control	-	-	**18 ºC**	**7.3**	**3.9**	**14**	**862±9.1**	**187±6.7**	**93±6.3**	**41±6.3**	**100 (7-9)**	**100**	**100**	**100**
December	Pa +Se+Ag	12.58	40% (5.03)	18 ºC*	7.8*	2.8*	13*	840±6.5*	141±6.5*	84±6.3*	43±4.5*	92.0±4.1* (12-14)	91.5±3.6*	65.8±2.5*	39.7±7.4*
2011			80% (10.06)	18 ºC*	7.5*	2.5*	12*	227±7.5*	42±6.3*	0	0	53.5±5.2* (15-17)	65.4±4.7*	36.4±4.1*	18.7±5.2*
	Control	-	-	**10 ºC**	**7.3**	**4.0**	**18**	**785±7.6**	**274±6.9**	**125±4.6**	**45±4.2**	**100 (7-9)**	**100**	**100**	**100**
January	Pa +Se+Ag	16.54	40% (6.61)	10 ºC*	7.7*	3.0*	18*	776±4.1*	236±6.5*	142±7.4*	65±4.7*	94.3±3.6* (14-18)	85.6±4.7*	76.5±6.3*	43.2±4.1*
2012			80% (13.22)	10 ºC*	7.4*	2.8*	16*	356±5.2*	96±8.6*	65±6.3*	36±5.2*	74.6±9.6* (15-19)	76.1±3.5*	41.2±4.1*	28.7±5.2*
	Control	-	-	**21 ºC**	**7.2**	**3.8**	**17**	**741±6.9**	**142±9.6**	**124±5.6**	**84±4.5**	**100 (7-9)**	**100**	**100**	**100**
February	Pa +Se+Ag	13.43	40% (5.37)	21 ºC*	7.9*	2.7*	15*	554±6.2*	87±3.6*	58±7.4*	32±4.1*	87.6±7.4* (13-15)	88.5±7.8*	45.6±6.3*	27.4±4.2*
2012			80% (10.74)	21 ºC*	7.7*	2.6*	12*	175±6.3*	46±7.8*	41±6.9*	0	65.9±6.5* (15-17)	65.6±6.5*	24.6±3.8*	0
	Control	-	-	**22 ºC**	**7.2**	**3.8**	**18**	**625±9.6**	**246±8.9**	**143±5.6**	**56±8.4**	**100 (7-9)**	**100**	**100**	**100**
March	Pa +Se+Ag	14.52	40% (5.80)	22 ºC*	7.4*	2.1*	16*	472±7.8*	263±7.8*	95±6.5*	47±7.4*	82.6±7.4* (12-14)	87.3±6.3*	65.9±7.4*	36.8±6.3*
2012			80% (11.60)	22 ºC*	7.2*	2.1*	12*	239±4.1*	142±5.6*	46±3.6*	0	54.1±4.7* (13-15)	45.2±6.5*	36.4±7.6*	0
	Control	-	-	**26 ºC**	**7.1**	**3.6**	**18**	**964±7.8**	**285±6.9**	**176±6.3**	**142±6.3**	**100 (7-9)**	**100**	**100**	**100**
April	Pa +Se+Ag	13.74	40% (5.49)	26 ºC*	7.7*	2.0*	17*	842±3.6*	174±6.5*	132±7.4*	76±8.4*	76.4±6.9* (11-13)	65.3±6.3*	47.7±9.4*	27.6±7.4*
2012			80% (10.98)	26 ºC*	7.8*	2.1*	12*	520±5.2*	54±7.8*	74±3.6*	34±6.5*	62.1±3.6* (12-15)	41.5±8.4*	36.5±6.5*	14.6±6.5
	Control	-	-	**28 ºC**	**7.1**	**3.5**	**17**	**1023±7.8**	**365±9.3**	**146±8.9**	**182±8.9**	**100 (7-9)**	**100**	**100**	**100**
