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RESEARCH ARTICLE

EGGS VIABILITY AND HISTOLOGICAL CHANGES IN OVARY OF *ARGAS* (*PERSICARGAS*) *PERSICUS* (ACARI: ARGASIDAE) AFTER TREATMENT WITH *ALLIUM SATIVUM* EXTRACT

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ABSTRACT

Agriculturists in developing countries are suffering from many diseases caused by tick infestations that reduce the productivity of their livestock. To diminish these losses, natural products (eco-friendly) for ectoparasite control with lower chance of improvement of resistance are required. This study aimed to examine the effect of different concentrations (0.5, 1.5, 2.5, and 4%) of *Allium sativum* (Garlic) extract on the viability of eggs laid by *Argaspersicus* females and their ovaries' development. It was found that the number of eggs laid by treated females were significantly($P<0.05$) decreased than those laid by normal females. There is a significant inverse relationship between the treatment concentration of garlic extract and the percentage of hatched eggs. Applying different concentrations (100, 200, 400, 500, and 600 ppm) of garlic extract on the eggs resulted in the percentage of unhatched eggs increasing significantly as garlic concentrations also increased.The average diameters of oocytes in treated females were decreased by 61% from the normal oocytes' average diameter, and the ovary appeared studded with previtellogenic primary oocytes. Histological changes observed in the treated ovaries include: the presence of vacuolization; alteration of oocyte morphology, which changed from rounded to elongate; disorganization of the yolk granules; and deformation of the chorion. These results demonstrate that garlic extract affected *A. persicus* oogenesis by interfering with the formation of yolk granules and egg shells. Our results suggest that garlicextract causes partial blockage of the vitellogenesis and other aspects of oogenesis as indicated by disrupted synthesis of yolk protein in the newly formed oocytes.

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INTRODUCTION

Argas (Persicurgas) persicus (Oken, 1818) is an ectoparasite of domestic poultry and wild birds in parts of Asia, Europe, Africa and Australia. It inhabits cracks and crevices in deserts, forest and steppe zones near human habitations (Hoogstraal, 1973). It is a vector of the poultry spirochaetosis*Treponema gallinarum* (Burroughs, 1947) and *Borrelia anserine* (El-kammah *et al.,* 1982) as well as Quaranfil virus in Egypt (Kaiser, 1966; El-kammah and AdbelWahab, 1981). The great destruction caused by these ticks to animal productivity has increased the need for their control. The rapid development of tick resistance against active substances in synthetic acaricides (Ghosh *et al.,* 2006; George, 2000), together with related environmental and health risks (Hodgson and Levi, 1996) associated with their application, increase the need for natural products as an alternate to synthetic acaricides. Compounds of plant origin have been used recently against all stages (adults, larva, and nymph) of economically important tick species with encouraging results (Mehlhorn *et al.,* 2005; Mehlhorn, 2008; Kamaraj *et al.,* 2010; Ghosh *et al.,* 2011; Semmler *et al.,* 2011; Sing *et al.,* 2013). Herbal preparation consisting of garlic extracts along with extracts of lemon and onion mixed with turmeric and camphor powder in Pongamia(Karanj) oil was applied for 5days to eliminate *Sarcoptesscabiei* infestation in piglets (Dwivedi and Sharma, 1986). Plant extracts like Allium plant extract have been proven to be potential alternatives to conventional insecticides;garlic (*Allium sativum*) acts as an antiparasitic agent as a result of the presence of its main component, allicim(Ankri and Mirelman,1999). It has larvicidal activities against filarial mosquito, *Culexquinquefasciatus* (Shrankhla*et al.,* 2011). It also has an antiprotozoal effect (Sharma *et al.,* 2009;Yakoob *et al.,* 2011). Essential oils of garlic produce high mortality(90-100%) on 10-day old *Rhipicephalusmicroplus* larva when used in certain concentrations (Martinez-Velazquez *et al.,*2011). Application of 10% garlic treated mite infestation in hens (Birrenkott *et al.,* 2000). Treatment of *Rhipicephalusmicroplus* females with 0.25% *Melia azedarach* (plant extract) resulted in reduction in ovary weight. In addition, histological techniques revealed morphological changes in the ovary such as

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deformation of the chorion; and the yolk granules were disorganized (Sousa *et al.,* 2013). Therefore, this study was conducted to investigate the potency of garlic extract as an acaricide on the reproductive potential and the histological morphology of the female ovary of *Argaspersicus*.

