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The Crucial Role of the Endosymbiont *Pantoea* sp. in Morphology and Mating of the Pistachio Green Stink Bug, *Brachynema germari* (Hemiptera: Pentatomidae)

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ABSTRACT

The pistachio green stink bug, *Brachynema germari* Kolenati (Hemiptera: Pentatomidae), is one of the most important pests of pistachio nuts in many pistachio-growing regions in Iran. This insect harbors a gammaproteobacterial symbiont, related to the genus *Pantoea,* in the numerous crypts of its posterior midgut, which is vertically transmitted by infection of the egg masses and orally acquired by newborn nymphs. In the present study, the effects of the symbiont on host morphology, emergence rates, and mating frequency of *B. germari* were explored. For this purpose, two symbiont elimination strategies, high temperature and egg surface sterilization, were used and their effects were compared. We found external morphological changes (e.g. abnormalities in notum and wings) as well as significantly fewer emergence rates (in all stages, except for the first instar) in the surface-sterilized and heat-treated insects compared with the controls. Also, the second, third, and fourth regions of the midgut exhibited remarkable morphological changes in the aposymbiotic insects compared with the controls. Besides, less mating frequency was observed in the aposymbiotic population compared with the control. Together, these results provided a close relationship between the bacterial symbiont and *B. germari* and suggested the importance of the symbiont for the morphogenesis, development, and reproduction of the insect host.

Keywords: Alimentary canal, Body-color, Emergence rates, Mating frequency, Morphogenesis.

INTRODUCTION

Mutualistic association with symbiotic microorganisms characterizes many, if not all, animal species (Raman, 1991). The most important symbiotic associations are generally categorized into three groups. The first category consists of obligate (or primary) mutualists that are housed within a special host organ (bacteriome) and relayed within transovarial transmission between the host generations (Moran *et al.*, 2008). These symbionts are essential for host survival and reproduction (Haine, 2008). In contrast to primary symbionts, facultative or secondary symbionts are generally not essential for their host. These symbionts infect their host sporadically (Sudakaran *et al.*, 2017), and are predominantly vertically transmitted, although horizontal transmission occasionally occurs (Haine, 2008). These symbionts can be located intra- or extracellularly, and invade various cells and tissues of hosts (Su *et al.*, 2013). The third group encompasses extracellular symbionts that are localized in the gut lumen or gut-associated crypts or caeca (Sudakaran *et al.*, 2017). These bacteria are often inherited vertically to newborn nymphs by egg smearing (Sudakaran *et al.*, 2017), coprophagy (Buchner, 1965), or special symbiont-containing capsules (Fukatsu and Hosokawa, 2002), jelly (Kaiwa *et al.*, 2014), or mucus

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(Hosokawa *et al.*, 2012), but can also be horizontally acquired (Kikuchi *et al.*, 2012a).

In general, the mutualistic symbionts significantly benefit their host by playing significant roles in digestion and detoxification of food (Su *et al.*, 2013), provisioning of essential nutrients (Akman *et al.*, 2002; Engel and Moran, 2013), defense against natural enemies (Brownlie and Johnson, 2009), tolerances to environmental stresses (Wernegreen, 2012), detoxification of noxious chemicals (Kikuchi *et al.*, 2012a), nitrogen recycling (Engel and Moran, 2013), promotion of plant adaptation (Frago *et al.*, 2012), diapause modification (Rahimi-Kaldeh *et al.*, 2019), and many others. Although many roles and effects of stinkbug symbionts have been investigated in detail, understanding of their roles in host insect morphogenesis and behavior has remained poorly understood. Recently, the morphogenesis of the midgut symbiotic organ and the process of symbiont colonization were investigated in the stinkbug *Plautia stali* Scott (Hem.: Pentatomidae) (Oishi *et al.*, 2019). On the other hand, the most attention among behavioral observations was given to the manipulation of wasp behavior and alteration of the wandering behavior of stinkbugs by symbionts. The endosymbiotic bacterium *Cardinium* sp. makes changes in the oviposition choice of the parasitoid wasp, *Encarsia pergandiella* Howard (Hym.: Aphelinidae) (Kenyon and Hunter, 2007). Exploring the wandering behavior in the stink bugs *Megacopta punctatissima* and *H. halys* showed negative and positive correlations between the supply of symbiont available and the number of wandering nymphs, respectively (Hosokawa *et al.*, 2008; Taylor *et al.*, 2014). However, the symbiotic and aposymbiotic newborn nymphs of *B. germari* and *A. heegeri* showed no significant differences in wandering behavior (Kashkouli *et al.*, 2019a, 2019b).