May	Pa +Se+Ag	12.73	40% (5.09)	28 ºC*	7.5*	1.9*	15*	875±5.6*	263±4.2*	84±7.8*	46±5.6*	72.6±3.2* (10-12)	87.4±7.4*	54.7±7.5*	36.5±9.5*
2012			80% (10.18)	28 ºC*	7.2*	1.8*	13*	624±8.9*	94±4.5*	65±6.3*	32±7.8*	42.2±7.4* (12-14)	56.7±6.5*	36.8±8.6*	21.4±7.4*
	Control	-	-	**31 ºC**	**7.1**	**3.1**	**18**	**1145±6.9**	**256±8.9**	**148±5.6**	**85±7.4**	**100 (7-9)**	**100**	**100**	**100**
June	Pa +Se+Ag	8.58	40% (3.43)	31 ºC*	7.4*	1.2*	16*	965±6.5*	174±4.2*	142±7.4*	32±6.5*	65.3±4.1* (9-11)	74.6±4.1*	47.6±6.5*	26.5±6.9*
2012			80% (6.86)	31 ºC*	7.8*	1.1*	12*	462±8.9*	85±5.8*	42±6.3*	25±7.8*	46.8±7.4* (10-13)	53.2±2.3*	36.7±5.6*	0
	Control	-	-	**32 ºC**	**7.1**	**3.3**	**17**	**1085±6.3**	**152±7.8**	**63±7.8**	**85±6.5**	**100 (7-9)**	**100**	**100**	**100**
July	Pa +Se+Ag	8.76	40% (3.50)	32 ºC*	7.8*	2.1*	16*	952±7.8*	86±6.5*	65±3.6*	41±6.9*	76.9±6.3* (8-11)	75.5±6.5*	63.7±4.7*	41.6±7.4*
2012			80% (7.00)	32 ºC*	7.6*	2.1*	13*	541±9.5*	81±6.3*	36±7.5*	0	56.7±7.4* (10-13)	53.6±5.6*	36.9±6.5*	32.8±6.5*
	Control	-	-	**34 ºC**	**7.2**	**3.6**	**17**	**845±8.9**	**176±8.6**	**84±6.9**	**65±4.6**	**100 (7-9)**	**100**	**100**	**100**
August	Pa +Se+Ag	9.15	40% (3.66)	34 ºC*	7.9*	2.5*	15*	632±6.5*	84±7.8*	47±8.5*	23±7.8*	75.6±6.9* (9-12)	87.4±4.6*	65.4±4.8*	41.6±9.8*
2012			80% (7.32)	34 ºC*	7.8*	2.4*	11*	354±3.6*	80±9.8*	38±5.6*	32±6.5*	47.4±3.6* (10-13)	51.6±3.6*	18.9±6.5*	0
	Control	-	-	**31 ºC**	**7.2**	**3.7**	**17**	**963±6.9**	**154±6.9**	**65±6.9**	**36±6.9**	**100 (7-9)**	**100**	**100**	**100**
September	Pa +Se+Ag	10.32	40% (4.12)	31 ºC*	7.4*	2.8*	16*	785±7.4*	96±6.5*	41±7.8*	0	89.4±6.4* (9-13)	89.5±4.1*	61.3±5.8*	35.6±7.4*
2012			80% (8.24)	31 ºC*	7.9*	2.6*	14*	362±6.5*	41±4.6*	26±6.5*	0	56.8±3.2* (12-13)	44.8±7.8*	22.6±4.7*	14.7±6.5*
	Control	-	-	**26 ºC**	**7.3**	**3.8**	**18**	**751±9.6**	**176±6.9**	**135±7.9**	**76±4.8**	**100 (7-9)**	**100**	**100**	**100**
October	Pa +Se+Ag	10.16	40% (4.06)	26 ºC*	7.2*	2.7*	15*	547±3.6*	142±7.8*	84±4.2*	47±6.9*	87.6±4.5* (10-13)	86.4±6.5*	62.8±6.5*	32.9±6.4*
2012			80% (8.12)	26ºC*	7.4*	2.6*	14*	248±9.5*	85±6.5*	46±3.6*	36±7.8*	56.1±8.5* (12-14)	54.6±6.5*	36.4±4.8*	21.5±4.2*

