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Does host stage affect the morphometry of *Aenasius arizonensis* (Girault), a solitary endoparasitoid of *Phenacoccus solenopsis* Tinsley?

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Abstract
We investigated the influence of three nymphal instars (1st, 2nd and 3rd) and adult stages of *Phenacoccus solenopsis* Tinsley on the morphometric parameters of *Aenasius arizonensis* (Girault). The study was conducted to identify the suitable host stages for quality improvement in mass production of *A. arizonensis* for biocontrol program against *P. solenopsis*. The female parasitoid parasitized all the stages of *P. solenopsis* except 1st instar nymphs, while the parasitism of 2nd instar nymphs yielded only males in the progeny. The length and breadth of parasitoid mummies (pupae); body size, antenna length, the length of fore-leg, middle-leg and hind-leg, and wing size of both male and female adults increased significantly with an advancement of host stage. The measurements through Scanning Electron Microscopy images also confirmed that the length of hind-leg increased with increase in host stage. The results of this study have implications for utilizing adult and 3rd instar nymphal stages in mass production *A. arizonensis* for larger sized progeny and devising an effective biological control strategy against *P. solenopsis*.

Keywords: *Phenacoccus solenopsis*, *Aenasius arizonensis*, morphometry, parasitism, scanning electron microscopy, progeny

1. Introduction

Mealybugs (Hemiptera: Pseudococcidae) with 1,970 species in 271 genera worldwide form the second largest family of the scale insects [5]. Over the past decade, *Phenacoccus solenopsis* Tinsley, an exotic mealy bug species have come forth as a dangerous threat to cotton cultivation in India [27]. During 2007, this mealy bug pest caused up to 30 to 40 percent loss in the yield of cotton in Punjab, India [12]. *P. solenopsis* is a polyphagous pest and has a wide morphological diversity, biological adaptations and ecological adaptations that allow it to feed on diverse host plants, including plant species of economic importance [11, 27]. Due to waxy material covering the mealy bug body and high rate of proliferation, it is difficult to manage this pest with insecticides.

Aenasius arizonensis (Girault) (Hymenoptera: Encyrtidae), a solitary nymphal and adult endoparasitoid has been identified as a key mortality factor of *P. solenopsis* under field conditions. It has been observed to be only ecological and fundamental host of *A. arizonensis* [37]. The parasitoid has neither been imported purposefully nor released artificially and is a classic example of fortuitous biological control in India [14, 30]. Field parasitism by *A. arizonensis* to an extent of 95 per cent has been reported in *P. solenopsis* [23, 31]. It can be qualified as an important biological control agent of *P. solenopsis* due to its salient attributes like adaptability to the environmental conditions, high host searching efficiency, high dispersal capacity, amenable to easy culturing in the laboratory, faster multiplication than the host, high female: male ratio in progeny and a synchronized life cycle with its host [28].

Mass production procedures in biological control programmes primarily emphasize on the generation of parasitoid adults possessing high fitness levels. Recognition of the key factors contributing to parasitoid's fitness holds relevance towards improving its performance under field conditions. Body size is an important index of fitness or quality in parasitoid's [20, 33]. Enhanced reproductive fitness *viz.*, host searching efficiency, reproductive longevity, daily fecundity and female-biased progeny sex ratio is attributable to larger body size in parasitoid's [20, 21, 33, 35]. A larger body size is advantageous for the reproductive fitness of female parasitoids due to the allocation of more resources to reproduction and efficient management of sperm transferred by males [32, 38].

Larger sized individuals also represent higher quality mates in parasitoids [7]. Moreover, the body size of mass-reared parasitoids correlates with their performance under field conditions [40].

Knowledge on the morphometry of *A. arizonensis* is an important prerequisite to optimize the mass production of high fitness parasitoids for their use in biological control programmes [2]. Most of the studies by earlier workers have documented morphometric parameters of *A. arizonensis* irrespective of host stage [2, 34]. Few studies have published the comparison of parasitoid adults emerged from different host stages on the basis of hind tibial length only [4, 13, 41]. The comprehensive information about whether and to what extent different host stages affects morphometry of this solitary endoparasitoid is still lacking. Moreover, the precise morphometric measurements through Scanning Electron Microscopy (SEM) have been documented for the first time. The information generated could be useful in improving the quality of this parasitoid in mass rearing system and implementing effective strategies for augmentative releases of quality individuals in biological control programmes against *P. solenopsis*.