The pistachio green stink bug, *Brachynema germari* Kolenati (Hemiptera: Pentatomidae) is one of the most important pests of pistachio nuts in Iran (Bigham and Hosseininaveh, 2010; Mehrnejad, 2001) that causes qualitative and quantitative damages. This pest has 3–5 generations per year (Ramzi and Hosseininaveh, 2010) and overwinters as an adult. The bug injury leads to desiccation, epicarp lesion, and kernel necrosis of nuts resulting in the dropping of the damaged nuts from the trees (Bagheri *et al.*, 2010; Mehrnejad, 2001). Also, this pest has been suggested to be a vector for fungal pathogen *Nematospora coryli* Peglion (Saccharomycetaceae), which is the causal agent of the diseases in pistachio nuts (stigmatomycosis) (Ershad and Barkhordary, 1974). Despite the agricultural importance of *B. germari*, its association with microorganisms is poorly understood. The previous study revealed that *B. germari* harbors a gamma-proteobacterial symbiont, related to the *Pantoea* genus, in the numerous crypts of its posterior midgut (Kashkouli *et al.*, 2019b). The symbiont is vertically transmitted by infection of the egg masses and orally acquired by newborn nymphs (Kashkouli *et al.*, 2019b). Upon hatching, the nymphs of stink bugs get into a resting status in aggregation and probe the eggs to acquire the symbiont (Kashkouli *et al.*, 2019b).

Two approaches, i.e. egg surface sterilization and heat treatment, seem possible to eliminate the symbiont of stinkbugs. Egg surface sterilization might be helpful for symbiont elimination because the symbiotic bacteria are present on the surface of the egg mass and newborn nymphs acquire them by probing the eggs (Taylor *et al*. 2017). High temperature could also be a helpful strategy for symbiont elimination because the symbionts are affected by environmental factors, especially temperature (Kashkouli *et al.*, 2018). Symbiont deprivations using these two elimination approaches illustrate the necessity of the symbionts for successful growth of *B. germari*, which symbiont eliminated insects suffered retarded growth, lower longevity, adult body weight, and a marked reduction in demographic parameters especially *r*, which is indicative of population decline (Kashkouli *et al.*, 2018, 2019b).

In the present study, we aimed to use these symbiont elimination approaches to evaluate the importance of the *Pantoea* symbiont in the

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insect morphology, emergence rates, and mating frequency of *B. germari*, and to compare the specific effects of these symbiont-manipulation techniques.

MATERIALS AND METHODS

Insect Rearing

The original population of *B. germari* was received from Pistachio Research Center (Rafsanjan, Iran), in which insect adults were collected from pistachio orchards around Rafsanjan, Kerman province, Iran (30° 21′ N, 56° 0′ E). The insects were reared on *Salsola kali* L. (insect second host plant) and pistachio nuts in the plastic cages (50×25×35 cm, covered with fine mesh net) for three generations before the experiments. A piece of wet cotton swab was supplied as a moisture resource to the insects. Rearing and all experiments were performed within incubators (Binder KBF 240, Germany), which were set at constant temperatures, 25 or 30°C, RH of 65±5%, and 16:8 (L:D) photoperiod. The egg masses laid by the 3rd generation within 24 hours were collected and subjected to the experiments.

Egg Surface Sterilization and Heat Treatment

The insects were subjected to egg surface sterilization and constant heat treatments according to the previously published method (Kashkouli *et al.* 2018). Briefly, for the heat treatment, non-manipulated eggs (183 eggs) were placed constantly in controlled temperature chambers at 30°C. In the egg surface sterilization treatment, the egg masses (168 eggs) were first treated with 96% ethanol for 5 min followed by treatment with bleach (12% NaOCl) for the maximum soaking time, 7 minutes, the eggs were then rinsed thoroughly in a separate 96% ethanol bath and then in a sterile water bath, approximately two days before hatching and then kept constantly in chambers at 25°C. In the control group, the original egg masses (83 eggs) were placed constantly in controlled temperature chambers at 25°C.

Experimental Design

The egg masses were monitored daily until all of them either reached the adult stage or died. To compare the emergence rates, the insects were checked daily during the life cycle and their growth stages were registered. The emergence rate for each stage was obtained by dividing the number with emergence/total eggs. The nominal emergence rate data were statistically analyzed using Fisher's exact probability test (SPSS v. 23).