Each experiment was replicated six times and the value of fecundity, hatchability and survival is the mean of six replicates. Product moment correlation coefficient and two way ANOVA was applied in between abiotic factors and fecundity/ hatchability/ survival of young snail Lymnaea acuminata. (*) significantly (P=.05) different from control. Abbreviation:Pa+Se+ Ag = Papain+ Serine +Agar.

Table 2.Effect of sub-lethal concentration (40% and 80% of 24 h LC_{50}) of papain + starch bait formulation on the reproduction of snail *L. acuminata*

Months	Treatment	24h LC_{50}	Sub-lethal dose of 24h LC_{50} mg/l	Abiotic Factors				Fecundity/20 snails/Day				Hatchability (%) (In days)	Survival (%)		
				Temp	pH	DO ppm	CO_2 ppm	24h	48h	72h	96h		24h	48h	72h
	Control	-	-	**24 ºC**	**7.3**	**3.8**	**18**	**825±6.3**	**365±6.5**	**142±3.2**	**74±5.6**	**100 (7-9)**	**100**	**100**	**100**
November	Pa+St+Ag	11.84	40% (4.73)	24 **ºC***	7.2*	2.7*	15*	765±4.8*	242±5.6*	86±5.6*	42±6.9*	85.6±6.5* (10-13)	88.0±6.5*	68.4±5.2*	47.1±3.5*
2011			80% (9.46)	24 **ºC***	7.1*	2.6*	14*	324±6.9*	86±4.8*	0	33±8.5*	65.8±2.5* (12-15)	62.1±3.6*	36.4±3.2*	25.6±5.6*
	Control	-	-	**18 ºC**	**7.3**	**3.9**	**14**	**862±9.1**	**187±6.7**	**93±6.3**	**41±6.3**	**100 (7-9)**	**100**	**100**	**100**
December	Pa +St+Ag	12.85	40% (5.14)	18 **ºC***	7.3*	2.8*	16*	588±5.8*	148±6.8*	76±5.4*	56±5.8*	94.3±6.4* (12-14)	90.3±6.5*	66.5±8.5*	42.3±7.4*
2011			80%(10.28)	18 **ºC***	7.4*	2.7*	12*	265±6.9*	94±6.9*	54±2.3*	0	63.5±9.8* (15-17)	64.7±9.6*	36.4±9.6*	0
	Control	-	-	**10 ºC**	**7.3**	**4.0**	**18**	**785±7.6**	**274±6.9**	**125±4.6**	**45±4.2**	**100 (7-9)**	**100**	**100**	**100**
January	Pa +St+Ag	16.75	40% (6.70)	10 **ºC***	7.3*	3.0*	13*	732±4.1*	251±4.8*	78±6.5*	41±5.8*	89.7±5.8* (14-18)	83.5±8.5*	58.4±7.4*	48.1±5.8*
2012			80%(13.40)	10 **ºC***	7.2*	2.9*	12*	576±3.6*	156±8.4*	62±9.6*	24±6.9*	71.2±6.5* (15-19)	64.1±7.4*	36.8±4.5*	28.7±3.5*
	Control	-	-	**21 ºC**	**7.2**	**3.8**	**17**	**741±6.9**	**142±9.6**	**124±56**	**84±4.5**	**100 (7-9)**	**100**	**100**	**100**
February	Pa +St+Ag	14.70	40% (5.88)	21 **ºC***	7.5*	2.8*	15*	645±9.6*	274±4.7*	142±4.7*	87±8.7*	87.4±8.4* (13-15)	68.2±6.5*	41.9±7.8*	36.4±5.8*
2012			80%(11.76)	21 **ºC***	7.9*	2.8*	12*	563±2.8*	142±5.8*	76±5.4*	0	65.8±6.9* (15-17)	42.6±4.5*	25.9±7.4*	0
	Control	-	-	**22 ºC**	**7.2**	**3.8**	**18**	**625±9.6**	**246±8.9**	**143±5.6**	**56±8.4**	**100 (7-9)**	**100**	**100**	**100**
March	Pa +St+Ag	14.25	40% (5.70)	22 **ºC***	7.8*	2.7*	13*	584±6.9*	362±6.9*	89±8.7*	74±4.8*	88.9±6.5* (12-14)	88.4±4.5*	54.8±7.8*	39.8±9.6*
2012			80%(11.40)	22 **ºC***	7.4*	2.6*	12*	332±2.8*	278±4.8*	67±6.9*	62±4.7*	65.2±3.6* (13-15)	74.2±2.5*	47.1±6.5*	24.6±6.3*