MATERIALS AND METHODS

Ticks

The soft tick, *Argas (Persicargas) persicus* (Oken) was collected from a domestic chicken house at Beni Suef Governorate, Egypt. To establish a laboratory colony, ticks were maintained at 27°C ± 1, 75% RH and 16 hrs day light. The ticks were held in transparent polyethylene tubes (25 × 100 or 13 × 100 mm) which were sealed at one end by a plaster of pairs plug (rearing tube) and covered at the other end with muslin cloth securely held by rubber bands (Kaiser, 1966). Domestic pigeons (*Columba livia*), from a commercial breeder in Cairo, were used for feeding the ticks. Both colony and host were kept in an insectary provided by the Department of Entomology, Ain Shams University. The pigeon host was tied to a wooden board with one wing stretched laterally. The inner wing feathers were plucked and ticks (adults and nymphs) were placed to feed on the wing for 15- 20 min. Following engorgement, ticks were transferred to a Petri dish containing a filter paper disk and observed until coxal fluid was emitted.

Allium sativum extract

Garlic (*Allium sativum*) extract was prepared according to Aboelhadid *et al*. (2013) by subjecting the fresh plant bulbs to steam distillation to obtain the extract. The obtained extract was purified chromatographically by HPLC according to Rembold *et al.* (1984)

Female treatment

Different concentrations of garlic extract were topically applied on the ventral side of the posterior half of female body. Ten microliters of the tested compound were applied to the mated female ticks on 3rd day after feeding. Ten pairs of treated females and normal males were placed separately in glass vials covered with muslin cloth and were kept at 27°Cand 75% RH in an insectary. Females used as control were treated with appropriate amount of distilled water. Eggs were collected daily for seven daysto follow their hatchability and to study the effect of the tested material on viability of eggs laid by treated females. To observe the general anatomical changes of treated female's ovary, 1.44% of garlic extract (LC_{50}) was applied to the mated females after 3 days of feeding as described before. Three days later, females were dissected in Phosphate buffered saline under the Optika microscope and ovaries were examined. The digital images were taken by an Optika camera and processed by Adobe Photoshop Elements version 3.0 (Adobe Systems).

Egg treatment

Newly laid eggs (0-1 h post oviposition) were immersed in 10ml distilled water solution containing one of five different doses (100, 200, 400, 500, or 600 ppm) of garlic extract. Thirty eggs were used for each dose and the experiment was repeated five times (30 × 5). Eggs were dipped for 1 minute in each solution. Eggs used as control were treated with appropriate amount of distilled water. They were incubated at 27°C and 75% RH in a laboratory until the eggs hatched to larvae after 7 - 14 days. Eggs which failed to hatch after 14 days were counted as unhatched eggs.

Histological study

Ovaries of 6-day old females from normal and treated groups were immersed in 3% glutaraldehyde then transferred to initial fixative. They were fixed overnight and given 3 brief rinses in a sucrose-cacodylate buffer (pH 7.2); post fixed 1hr in 1% osmium tetroxide in 0.1M cacodylate buffer, rinsed 3 times in distilled water, and dehydrated through a graded series of ethanol. Then specimens were infiltrated with Spurr's epoxy resin in a graded series of absolute alcohol-Spurr's resin mixtures, then embedded in freshly prepared Spurr's resin at 70°C for 72hrs. Semithin sections were stained with toluidine blue. Specimens were examined and photographed by equipped Leica light microscope with a camera using objective lens 40X and 100X with 20, 50 and 100 µm scales. Oocytes were measured with the image analysis software, ImageJ (Rasband, 1997).

Statistical Analysis

The obtained data was subjected to analysis of variance (ANOVA) followed by *post-hoc* analysis (Tukey's HSD test) with the help of SPSS version19 for Windows, in which the equation of the standard deviation, standard errors, and probabilities (p) were used. The level of significance was expressed as significant ($P<0.05$) and non-significant ($p > 0.05$).Probit analysis was used for the calculation of the LC50 value.

RESULTS

Effect of different concentrations of *Allium sativum* (Garlic) extract on viability of eggs laid by *Argas persicus* females treated on 3rd day after feeding

Females on 3rd day after feeding were treated with different concentrations of garlic extract (0.5, 1.5, 2.5 and 4%) and kept at 27°C and 75% RH. The eggs laid by treated female ticks were counted in comparison with the controlgroup. The eggs from normal and treated females were examined for their hatchability after 7 – 14 days incubation at the same conditions. Treated females exhibit a significant reduction ($P<0.05$) on the number of laid eggs by 38.5%, 61.2%, 58.7%, and 60.8% at 0.5, 1.5, 2.5 and 4% of garlic extract concentrations, respectively, compared to the normal females. In addition, the treatment of females with 0.5, 1.5, 2.5 and 4% of garlic extract resulted in significant reduction ($P<0.05$) in the percentage of hatched eggs (fertility) by 15.6%, 53.5%, 62.8%, and 92.3%, respectively. Basically, the fertility decreased significantly ($P<0.05$) when the concentrations used were increased (Table 1).