2. Materials and methods

2.1 Rearing of *P. solenopsis*

The culture of *P. solenopsis* was maintained on sprouted potatoes (*Solanum tuberosum* L.) for multiple generations in an environmental chamber at 27 ± 2 ^{0}C, 70 ± 5 % relative humidity and under a 14L: 10D photoperiod according to the procedures outlined by Nagrare *et al*. (2011) [28]. Potato sprouts of length 2.5-5 cm were placed in glass jars (20 x 15 cm) and inoculated with gravid females using a soft camel hair brush. The moist soil was kept at the base of each jar to maintain the turgidity of inoculated potato sprouts. The jars were covered with muslin for the establishment of mealybug colonies. The culture of *P. solenopsis* thus maintained was further used for rearing of *A. arizonensis*.

2.2 Rearing of *A. arizonensis*

Stock culture of *A. arizonensis* was maintained on the *P. solenopsis* at 27 ± 2 ^{0}C, 70 ± 5 % relative humidity and under a 14L: 10D photoperiod. Field collected parasitized mealybugs (mummies) were obtained from *P. solenopsis* infested cotton, *Hibiscus* sp. and weed hosts (*Abutilon* sp., *Sida* sp. and *Parthenium* sp.) and kept in glass vials plugged with cotton wool. The naive parasitoid adults obtained from mealy bug mummies were sexed [16] and were released in glass jars having healthy colonies of mealy bug maintained on sprouted potatoes. After parasitism, the mummies were collected and kept in glass vials plugged with cotton wool. Honey streak on a paper strip was utilized as a source of food for the emerging adult parasitoids in glass vials. The newly emerged *A. arizonensis* adults were employed for further experiments.

We allowed 2-day old mated female of *A. arizonensis* to parasitize the three nymphal instars (1st, 2nd and 3rd) and adult stages of *P. solenopsis* at parasitoid: host ratio of 1: 10 kept separately in glass jars (20 x 15 cm) covered with muslin. A cotton swab dipped in 10 percent honey solution was hung in each jar as a source of food for *A. arizonensis* adults. Five replicates of each treatment were maintained. The mealy bugs were inspected daily for mummy formation. Fifty randomly selected mummified Puparium of each host stage were collected from stock cultures and kept separately in glass vials (4 x 3 cm) prior to adult emergence for morphometric observations. The parasitoid adults, thus emerged from different host stages of *P. solenopsis* were killed by refrigeration and then used for further morphometric studies.

2.3 Measurement of *A. arizonensis* mummies and adults

The present studies on morphometry of *A. arizonensis* were conducted at Acarology laboratory, Department of Entomology, Punjab Agricultural University, Ludhiana during 2015. The morphometric parameters of mummies and *A. arizonensis* adults that emerged from different stages of *P. solenopsis* were measured. The length and breadth of mummies; body length and width, head width, antenna length, length of fore-leg, middle leg and hind-leg, wing length and width of both male and female *A. arizonensis* adults that emerged from several instars of *P. solenopsis* were measured with an accuracy of 0.01 mm using stereo-zoom microscope (SZ40, Olympus, India) equipped with an ocular micrometer.

An analysis of variance (ANOVA) for a complete randomized design (CRD) was utilized to analyze the data on morphometric parameters of mummies and *A. arizonensis* adults. Statistical analyses were realized by using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp Armonk, NY, USA). The significance of differences was tested by F-tests, and the significance of differences between treatment means was compared using Tukey student's range (HSD) test at 5 percent probability level. The morphometric parameters of *A. arizonensis* female adults that emerged from 3rd instar nymphal and adult host stages were compared using Student's t-test to calculate the level of significance.

2.4 Sample processing for Scanning Electron Microscopy

The samples of *A. arizonensis* adults were fixed in 2.5 % Glutaraldehyde at 4^o C overnight. The fixative was drained and 3 items of washing were performed with 0.1M Sodium Cacodylate buffer for 15 min at 4^o C. The wash buffer was drained and 1 % Osmium tetraoxide was added and kept for 1-2 hours at 4^o C. Osmium tetraoxide (1 %) was drained and 3 items of washing were performed with 0.1M Sodium Cacodylate buffer for 15 min at 4^o C. It was followed by washings with 30, 50, 70, 80, 90, 95 percent Ethanol. They were incubated for 15 minutes at 4^0 C after each washing. The samples were then washed thrice with 100 percent Ethanol followed by incubation for 20 minutes at room temperature each time. The samples of *A. arizonensis* were placed in a vacuum desiccator overnight. After drying, the samples were mounted on aluminium stubs (25 x 40 mm) with adhesive tapes. Sputter coating of mounted samples was done with gold: palladium (60: 40) using Ion Sputter Coater (Hitachi E-1010, Japan).