	Control	-	-	**26 ºC**	**7.1**	**3.6**	**18**	**964±7.8**	**285±6.9**	**176±6.3**	**142±6.3**	**100 (7-9)**	**100**	**100**	**100**
April	Pa +St+Ag	13.57	40% (5.42)	26 **ºC***	7.5*	2.5*	18*	951±4.8*	361±6.9*	176±7.9*	85±6.9*	87.6±7.4* (11-13)	84.5±4.5*	54.6±7.8*	36.9±4.1*
2012			80%(10.84)	26 **ºC***	7.3*	2.0*	16*	521±5.9*	165±5.8*	142±8.4*	53±5.8*	56.8±9.5* (12-15)	62.3±6.3*	39.8±6.5*	18.7±5.2*
	Control	-	-	**28 ºC**	**7.1**	**3.5**	**17**	**1023±7.8**	**365±9.3**	**146±8.9**	**182±8.9**	**100 (7-9)**	**100**	**100**	**100**
May	Pa +St+Ag	12.30	40% (4.92)	28 **ºC***	7.7*	1.8*	17*	972±4.8*	285±3.6*	185±6.9*	92±3.6*	88.6±6.5* (10-12)	89.2±7.8*	62.1±8.5*	47.6±6.3*
2012			80%(9.84)	28 **ºC***	7.6*	1.5*	15*	647±6.9*	169±2.5*	69±7.8*	64±7.9*	65.8±8.4* (12-14)	73.6±5.8*	58.3±4.7*	41.0±8.5*
	Control	-	-	**31 ºC**	**7.1**	**3.1**	**18**	**1145±6.9**	**256±8.9**	**148±5.6**	**85±7.4**	**100 (7-9)**	**100**	**100**	**100**
June	Pa +St+Ag	9.85	40% (3.94)	31 **ºC***	7.7*	1.5*	19*	1036±5.8*	287±6.3*	162±5.8*	132±9.5*	91.5±9.5* (9-11)	88.9±6.9*	63.8±7.4*	52.4±8.7*
2012			80% (7.88)	31 **ºC***	7.5*	1.3*	18*	792±6.9*	146±5.6*	94±6.9*	84±6.9*	76.4±7.9* (10-13)	56.8±7.8*	37.4±6.5*	17.6±3.5*
	Control	-	-	**32 ºC**	**7.1**	**3.3**	**17**	**1085±6.3**	**152±7.8**	**63±7.8**	**85±6.5**	**100 (7-9)**	**100**	**100**	**100**
July	Pa +St+Ag	9.16	40% (3.66)	32 **ºC***	7.6*	1.6*	17*	934±5.7*	184±6.3*	86±6.9*	75±7.4*	84.7±6.8* (8-11)	79.5±6.5*	58.6±4.1*	23.6±4.7*
2012			80% (7.32)	32 **ºC***	7.8*	1.5*	15*	463±6.9*	83±4.8*	65±8.5*	0	57.6±6.8* (10-13)	46.2±4.8*	28.9±2.5*	14.8±7.4*
	Control	-	-	**34 ºC**	**7.2**	**3.6**	**17**	**845±8.9**	**176±8.6**	**84±6.9**	**65±4.6**	**100 (7-9)**	**100**	**100**	**100**
August	Pa +St+Ag	9.25	40% (3.70)	34 **ºC***	7.9*	2.2*	16*	826±58*	78±5.8*	86±4.8*	0	92.6±4.7* (9-12)	84.1±9.6*	76.1±3.2*	56.9±4.1*
2012			80% (7.40)	34 **ºC***	7.8*	2.6*	14*	365±8.4*	65±3.6*	0	0	74.5±6.8* (10-13)	56.8±7.9*	38.4±7.4*	21.3±3.2*
	Control	-	-	**31 ºC**	**7.2**	**3.7**	**17**	**963±6.9**	**154±6.9**	**65±6.9**	**36±6.9**	**100 (7-9)**	**100**	**100**	**100**
September	Pa +St+Ag	10.20	40% (4.08)	31 **ºC***	7.1*	2.7*	16*	782±2.5*	142±8.9*	74±5.8*	86±4.9*	89.5±6.9* (9-13)	86.1±6.5*	54.2±7.8*	38.1±8.5*
2012			80% (8.10)	31 **ºC***	7.3*	2.7*	13*	362±6.9*	76±8.4*	51±6.5*	0	65.2±4.8* (12-13)	58.4±9.6*	45.2±5.8*	32.6±5.2*
	Control	-	-	**26 ºC**	**7.3**	**3.8**	**18**	**751±9.6**	**176±6.9**	**135±7.9**	**76±4.8**	**100 (7-9)**	**100**	**100**	**100**
October	Pa +St+Ag	10.76	40% (4.30)	26 **ºC***	7.2*	2.8*	14*	541±2.8*	87±2.8*	46±2.5*	76±9.6*	88.4±8.4* (10-13)	87.3±4.7*	56.8±6.5*	46.5±4.1*
2012			80% (8.60)	26 **ºC***	7.5*	2.6*	12*	254±8.4*	46±4.8*	0	52±8.4*	65.6±6.9* (12-14)	56.7±8.4*	42.6±3.2*	36.1±7.8*