Effect of direct application of *Allium sativum* extract on viability of eggs

Eggs at day of deposition were dipped for one minute in water solutions containing one of five different concentrations (100, 200, 400, 500, or 600 ppm) of garlic extract. Thirty eggs were used for each concentration and the experiment was repeated

five times. They were incubated at 27°C and 75% RH in laboratory until the eggs hatched to larvae after 7 - 14 days.

Table 1. Effect of different concentrations of *Allium sativum* (Garlic) extract on viability of eggs laid by *Argas persicus* females treated on 3rd day after feeding

Conc. (%)	No. of eggs laid	% Hatched eggs (% Fertility)	% Unhatched eggs
0.5	60.7 ± 1.2 [b]	83.55 ± 1.47 [b]	16.43 ± 1.48 [b]
1.5	38.3 ± 0.88 [c]	46.02 ± 2.28 [c]	54 ± 2.31 [c]
2.5	40.7 ± 1.2 [c]	36.83 ± 2.19 [d]	63.17 ± 2.19 [d]
4	38.7 ± 1.2 [c]	7.60 ± 2.74 [e]	92.33 ± 2.60 [e]
Control	98.7 ± 0.88 [a]	99.00 ± 1.17 [a]	2.33 ± 0.89 [a]

*Data are presented as mean ± SE
*Means bearing different letters within column are significantly different (P<0.05) ANOVA, Tukey's HSD test.

The numbers of hatched eggs corresponding to each treatment group are presented in Table 2. Dipping the eggs in garlic extract with concentrations of 100, 200, 400, 500 or 600 ppm has a highly significant effect (P<0.05) on eggs hatchability compared to the untreated group, where the percentage of unhatched eggs were increased significantly with increases of garlic extract concentrations (Table 2).

Table 2. Hatchability of *Argaspersicus* eggs treated by dipping technique with different concentrations of *Allium sativum* (Garlic) extract

Conc. (ppm)	No. of Treated eggs	% Hatched eggs	% Unhatched eggs
100	30	88.89 ± 2.94[ab]	10 ± 3.87[ab]
200	30	77.78 ± 2.94[b]	22.23 ± 2.93[b]
400	30	46.67 ± 3.85[c]	53.33 ± 3.83[c]
500	30	14.44 ± 2.22[d]	85.53 ± 2.23[d]
600	30	1.11 ± 1.11[e]	98.9 ± 1.1[e]
Control	30	96.67 ± 3.3[a]	3.33 ± 1.92[a]

*Data are presented as mean±SE
*Means bearing different letters within a column are significantly different (P<0.05) ANOVA, Tukey's HSD test.

Histological effects of *Allium sativum* (Garlic) extract on ovary development of *Argaspersicus* females treated on 3rd day after feeding

General anatomy

Mated females were treated by the LC_{50} concentration of garlic extract after three days of feeding. Three days later, females were dissected under the Optika microscope, and their ovaries were examined. The ovary in normal *A. persicus*is a hollow, broad, strap-like tube that lies transversal across the posterior half of the body. It is covered dorsally by the postero-lateral diverticula of the stomach, and its ventral surface overlies the anterior portion of the rectal sac. Its entire length is approximately one-half of the body width. After mating and feeding, the oocytes protrude into haemocoel, giving the ovary a grape-like appearance (Figure 1A). On the other hand, ovaries of *A.persicus* females treated with LC_{50} concentration of garlic extract were not able to produce normal vitellogenic oocytes and appeared to be filled with young oocytes, revealing the blockage of the developmental process of oogenesis in *A.persicus* (Figure 1B). The average diameter of the oocytes inside the treated females was 0.209±0.07 mm, which decreased by 61% from the normal oocytes that had an average diameter of 0.533±0.17 mm. (Figure 1).