After the adult emergence, each female was paired with a male from the same treatment. The morphometric features of the emerged females and males were measured separately. For this experiment, 34 control, 28 surface-sterilized, and 19 heat-treated females and 30 control, 21 surface-sterilized, and 16 heat-treated males were used. The lengths and widths of body, head, scutellum and the lengths of antenna and proboscis were measured using graticules (linear micrometer eyepieces, Olympus) under a stereomicroscope, 3-4 days after emergence. To record body color, the insects were photographed using a digital microscope (BMZ-04-DZ, Behin Pajouhesh. ENG. CO. Iran) with the same light and distance settings.

To monitor mating frequency, 41 control and 42 surface-sterilized females were allowed to copulate with males from the same treatments. The female mating frequency was registered every day by recording the number of females mating during preoviposition and oviposition periods. Differences in the morphometric features and female mating frequencies were analyzed using Analysis Of Variance (ANOVA) (SPSS v. 23). The graphs were plotted using Graph Pad Prism version 7.01.

Insect Dissection and Internal Body Examination

The insects were cold immobilized and the surface contaminations were removed by

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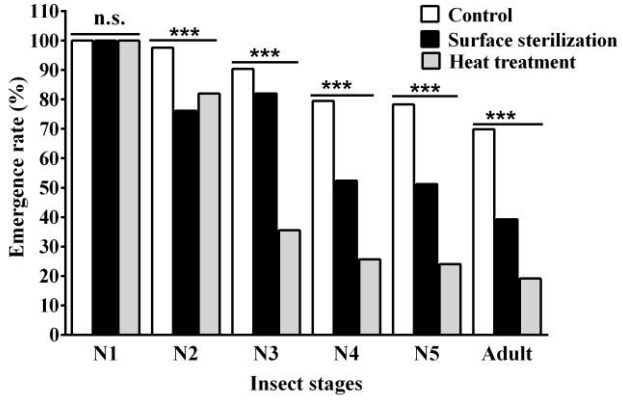

Figure 1. Effects of Surface Sterilization and Heat Treatment on the Emergence Rates of Insect. The emergence rates of immature stages and adult in the control, surface-sterilized, and heat-treated *B. germari.* The emergence rate for each stage was obtained by dividing the number with emergence/total eggs. ns: Not significant, ***: P≤ 0.001.

immersing the preserved insects in 70% ethanol (2 minutes) and rinsing with sterile de-ionized water. Then, their abdomen was incised on both sides using forceps with fine tips and the alimentary canal was pulled out carefully in sterile de-ionized water. For photographing the gut, 24 control and 16 surface-sterilized adult insects were photographed by the digital microscope and the same light and distance settings.

For DNA extraction and PCR experiments, the fourth midgut region of the adults was dissected and placed into separated 1.5 mL tubes.

DNA Extraction and PCR

The fourth midgut region of 12 control, 15 surface-sterilized, and 9 heat-treated adults were individually subjected to DNA extraction using the CinnaPure DNA kit for tissues and bacteria, according to the manufacturer's instructions (Sinaclon, Tehran, Iran). Subsequently, the extracted DNA from each sample was used for PCR. Bacterial symbiont *16S rRNA* gene was amplified using general primers 16SA1 (**5'**-AGAGTTTGATCMTGGCTCAG-**3'**) and 16SB1 (**5'**-TACGGYTACCTTGTTACGACTT-**3'**) (Hosokawa *et al*., 2006). PCR was carried out using an Eppendorf 5331 MasterCycler Gradient Thermal Cycler (VWR, Radnor, USA) by using Ampli***Taq***Gold DNA polymerase (Applied Biosystems) and its supplemented buffer system. The cycle parameters were as follows: 10 minutes at 95°C, followed by 30 cycles of 95 °C for 30 seconds, 1 minutes at 55°C, and 72°C for 1 minutes, and a final extension time of 10 minutes at 72°C (Kashkouli *et al*., 2019b). The PCR products were subjected to 1% agarose gel electrophoresis (130 V, 30 minutes).

RESULTS

Effects of Surface Sterilization and Heat Treatment on the Emergence Rates of Insect

Except for the first instars, notable differences in the emergence rates were observed among different treatments, in which significant declines were observed in the emergencies of the heat-treated and surface sterilized insects compared to the control (Figure 1). Comparing different treatments, the emergence rates in the heat-treated insects (the third and subsequent instars, as well as adults) were drastically reduced (Figure 1). The overall rate of

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emergence in the control adults was about 1.78- and 3.65-folds higher than that of the surface-sterilized and heat-treated adults, respectively (Figure 1).