Each experiment was replicated six times and the value of fecundity, hatchability and survival is the mean of six replicates. Product moment correlation coefficient and two way ANOVA was applied in between abiotic factors and fecundity/ hatchability/ survival of young snail Lymnaea acuminata. (*) significantly (P=.05) different from control. Abbreviation: Pa+St+ Ag = Papain+ Starch +Agar.

Table 3. Effect of sub-lethal concentration (80% of 24 h LC_{50}) of papain+serine in bait formulation on the certain biochemical changes (ovotestis) and AChE activity (nervous tissue) of snail *L. acuminata*.

Months	Treatment	24h LC_{50}	Sub-lethal dose mg/l	Protein	Amino Acid	DNA	RNA	AChE
November 2011	Control	-	-	84.12±0.89 (100)	62.45±0.84 (100)	89.54±0.85 (100)	96.21±0.96 (100)	1.65±0.06 (100)
	Pa+Se+Ag	11.14	8.90	64.52±0.21* (74.32)	51.29±0.74* (82.12)	65.30±0.54* (72.92)	63.87±0.41* (66.38)	0.93±0.05* (56.36)
December 2011	Control	-	-	82.91±0.81 (100)	62.47±0.88 (100)	90.84±0.85 (100)	95.31±0.98 (100)	1.63±0.06 (100)
	Pa +Se+Ag	12.58	10.06	41.41±0.54* (49.94)	58.71±0.45 (93.98)	61.75±0.54* (67.97)	54.21±0.64* (56.87)	0.68±0.03* (41.71)
January 2012	Control	-	-	83.96±0.80 (100)	65.45±0.64 (100)	81.84±0.95 (100)	95.58±0.86 (100)	1.62±0.06 (100)
	Pa +Se+Ag	16.54	13.22	35.51±0.54* (42.29)	56.83±0.51 (86.82)	54.16±0.65* (66.17)	43.37±0.32* (45.48)	1.28±0.04* (79.01)
February 2012	Control	-	-	82.90±0.82 (100)	66.45±0.74 (100)	88.34±0.89 (100)	94.61±0.86 (100)	1.64±0.06 (100)
	Pa +Se+Ag	13.43	10.74	32.04±0.64* (38.64)	32.37±0.65* (48.71)	44.43±0.62* (50.29)	49.40±0.63* (52.21)	1.03±0.04* (62.80)
March 2012	Control	-	-	85.97±0.81 (100)	67.15±0.74 (100)	88.58±0.95 (100)	96.78±0.76 (100)	1.63±0.04 (100)
	Pa +Se+Ag	14.52	11.60	24.63±0.98* (28.64)	47.18±0.54* (70.26)	46.93±0.74* (52.98)	42.32±0.41* (43.72)	0.97±0.06* (59.50)
April 2012	Control	-	-	85.38±0.83 (100)	65.35±0.94 (100)	87.72±0.95 (100)	95.21±0.96 (100)	1.64±0.03 (100)
	Pa +Se+Ag	13.74	10.98	22.47±0.84* (26.31)	36.10±0.65* (55.24)	51.03±0.64* (58.17)	32.47±0.74* (34.10)	1.49±0.07* (90.85)
May 2012	Control	-	-	86.91±0.84 (100)	64.65±0.84 (100)	87.98±0.75 (100)	98.68±0.98 (100)	1.66±0.06 (100)
	Pa +Se+Ag	12.73	10.18	34.76±0.65* (39.99)	28.41±0.97* (43.94)	45.25±0.45* (51.43)	36.51±0.65* (36.99)	0.84±0.03* (50.60)