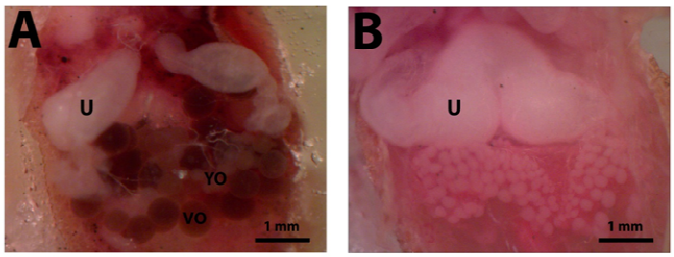

Fig.1. Photomicrograph of a dissected normal *A.persicus* female (A) showing Uterus (U), Vitellogenic oocyte (VO), young oocyte (YO). (B): dissected treated *A. persicus* female with garlic extract on day three after feeding showing immature oocytes. Scale bar = 1 mm

Structure of the developing oocytes

A histological examination of *A.persicus* females six days after feeding reveals three developmental stages of primary oocytes; young oocytes, previtellogenic oocytes, and vitellogenicoocytes (Figure 2A). The young oocytes result immediately from oogonial division. They are oval, elongated or polygonal and are facing the ovarian lumen (Figure 2A, D).The nuclei are large with irregular or rounded margins in addition to a comparatively large nucleolus (Figure 2D). The oocyte does not have evident granules in the cytoplasm. In this phase oocytes measure 112±1.66 X 78±1.40 µm. Previtellogenic oocytes are marked by great cytoplasmic growth that is rich in coarse and evenly distributed granules. At this stage, it is possible to identify the chorion surrounding the oocyte, which has a sky blue color and connected to ovarian wall by a thin stalk, funicle cell, formed of elongated epithelium cells (Figure 2C). In this phase, oocytes measure 154±1.83 X 95±3.60 µm.Vitellogenic oocytes are marked by the appearance of large yolk granules, and the chorion is thicker, with an inner vitelline membrane (Figure 2B). In this phase, the oocytes' diameter was 316 ± 0.8 µm. The protoplasm of the egg is differentiated into a densely stained peripheral layer, the periplasm, and an inner vacuolated cytoplasm or cytoplasmic network containing the yolk granules (Figure 2B). By this stage, the nucleus becomes diffused and is difficult to detect among yolk bodies.

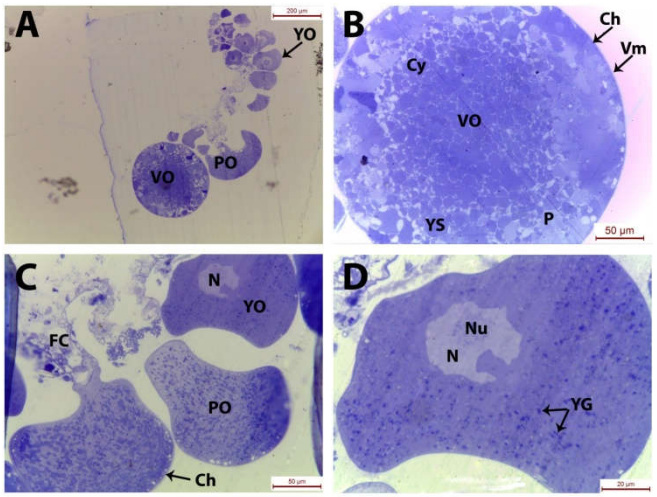

Fig. 2. Semithin sections through ovaries of female *A. persicus*6 days after feeding stained with toluidine blue, from the control group. (A): young oocytes (YO), Previtellogine oocytes (PO) and Vitellogenic oocytes (VO). (B): Vitellogenic oocytes (VO), Chorion (Ch), vitelline membrane (Vm), protoplasm (P), cytoplasm (CY) and yolk spheres (YS). (C): Funicle cell (FC), Previtellogine oocytes (PO), young oocytes (YO), Nucleus(N),Chorion (Ch). (D): young oocytes (YO), Nucleus (N), Nucleolus (Nu) and yolk granules (YG). Scale bars=20, 50, or 200 µm

The histological examination of *Argaspersicus* females treated with LC_{50} concentration of garlic extract reveals malformed oocytes. Affected oocytes measure 218±3.28 X 106±1.03 µm. Oocytes showed an alteration of morphology, which changed from rounded to elongated (Figure 3A) lysis of nucleolus (Figure 3B, C).Vacuoles can be observed in addition to cellular and chorion deformation (Figure 3B,C,D).