Surface Sterilization and Heat Treatment Effects on the External Morphology of Insect

By comparing external morphology of treated and untreated insects, it was revealed that all control insects had normal cuticle formation (Figure 2), while some surface-sterilized (10.61%) and heat-treated (31.43%) insects had apparent difficulties while molting (Figure 2). In addition, all abnormal surface-sterilized and 45.45% of abnormal heat-treated insects died during molting.

In the constant heat condition, the insects had a softer cuticle and a lighter green (greenish-yellow) body-color instead of intensive green observed in the control and surface-sterilized insects (Figure 2).

While morphometric analyses revealed no significant differences between females in the different treatments, significant differences in the lengths of body and antenna and the widths of body and head were observed in male individuals (Figure 3).

Effects of Surface Sterilization on the Morphogenesis of Midgut

Dissection of insect bodies was done and the close relationship between the alimentary canal and ovary was observed (Figure 4). The gut morphogenesis was compared among control and surface-sterilized populations. Generally, the midgut of *B. germari* is a large and distinct part of the insect alimentary canal and morphologically divided into four distinct regions (V1-V4) connected to hindgut (Figure 5). The first section (V1) was dilated with a stomach-like shape providing necessary space for ingested food (Figure 5). The second region (V2) was a narrow tube, while the third section (V3) was wider and

shorter than the V2 (Figure 5). The fourth part (V4) was the shortest midgut portion and was characterized by many well-developed crypts, which were arranged in rows, and fused into a helical-shaped structure (Figure 5).

The V2-V4 regions of the surface-sterilized insect midguts exhibited morphological differences (Figure 5). The V2 and V4 regions were atrophied in the surface-sterilized insects (Figure 5-c), whereas these regions were enlarged in the symbiotic insects (Figure 5-a). In addition, whole or some parts of the V4 region of control insects were light yellow, while midgut fourth region of the surface-sterilized insects was constantly colorless (Figures 5-a and -c). The V3 region, by contrast, was larger in the surface-sterilized insects (Figure 5-c) compared with the symbiotic insects (Figure 5-a). The morphological differences were consistent across all the examined individuals.

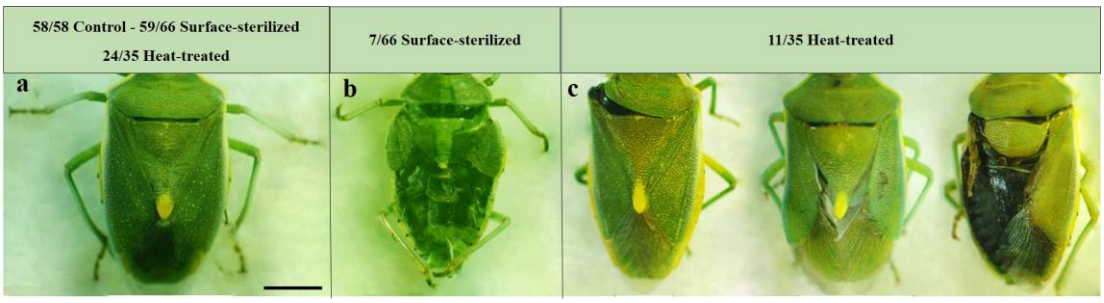

Figure 2. Effects of surface sterilization and heat treatment on the morphology of *B. germari.* Normal adult emerged from all control, 89.39% of surface-sterilized, and 68.57 of heat-treated insects **(a)**. Abnormal insects emerged from 10.61% of surface sterilized egg masses **(b)** and 31.43 of heat-treated insects **(c)**. Bars show 2 mm.1