June 2012	Control	-	-	87.95±0.83 (100)	67.65±0.74 (100)	86.81±0.95 (100)	96.76±0.88 (100)	1.66±0.03 (100)
	Pa +Se+Ag	8.58	6.86	37.63±0.65* (42.78)	19.51±0.69* (28.83)	38.02±0.63* (43.79)	34.84±0.47* (36.00)	0.94±0.01* (56.62)
July 2012	Control	-	-	81.91±0.82 (100)	66.55±0.54 (100)	86.34±0.75 (100)	93.41±0.86 (100)	1.67±0.06 (100)
	Pa +Se+Ag	8.76	7.0	38.54±0.74* (47.05)	24.45±0.87* (36.73)	36.56±0.76* (42.34)	38.21±0.54* (40.90)	1.06±0.01* (63.47)
August 2012	Control	-	-	82.98±0.85 (100)	68.46±0.89 (100)	87.56±0.85 (100)	96.25±0.99 (100)	1.67±0.06 (100)
	Pa +Se+Ag	9.15	7.32	35.50±0.41* (42.78)	31.54±0.63* (46.07)	42.63±0.84* (48.68)	46.52±0.62* (48.33)	1.24±0.02* (74.25)
September 2012	Control	-	-	81.47±0.89 (100)	68.42±0.88 (100)	87.54±0.85 (100)	96.21±0.96 (100)	1.68±0.06 (100)
	Pa +Se+Ag	10.32	8.24	47.62±0.36* (58.45)	46.51±0.25* (67.97)	41.85±0.74* (47.80)	54.41±0.64* (56.55)	0.98±0.03* (58.33)
October 2012	Control	-	-	83.93±0.81 (100)	67.15±0.84 (100)	86.73±0.97 (100)	97.64±0.86 (100)	1.68±0.04 (100)
	Pa +Se+Ag	10.16	8.12	52.41±0.74* (62.44)	41.25±0.52* (61.42)	54.21±0.64* (62.50)	56.54±0.97* (57.90)	1.14±0.04* (67.85)

*Each experiment was replicated six times and the value of protein, amino acid, DNA, RNA and AChE is the mean±SE of six replicates. Values in parentheses indicate per cent change with control taken as 100%. Two way ANOVA was applied in between control and treated group. *-significant (P=.05) different from control.*

⁎-Significant (P= .05) when t-test was applied between treated and control groups. Protein, Amino acid, DNA and RNA levels- µg/mg. Acetylcholinesterase activity- µmole 'SH' hydrolyzed/ min/mg protein.

Abbreviation: Pa+Se+Ag= Papain+Serine+Agar.

Table 4. Effect of sub-lethal concentration (80% of 24 h LC_{50}) of papain + starch in bait formulation on the certain biochemical changes (ovotestis) and AChE activity (nervous tissue) of snail *L. acuminata.*