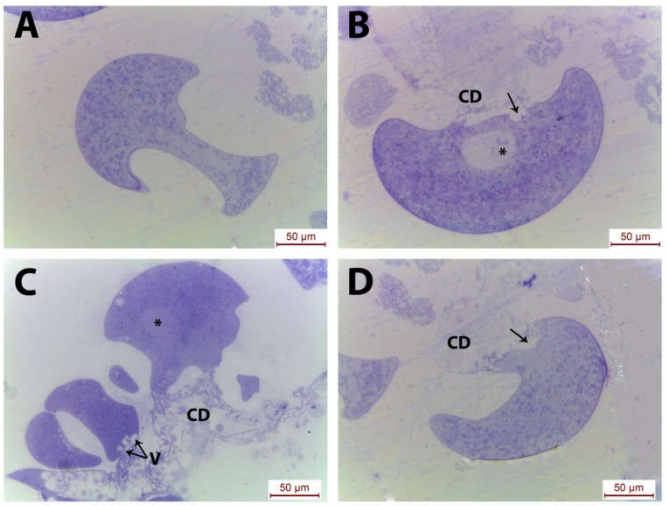

Fig. 3. Semithin sections through ovaries of female *A. persicus*6 days after feeding stained with toluidine blue, from the group treated with garlic extract at LC_{50} concentration. (A): Showing alteration of morphology. (B): Cellular deformation of funicle cells (CD), lysis of nucleolus (*). (C) Cellular deformation of funicle cells (CD), lysis of nucleolus (*) and vacuoles (V). (D) Cellular deformation of funicle cells (CD), deformation of chorion (arrow) and lysis of yolk spheres. Scale bar =50 µm

DISCUSSION

The present study demonstrated that topical application of *Argas persicus* females three days after feeding with *Allium sativum* (garlic) extract reduced eggs viability that was expressed as an impairment of eggs' hatchability. A number of plants can synthesize secondary metabolites, which are used by the plants for their protection against pathogens and pests (Dubey, 2011; Isman, 2015; Benelli, 2015a, b). The pesticidal effects of plant substances have been used against arthropods (Karunamoorthi *et al*., 2009a, b; Benelli *et al*., 2015a, b).Garlic oil along with diatomaceous earth has a toxic effect against stored-product pests (Yang *et al*., 2010). Garlic is produced in hundreds of thousands tons annually in Egypt, and it's cheap in price. *A.sativum* extract has a wide safe margin and is easy to apply on animals with low cost, but the only disadvantage of its use is the vigor odor (Aboelhadid *et al.*, 2013). This study's findings show that garlic extract could be an alternative tick control method with some benefit to chemical acaricides. Reduction of female fertility after treatment with garlic extract was reported for different insect species. Treatment of female *Dermanysuusgallinae* mites with pure garlic juice inhibited reproduction (Maurer *et al.*, 2009). Rim and Jee (2006) reported that aqueous garlic extract has a toxic effect on hatching eggs of *Aedesaegypti*. Using extracts of *Artocarpusaltilis* and *Azadirachtaindica* against adult female ticks of *Boophilusmicroplus* has a severe effect on the reproductive physiology of female ticks (Williams, 1993). Birrenkot and his colleagues (2000) reported that repeated topical application of garlic juice (10%) on hens infested with mite *Ornithonyssussylvarium* significantly reduced the level of infestation. Topical application of adult *B. microplus* females with *Melia azedarach* extract caused a partial or total inhibition of egg production and embryogenesis (Borges *et al*., 2003). Shyma *et al*. (2014) reported that treatment of *B.microplus* with garlic cloves and papaya seed extract produced complete failure of eclosion of eggs from the treated ticks, even at lower concentrations.