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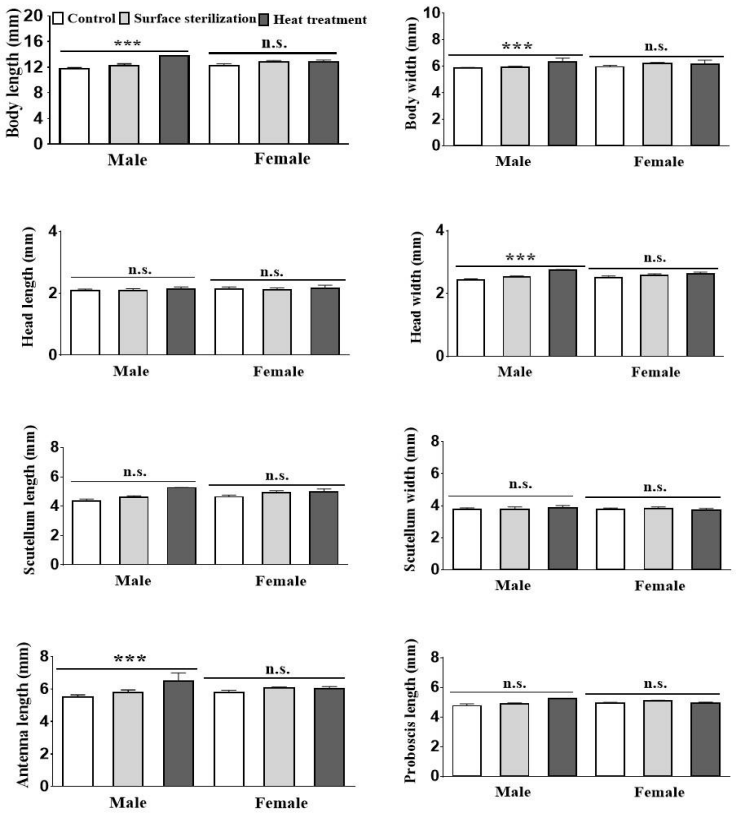

Figure 3. Comparisons of the lengths and widths of different body parts. The control, surface-sterilized, and heat-treated populations were compared. Comparisons were perfomed among females and males separately. The analyses were done by one-way ANOVA (***: P≤ 0.001, ns: Not significant).

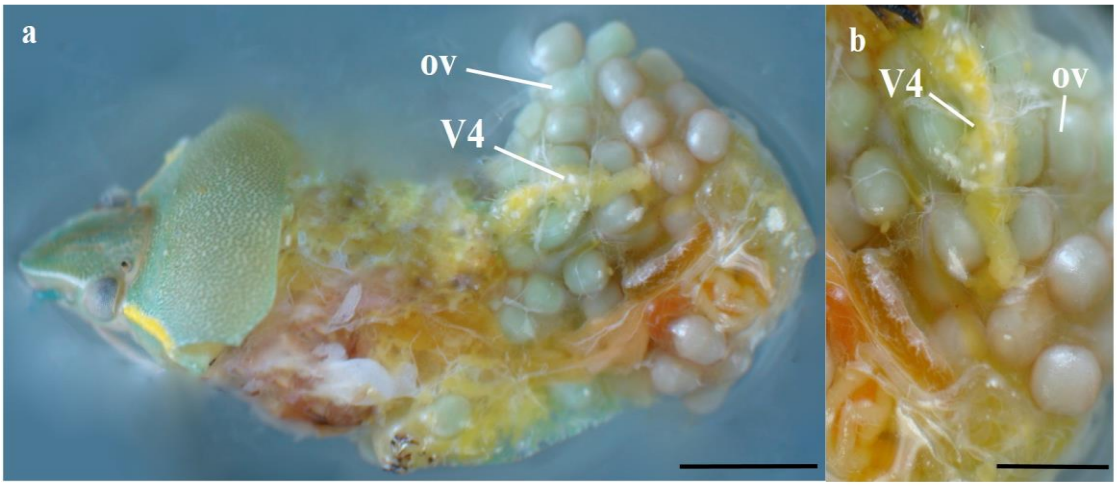

Figure 4. Close relationship between ovary (ov) and the fourth midgut region (V4). Dissected adult of *B. germari* showing anatomies of alimentary canal and ovary, bars show 2 mm **(a)**. More detailed view of this relationship, bar show 1 mm **(b)**.