2.5 Scanning Electron Microscopy sample imaging

Scanning Electron Microscope (S-3400N, Hitachi, Japan) equipped with an Energy-dispersive detector (Thermo Noran System SIX) was used to generate high-resolution pictures and accurate measurements for hind-leg of A. *arizonensis* male and female adults that emerged from 3rd instar nymphs and adult stages of *P. solenopsis*. Scanning Electron Microscopy was conducted at Electron Microscopy and Nanoscience Laboratory, Punjab Agricultural University, Ludhiana during 2015. Sputter coated parasitoid specimens were imaged using an objective lens aperture of diameter 500 µm with a working distance (10.0 to 10.9 mm) under high accelerating voltage conditions (15 kV). Images with a resolution (1280 x 960 pixels) each were recorded at magnifications (60 to 110x).

3. Results and Discussion

3.1 *A. arizonensis* mummies

A. arizonensis females did not parasitize the 1st instar nymphs of *P. solenopsis*, so the measurements of mummies were possible from 2nd and 3rd instar nymphs as well as adults of the mealybug host. The length ($F_{2,147}$ = 2355.91; P<0.0001) and width ($F_{2,147}$ = 3189.41; P<0.0001) of *A. arizonensis* mummies increased significantly with an advancement in host stage (Table 1). Significantly longer mummies (3.49±0.01 mm) were recorded in adult host stage, followed by 3rd instar (2.48±0.02 mm) and 2nd instar nymphs (1.80±0.01 mm). Similarly, mean width was also found to be significantly higher (1.88±0.01 mm) in mummies from adult host stage followed by 3rd instar (1.54±0.01 mm) and 2nd instar (0.87±0.01 mm) nymphal host stages (Table 1).

In the present study, the dimensions of *A. arizonensis* mummies increased with an increase in host stage. The differences in measurements among mummies of different host stages may be due to the variation in the body size of mealybug at different life stages. The increase in body size of mealybug, *P. solenopsis* with the advancement of age in an increasing order of 1st instar nymphs<2nd instar nymphs<3rd instar nymphs<adult has been reported by Hodgson *et al*. (2008)[18] and Dhawan and Saini (2009)[10]. No information is available in the literature regarding the effect of different host stages on the size of *A. arizonensis* pupae (mummies). Irrespective of host stage, Sangle *et al*. (2012) [34] and Aga *et al*. (2016)[2] reported that length and width of *A. arizonensis* (= *A. bambawalei*) pupae varied from 3.37 to 4.43 mm and 1.19 to 2.56 mm, respectively; which corroborate with the present findings with slight deviations which may be due to differences in the host stage and/or environmental conditions.

3.2 *A.* arizonensis male adult progeny

A significant increase in morphometric parameters for *A. arizonensis* (male) adults such as body length ($F_{2,51}$ = 106.77; P<0.0001), body width ($F_{2,51}$ = 89.64; P<0.0001), antenna length ($F_{2,51}$ = 60.65; P<0.0001), forewing length ($F_{2,51}$ = 121.83; P<0.0001) and width ($F_{2,51}$ = 135.59; P<0.0001), hindwing length ($F_{2,51}$ = 121.77; P<0.0001) and width ($F_{2,51}$= 104.11; P<0.0001) were recorded with an advancement of host stage. The length of fore-leg ($F_{2,51}$ = 79.35; P<0.0001), middle-leg ($F_{2,51}$ = 93.94; P<0.0001) and hind-leg ($F_{2,51}$ = 181.07; P<0.0001) was also recorded to be significantly longer in male adults that emerged from parasitized *P. solenopsis* adults followed by the 3rd and 2nd instar nymphs (Table 2). The head width of *A. arizonensis* male adults was significantly higher in adults that emerged from adult host stage ($F_{2,\ 51}$ = 73.15; P<0.0001). However, it was not statistically different in adults from 3rd instar and 2nd instar host nymphs (Table 2).

3.3 *A. arizonensis* female adult progeny

It is worthwhile to mention here that no female adults emerged from the mummies of 2nd instar *P. solenopsis* nymphs, so the measurements were possible only in *A. arizonensis* female wasps that emerged from 3rd instar nymphal and adult host stages (Table 3). Female wasps that emerged from *P. solenopsis* adults exhibited significantly higher mean values for body length (t_{58} = 41.47; P<0.0001) and width (t_{58} = 10.68; P<0.0001), head width (t_{58} = 17.95; P<0.0001), antenna length (t_{58} = 15.45; P<0.0001), forewing length (t_{58} = 17.63; P<0.0001) and width (t_{58} = 7.18; P<0.0001), hindwing length (t_{58} = 12.69; P<0.0001) and width (t_{58} = 39.02; P<0.0001) as compared to those which emerged from 3rd instar nymphs. Significantly longer fore-leg (t_{58} = 28.74; P<0.0001), middle-leg (t_{58} = 11.71; P<0.0001) and hind-leg (t_{58} = 10.49; P<0.0001) was recorded for female wasps that emerged from adult host stage in comparison to those which emerged from 3rd instar *P. solenopsis* nymphs (Table 3).