In addition, immersions of newly laid eggs of *A. persicus* in solution containing different concentration of garlic extract have a significant effect in reducing egg viability. Egg immersion in tested compounds enablesthe penetration to occur through the whole surface of the egg (including micropile) during the time of immersion (1minute) and afterwards. Application of 10% garlic extract in aqueous solution on eggs of *B. microplus* for 2 minutes prevents the eggs from hatching. In addition, the eggs appeared with abnormalities (Aboelhadid *et al*., 2013). Govindarajan and Rajeswary **(**2015) reported ovicidal activity of leaf and seed extract of *Albizia lebbeck* against *Culex quinquefasciatus*, *Aedesaegypti*, and *Anopheles stephensi*. Lázaro *et al*. (2012) found that dipping the eggs of *Rhipicephalusmicroplus* in 10 ml aqueous extracts of *Baccharistrimera* (50, 100, 150, or 200 mg of fresh leaves per ml) caused 100% inhibition in eggs' hatchability. Garlic essential extract consists of diallyl, dimethyl, allylmethyl, mono/di/trisulphides, and a few minor compounds. These components are diallyltrisulfide (33.57%), followed by diallyidisulphide (30.93%) and methyl allyl trisulfide (11.28%). These organosulfur compounds possess remarkable toxicity on insects (Kimbaris *et al*., 2009). The mode of action of garlic extract exerts its effect by oxidation of thiol groups present in essential proteins, causing inactivation of enzymes (Ghannoum, 1988). Among the plant derived lectins, mannose-binding lectins isolated from garlic bulb (Smeets *et al*., 1997) has gained attention. These derivative compounds were found to be toxic to *Spodopteralittoralis* (Sadeghi *et al*., 2008) and sap-sucking insects from the order Homoptera (Mondal *et al*., 2011). The insecticidal action of garlic lectins may be due to an interaction with several glycosylated receptor proteins in the midgut of insects that inhibits nutrient absorption, leading to death. It also gets accumulated into ovarioles and haemolymph, which causesinterference in the reproduction and the development of the insect (Upadhyay and Singh, 2012). Accordingly, the acaricide effect of garlic extract on tick is recommended to be due to the effect of its different constituents of sulphur compounds and lectins.

Balashov (1972) classified oogenesis in *Argaspersicus*, *Hyalommaasiaticum*, and *Ixodesricinus* into five oocyte growth stages. Stage one is a time for little development in which oocytes are formed by oogonial division and immediately enter prophase of the first maturation division without interkinesis. Stage two is considered as the great growth that ends with appearance of first yolk granules in the cytoplasm. Stage three and four are characterized by the appearances of yolk granules and end with ovulation. Stage five begins with mature eggs coming into the lumen of the ovary. In the present study, young oocytes greatly resemble stage one in Balashov's classification (1972), during which oocyte nucleus and cytoplasm increased only slightly (Diehl *et al*., 1982). Previtellogenic oocytes correspond to stage two in Balashov's classification in which the cytoplasm greatly increased and lasts till appearance of the first yolk granules. Vitellogenic oocytes are characterized by the formation of yolk granules that completely mask the nucleus, which has shifted to the oocyte periphery. They are similar to stage three and

four in Balashov's classification. However, stage five of Balashov's classification was not studied, since it only occurs in the ovarian lumen or in the oviducts (Balashov, 1972). Similar stages of oocytes development were also described in *Ornithodorosmoubata* (Wagner-Jevseenko, 1958), *Argasarboreus* (Khalil, 1969; Elshoura *et al*., 1989), *Dermacentorandersoni* (Brinton and Oliver 1971), and *Rhipicephalussanguineus* (Matos *et al*., 2014). The results of the present study show that treatment of female *A. persicus* with garlic extract 3 days after feeding resulted in decreasing the number of mature eggs, cellular deformation, chorion deformation, alteration in morphology of funicle cells and oocytes, lysis of nucleolus, and the appearance of vacuoles. Similar results wereachieved by Arnosti *et al*. (2011) in a study where the ovaries of *R. sanguineus* were exposed to acid esters of castor oil. The lack of yolk spheres in oocytes from treated females is explained by the effect of the tested compound on vitellogenin synthesis or its uptake by the oocytes. These effects have been observed in *chrysoperlacarnea* after treatment with botanical insecticide *Azadirachtin* (Medina *et al.* 2004) and also in *B. microplus* after treatment of engorged females with *Melia azedarach* extract (Sousa *et al*., 2013). Friesen and Kaufman (2002 & 2004) evaluated the inhibitory effect of cypermethrin and 20-hydroxecdysone on *Amblyommahebraeum*. This acaricide caused suppression of vitellogenesis and egg development. Vacuoles and cellular deformation caused by garlic extract indicates cell intoxication. Vaculaization is an attempt by the cell to isolate the toxic substance or even the cytoplasmic damage, so that the cell can still perform its metabolic process. In addition to toxic substance and debris, many vacuoles can also contain entire organelles that are unable to perform their metabolic function (Arnosti *et al*., 2011). As discussed by Weathersbee III and Tang (2002), the disruption of reproductive capability could lead to substantial population decline over time.

Conclusion

Regarding the result of this study, it could be implied that application of garlic extract in the field might delay tick development, and multiple applications would increase potential effects on the viability of eggs development for a more effective control strategy.

Conflict of Interest: The authors declare that they have no conflict of interest.

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