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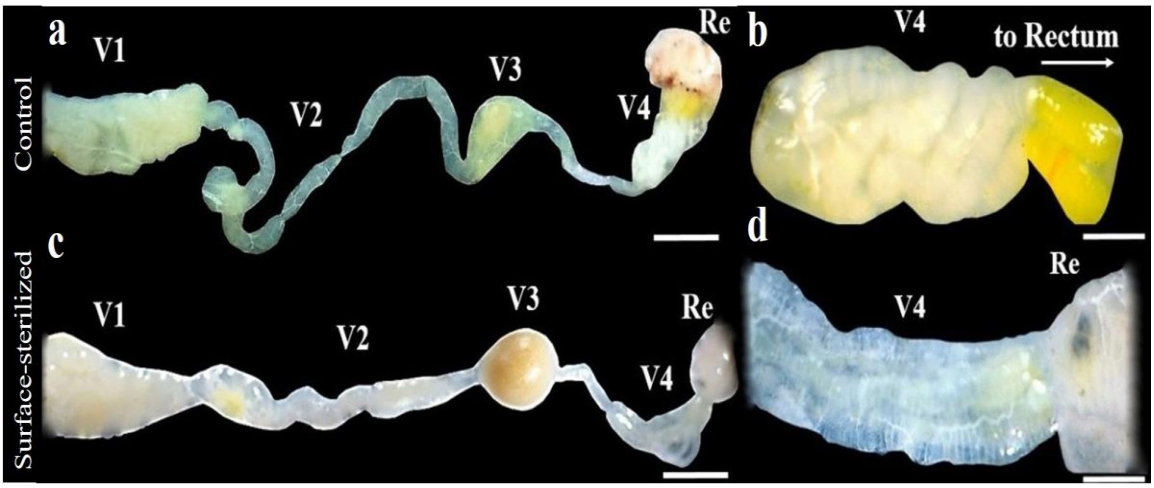

Figure 5. Gut morphologies of the symbiotic (a-b) and the surface-sterilized (c-d) *B. germari***.** Different midgut regions, V1-V4 and rectum (Re) were shown (a-c) (bar show 2 mm). More detailed view of V4 is also observed (b-d) (bar shows 500 µm). The V2 and the V4 regions were enlarged in symbiotic insects, whereas these regions were atrophied in the surface-sterilized insects. The V3 region, by contrast, was larger in the surface-sterilized compared with the control i.

Effects of Surface Sterilization on Female Mating Frequency

The female mating frequency was assessed between the control and surface-sterilized insects. Results showed that many of the surface-sterilized insects did not copulate, while the control insects could copulate naturally (Figure 6).

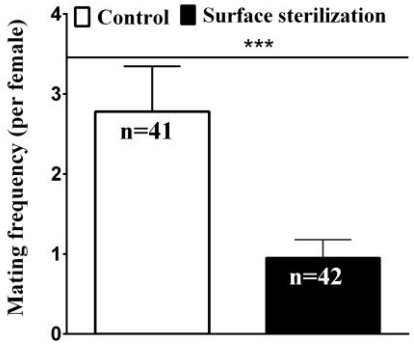

Figure 6. Mating frequencies of the symbiotic and the surface-sterilized *B. germari*. The female mating frequencies were registered daily by recording the number of females mating during preoviposition and oviposition periods. Independent sample t-test (***: P≤ 0.001) was used for comparing the means.

PCR Analyses

In all control insects (12/12), strong gel bands were detected (Figure 7). Weak gel bands of *16S rRNA* gene were observed in 6.66% (1/15) of the surface-sterilized and all heat-treated insects (12/12) (Figure 7). No gel band was observed in 93.33% of the samples from surface-sterilized insects.

DISCUSSION

In the previous study, it was confirmed that a Gammaproteobacteial symbiont, related to the genus *Pantoea* (Under accession numbers of KR261608.1 and KX258232 for the *16S rRNA* and *groEL* sequences, respectively), colonizes the host midgut crypts and is vertically transmitted via egg surface contamination (Kashkouli *et al.*, 2018, 2019b). The egg surface sterilization and heat treatment (symbiont elimination approaches) reduced the

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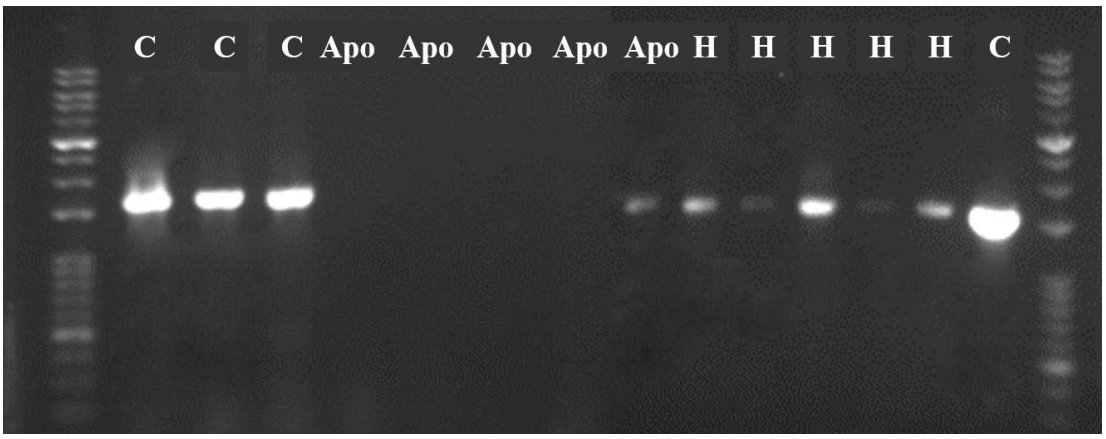