Months	Treatment	24h LC_{50}	Sub-lethal dose mg/l	Protein	Amino Acid	DNA	RNA	AChE
November 2011	Control	-	-	84.12±0.89 (100)	62.45±0.84 (100)	89.54±0.85 (100)	96.21±0.96 (100)	1.65±0.06 (100)
	Pa+St+Ag	11.84	9.46	42.38±0.35* (50.38)	52.32±0.65* (83.77)	62.30±0.69* (69.57)	48.65±0.25* (50.56)	0.97±0.02* (58.78)
December 2011	Control	-	-	82.91±0.81 (100)	62.47±0.88 (100)	90.84±0.85 (100)	95.31±0.98 (100)	1.63±0.06 (100)
	Pa +St+Ag	12.85	10.28	40.52±0.52* (48.87)	48.21±0.41* (77.17)	54.74±0.85* (60.25)	57.64±0.54* (60.47)	0.84±0.05* (51.53)
January 2012	Control	-	-	83.96±0.80 (100)	65.45±0.64 (100)	81.84±0.95 (100)	95.58±0.86 (100)	1.62±0.06 (100)
	Pa +St+Ag	16.75	13.40	39.62±0.69* (47.18)	45.62±0.62* (69.70)	46.52±0.65* (56.84)	47.62±0.74* (49.82)	0.65±0.01* (40.12)
February 2012	Control	-	-	82.90±0.82 (100)	66.45±0.74 (100)	88.34±0.89 (100)	94.61±0.86 (100)	1.64±0.06 (100)
	Pa +St+Ag	14.70	11.76	51.56±0.82* (62.19)	52.14±0.65* (78.46)	51.23±0.45* (57.99)	39.51±0.65* (41.76)	0.86±0.03* (52.43)
March 2012	Control	-	-	85.97±0.81 (100)	67.15±0.74 (100)	88.58±0.95 (100)	96.78±0.76 (100)	1.63±0.04 (100)
	Pa +St+Ag	14.25	11.40	54.21±0.75* (63.05)	47.96±0.42* (71.42)	58.31±0.65* (65.82)	36.53±0.41* (37.74)	1.02±0.03* (62.57)
April 2012	Control	-	-	85.38±0.83 (100)	65.35±0.94 (100)	87.72±0.95 (100)	95.21±0.96 (100)	1.64±0.03 (100)
	Pa +St+Ag	13.57	10.84	44.83±0.54* (52.50)	43.62±0.58* (66.74)	38.96±0.84* (44.41)	43.18±0.36* (45.35)	0.64±0.04* (39.02)
May 2012	Control	-	-	86.91±0.84 (100)	64.65±0.84 (100)	87.98±0.75 (100)	98.68±0.98 (100)	1.66±0.06 (100)
	Pa +St+Ag	12.30	9.84	41.99±0.75* (48.31)	53.74±0.28* (56.77)	46.56±0.92* (52.92)	28.93±0.74* (29.31)	0.76±0.02* (45.78)

June 2012	Control	-	-	87.95±0.83 (100)	67.65±0.74 (100)	86.81±0.95 (100)	96.76±0.88 (100)	1.66±0.03 (100)
	Pa +St+Ag	9.85	7.88	33.12±0.41* (37.65)	41.63±0.65* (61.53)	36.51±0.74* (42.05)	37.63±0.36* (38.89)	0.53±0.01* (31.92)
July 2012	Control	-	-	81.91±0.82 (100)	66.55±0.54 (100)	86.34±0.75 (100)	93.41±0.86 (100)	1.67±0.06 (100)
	Pa +St+Ag	9.16	7.32	35.41±0.51* (43.23)	38.48±0.62* (57.82)	42.13±0.74* (50.51)	35.18±0.24* (37.66)	1.25±0.04 (74.85)
August 2012	Control	-	-	82.98±0.85 (100)	68.46±0.89 (100)	87.56±0.85 (100)	96.25±0.99 (100)	1.67±0.06 (100)
	Pa +St+Ag	9.25	7.40	32.65±0.36* (39.34)	46.32±0.36* (67.65)	38.75±0.65* (44.24)	41.91±0.62* (43.54)	0.92±0.03* (55.08)
September 2012	Control	-	-	81.47±0.89 (100)	68.42±0.88 (100)	87.54±0.85 (100)	96.21±0.96 (100)	1.68±0.06 (100)
	Pa +St+Ag	10.20	8.10	28.47±0.76* (34.94)	41.84±0.62* (61.15)	51.62±0.47* (58.96)	47.36±0.36* (49.22)	0.87±0.04* (51.78)
October 2012	Control	-	-	83.93±0.81 (100)	67.15±0.84 (100)	86.73±0.97 (100)	97.64±0.86 (100)	1.68±0.04 (100)
	Pa +St+Ag	10.76	8.60	24.32±0.64* (28.97)	47.95±0.41* (71.10)	48.65±0.65* (56.04)	46.32±0.38* (47.43)	0.76±0.03* (45.23)

*Each experiment was replicated six times and the value of protein, amino acid, DNA, RNA and AChE is the mean±SE of six replicates. Values in parentheses indicate per cent change with control taken as 100%.Two way ANOVA was applied in between control and treated group. *-significant (P=.05) different from control. ⁎-Significant (P= .05) when t-test was applied between treated and control groups. Protein, Amino acid, DNA and RNA levels- µg/mg. Acetylcholinesterase activity- µmole 'SH' hydrolyzed/ min/mg protein.*

Abbreviation: Pa+St+Ag= Papain+ Starch+Agar.