Figure 7. Effects of the egg surface sterilization and heat treatment on the symbiont of the stink bug, *Brachynema germari*. Strong gel bands were detected in Control (C), while weak bands were observed in an Aposymbiont (Apo) and all Heat-treated (H) insects.

bacterial symbiont titers about 24.18- and 3.87-folds lower than the control analyzed by qPCR (Kashkouli *et al.*, 2018). Here, the PCR detections of *16S rRNA* gene of heat-treated and surface-sterilized insects also showed the symbiont reductions in these treatments compared with the control. Comparing these two elimination approaches, the surface sterilization eliminated the symbiont almost completely, while partial symbiont removal was observed in the heat-treated insects. Therefore, we compared the surface-sterilized and the control insects in the mating frequency and internal morphogenesis experiments to better understand the role of the symbiont.

In the present study, the effects of the *Pantoea* symbiont on morphology, emergence rates, and mating frequency of *B. germari* were investigated. Here, we observed lower emergence rates as well as morphological changes in the surface-sterilized and heat-treated populations compared with the symbiotic sibling population. In addition, the mating frequency was affected by the symbiont. Previous studies have suggested that in mutualistic associations, symbionts alter morphogenesis, development, physiology, and behavior of the host to enhance transmission, stability, and functioning of itself (Su *et al.*, 2013).

We found that the symbiont was involved in the normal formations of the notum and wing of *B. germari*. Also, significantly fewer emergence rates were observed in surface-sterilized and heat-treated *B. germari*. Aposymbiotic insects of *Adomerus triguttulus* (Hosokawa *et al.*, 2013), *Plautia stali* (Hosokawa *et al.*, 2016), and *M. punctatissima* (Hosokawa *et al.*, 2006) also exhibited lower adult emergence rate than the symbiotic insects. Therefore, it can be concluded that the insect molting was affected by the symbiont. *Nezara viridula* L. (Hem.: Pentatomidae) nymphs had difficulties during final molting, some of which exhibited abnormal cuticle formation under simulated warming condition (Musolin *et al.*, 2010). A similar result was also observed in *Wolbachia*-eliminated butterfly, *Eurema hecabe* L. (Lep.: Pieridae), as the adult insects emerged with deformed wings (Narita *et al.*, 2007).

Comparing the effects of surface sterilization and heat treatments, we observed that the emergence rates of the heat-treated third, fourth, and fifth in stars, as well as adults, dropped off dramatically compared with surface-sterilized insects. This phenomenon can be concluded as the effect of heat treatment itself. In addition,

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the adult insects reared under heat treatment were apparently in a weaker physical shape; exhibited a softer cuticle and greenish-yellow body coloration (not to be confused with genetic color morphs), whereas other insects had a normal green color. Although the aposymbiosis-associated color changes (Fukatsu and Hosokawa, 2002; Hosokawa *et al.*, 2006, 2013, Kikuchi *et al.*, 2009, 2012b) have been reported for several hemipteran bugs, in the present study, it seems that the body color differentiation was related to the heat treatment itself rather than the symbiont. On the other hand, abnormal aposymbiotic individuals displayed more severe deformation than the heat-treated ones. This differentiation can be related to the different levels of symbiont elimination in the surface sterilization and heat treatments.

In the surface-sterilized *B. germari*, the V2, V3 and V4 regions of midgut exhibited remarkable morphological differences. The V2 and V4 regions were enlarged in symbiotic insects, whereas these regions were atrophied in the surface-sterilized insects. The V3 region, by contrast, was larger in the surface-sterilized insects than that in the symbiotic insects. Enlargement of the V2 and the V4 regions in symbiotic insects may reflect induction/suppression of many genes and functions involved in symbiosis, while enlargement of V3 region in the surface-sterilized insects may be, although speculative, due to resource allocation between the adjacent midgut regions (Futahashi *et al.*, 2013). Similar results were observed in *Riptortus pedestris* Fabricius (Hem.: Alydidae), in which the gut morphology was affected by the symbiont (Futahashi *et al.*, 2013). Symbiont-induced morphogenetic have been documented from various mutualistic associations, such as formation of the symbiotic structure in the host midgut by the symbiont *Burkholderia* (Kikuchi *et al.*, 2011), normal morphogenesis of symbiotic light organ in the *Euprymna squids–Vibrio fischeri* luminescent symbiosis (Nyholm and Mcfall-ngai, 2004), nodule formation of root in the legume–*Rhizobium* nitrogen-fixing symbiosis (Oldroyd and Downie, 2008), and others. On the other hand, while the V4 is a yellow-colored region in symbiotic insects, this region in the surface-sterilized insects is yellowish-white. Symbiont-deprived *Sibaria englemani* also exhibited qualitative differences in the morphology of symbiont containing caeca, including increased translucency of crypts (Bistolas *et al*., 2014). It is of interest whether or not the *B. germari* symbiont is involved in the formation of the alimentary canal; future experimental and developmental studies may help us to better understand this relationship.

Here, we observed the close relationship between the V4 and ovary. As it has been proven that the gut symbiont deprivation led to a reduction in the number of eggs laid by females (Kashkouli *et al.*, 2018, 2019b), the gammaproteobacterial symbiont of *B. germari* might have a role in the egg formation, ovary development, or maturation. The necessity of bacterial symbionts for ovary development and oogenesis in insects have been reported for some insects including *R. pedestris* (Lee *et al.*, 2017), *E. hecabe* (Narita *et al.*, 2007), and *Asobara tabida* Nees (Hymenoptera, Braconidae) (Dedeine *et al.*, 2001), for which *Wolbachia* was found to be required for egg maturation and successful oogenesis.

Here, we report that in *B. germari*, the mating frequency was affected by the symbiont as the significantly lower rate of mating was observed in the surface-sterilized insects. As the symbionts and the hosts both benefit from the reproduction multiplicity, it might have been evolutionarily favored by host acting on both the partners (Su *et al.*, 2013).

These results besides previous investigation (Kashkouli *et al.*, 2018, 2019b) suggest that the overall fitness of *B. germari* is highly dependent on the presence of this symbiotic association. The developmental processes and mechanisms underlying the morphological and behavioral changes deserve further studies. As the pistachio green stink bug, *B. germari* is an abundant

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and serious pest of pistachio nuts in Iran, further investigations may lead to symbiosis-based management tactics such as application of a sterilizing agent targeting the bacterial symbionts on the egg surface of this important pest.

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نقش اساسی درون‌همزیست *Pantoea* sp. در ریخت‌شناسی و جفت‌گیری سن سبز پسته، *Brachynema germari* (Hemiptera: Pentatomidae)

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چکیده

سن سبز پسته، *Brachynema germari* Kolenati (Hemiptera: Pentatomidae)، یکی از مهمترین آفات پسته در بسیاری مناطق پرورش پسته ایران است. این حشره دارای همزیست gammaproteobacteria، مربوط به جنس *Pantoea*، در تعدادی کریپت‌های بخش انتهایی معده خود می‌باشد که این باکتری‌ها به‌صورت عمودی با آلوده‌سازی دسته‌های تخم انتقال می‌یابند و پوره‌های تازه متولد شده به‌صورت دهانی آنها را دریافت می‌کنند. در مطالعه حاضر، اثرات همزیست بر ریخت-شناسی، نرخ ظهور و تعداد جفت‌گیری *B. germari* بررسی شده است. برای این منظور، دو استراتژی حذف همزیست یعنی تیمار حرارتی و ضدعفونی سطحی دسته‌های تخم استفاده شده و اثرات آنها مورد مقایسه قرار گرفتند. نتایج نشان دهنده تغییرات ریخت‌شناسی خارجی (بدشکلی در نوتوم و بال‌ها) به‌همراه کاهش معنادار نرخ ظهور (در همه مراحل به جز پوره سن اول) در حشرات ضدعفونی سطحی و تیمار حرارتی شده در مقایسه با کنترل بود. همچنین، بخش‌های دوم، سوم و چهارم معده تغییرات ریخت‌شناسی قابل توجهی در حشرات بدون همزیست در مقایسه با کنترل نشان دادند. به‌علاوه، تعداد جفت‌گیری کم‌تری در جمعیت بدون همزیست در مقایسه با کنترل مشاهده شد. نتایج این پژوهش ارتباط نزدیک بین باکتری همزیست و *B. germari* را نشان داد و اهمیت همزیست برای مورفوژنزیز، نمو و تولیدمثل حشره میزبان را پیشنهاد نمود.

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