Seasonal fluctuations in the secretary neuroendocrine cells of *Aplysiacalifornica*inhibited the protein Kinase A and C which play a significant role in regulation of egg laying hormone [29]. According to them cAMP and diacylglycerol second messenger pathways are regulated on a seasonal basis [29]. It may be possible that in present study variation in fecundity in snail fed to bait formulations of papain may be due to regulated by cAMP/ diaceylglycerol. Dissolved oxygen is one of the major components required by snail's metabolic activity [30]. In normal condition metabolic demand for oxygen increases substantially with temperature [31]. At higher temperature the increasing rate of snail's metabolism caused high release of CO_2, which affects the pH of water [32]. This was evident from the elevated concentration of CO_2 and decrease in pH of water in summer season. It is clear from the result section that there was positive correlation between fed to bait formulations containing 40% and 80% of 24h LC_{50} and different abiotic factors such as temperature/ pH/ dissolved O_2/ CO_2 of water. It indicates that the effect of drugs in aquatic medium is significantly altered with seasonal variation in abiotic factors in each month of the year2011-12. It seems that there was a cumulative effect of these abiotic factors on the level of protein, amino acids and nucleic acids in ovotestis of *L. acuminata*. These effects may be direct or indirect through caudo dorsal cells (CDC_s), which release ovulation hormone and ultimately affect the reproduction of snails in different months of the year [7,33].

The reduction in protein and amino acids levels may be due to indirect interference of the environmental abiotic factors with protein synthesis or due to direct interference of test component papain. The synthesis of protein in any of a tissue can be affected by; firstly, it either affects the RNA synthesis at the transcription stage, or secondly it somehow affects the uptake of amino acids in the polypetide chain. Both these possibilities may account for the lower protein content in the affected tissue. In the first case, the RNA synthesis would be inhibited resulting in reduced RNA as well protein content. In the second case, only the protein content would be affected [34]. In the present study there is a significant reduction in DNA/RNA/protein/amino acids in ovotestis bait fed snails. It indicates that papain affects the protein synthesis at transcriptional level.

pH is one of the crucial environmental factor that have significant effect on number of enzymes involved in protein synthesis [35]. Change in the level of DNA and RNA in ovotestis of *L. acuminata* were significantly influenced by the water temperature [36]. In snail *Helix aspersa* elevated temperature from 5 to 25ºC induces spermatogenic, DNA synthesis and formation of spermatid and spermatozoa [37]. Instead of it reduction in amino acids level in ovotestis also indicates that amino acid pool was affected as there was reduction in amino acid synthesis. If it hit protein synthesis at translation level there must be higher amino acid in ovotestis. The synthesis of DNA and RNA are influenced by the intracellular pH physiological range. The activity increases with increasing pH from 7.0-8.0. The process of cellular growth and divisions requires the synthesis of nucleic acids and protein [38,39]. Increase in pH from 7-8 caused a significant increase in DNA and protein level in ovotestis of *L. acuminata* [38].

It is clear from the result section that the sub-lethal treatment of papain in bait formulation ($24hLC_{50}$) caused a significant inhibition of AChE activity in the nervous tissue of *L. acuminata.* Jaiswal et al. [40] noted that papain is uncompetitive inhibition of AChE. AChE inhibition result in accumulation of acetylcholinesterase at the nerve synapses so that the post synaptic membrane is in a state of permanent stimulation producing paralysis, ataxia and general lack of coordination in neuromuscular system and eventual death [40,41]. There was a significant positive correlation between the AChE activity and the fecundity of snail. It

indicates that the reproductive capacity of snail up to some extent is mediated through cholinergic stimuli in the brain of snail.

4. CONCLUSION

From the above discussion it can be stated that papain significantly altered the reproductive capacity of snails. The anti-reproductive action of papain against *L. acuminata* significantly altered with respect to the change in the abiotic factors in different months of the year. In this way proper concentration of papain can be used in different months to reduce the population of vector snail below a threshold level, so that incident of fasciolosis can be effectively controlled.

COMPETING INTERESTS

Authors have declared that no competing interests exists.

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