3.4 Scanning Electron Microscopy measurements of *A. arizonensis*

The measurements through SEM images revealed relatively longer (1009 µm) hind leg for *A. arizonensis* male adults that emerged from adult host stage, followed by those which emerged from 3rd instar nymphs of *P. solenopsis* (708 µm) (Figure 1). However, it was lowest (605.1 µm) in adults from 2nd instar nymphs. A similar trend was observed in the case of female adults; wherein, the length of hind leg was also longer (1635 µm) in adults that emerged from adult host stage as compared to those which came out from 3rd instar *P. solenopsis* nymphs (1461 µm) (Figure 2). Present results revealed for the first time the precise measurements of *A. arizonensis* adults that have emerged from different stages of *P. solenopsis* through SEM images. The length of hind-leg increased with an advancement in host stage.

The quantity of nutritional resources available during larval development is an important determinant of body size in parasitoids [19, 26]. These nutritional resources are often considered as an index of host quality which varies with host stage/size [15, 36]. Moreover, parasitoids may differ in their strategy for utilizing the host body resources in accordance with the host stages. Idiobionts modify their brood/ clutch size in response to the fixed quantity of host body resources available for the survival and development of its progeny in non-feeding host stages (eggs or pupae) [3, 39]. In contrast, koinobionts like *A. arizonensis* use alternative cues, such as host nutritional status and future growth potential, as the basis for assessment of host quality. Such parasitoids maximize their progeny fitness by preferentially laying eggs in larger, more resourceful hosts and delaying the disruption in the acquisition of food by the host after parasitism; thereby, providing a window of opportunity to the parasitoid progeny for completing their development in feeding and fully functional host[15,29].

It is worthwhile to mention here that no parasitism was observed in 1st instar nymphal stage of the mealybug host, *P. solenopsis*. Less preference for 1st instar mealybug nymphs may be due to inadequate/ reduced body resources available at younger stages of the host to the parasitoid progeny [8, 9]. Godfray (1994) [15] also indicated that higher fitness costs were involved in the parasitism of small hosts by koinobiont parasitoids, as the development time is often lengthened and survival is often reduced. The present results on less preference for younger stages are consistent with the findings of Fand *et al*. 2011 [13], He *et al*. 2015 [17] and Zhang *et al*. 2016 [42]. Similar findings have also been documented in other parasitoids like *Aenasius vexans* Kerrich on *Phenacoccus herreni* Cox and Williams [6] and *Anagyrus loecki* Noyes and Menezes on *Phenacoccus madeirensis* Green [8].

Our studies revealed that parasitism of 2nd instar nymphal stage of *P. solenopsis* yielded only *A. arizonensis* male adults in the progeny. The present results corroborate with the studies conducted by Fand *et al*. (2011) [13] and Abdin *et al*. (2013) [1], who also reported that the progeny of *A. arizonensis* (= *A. bambawalei*) from 2nd instar *P. solenopsis* nymphs produced primarily males. Similar findings have also been reported in other parasitoid species like *Aphidius ervi* (Haliday) on *Acyrtosiphon pisum* Harris [36], *Leptomastix epona* (Walker) on *Pseudococcus viburni* (Signoret) [22] and *A. vexans* on *P. herreni* [6].

Our findings revealed that all the morphometric parameters of both male and female *A. arizonensis* adults increased with a subsequent increase in host stage, which was offered initially to them for parasitism. Larger sized hosts might have provided more resources for the development of the parasitoid which could be a possible reason for the emergence of larger sized *A. arizonensis* adults from later host stages of *P. solenopsis*. Larger sized parasitoids exhibit enhanced reproductive fitness and female-biased sex ratio [20, 21, 33, 35] which is highly desirable for improving mass rearing system and increasing the efficiency of any biological control programme. A positive influence of host stage on adult body size has also been documented in several other parasitoids like *Aphidius sonchi* Marshall on *Hyperomyzus lactucae* (L.) [25], *Ephedrus californicus* Baker on pea aphids [24], *A. ervi* on *A. pisum* [36], *A. vexans* on *P. herreni* [6].

Irrespective of host stage, Sangle *et al*. (2012) [34] and Aga *et al*. (2016) [2] have documented various body appendage measurements of *A. bambawalei* (= *A. arizonensis*) adults, which corroborate with the present findings with slight deviations. The present results are also in agreement with the findings of Zhang *et al*. (2014) [41] and Badshah *et al*. (2016) [4] who also observed that the hind tibia length of *A. arizonensis* (= *A. bambawalei*) adults that emerged from the adult stage of *P. solenopsis* were significantly higher as compared to 3rd instar host stage. However, Fand *et al*. (2011) [13] reported that the hind tibial length was significantly higher in *A. bambawalei* adults from 3rd instar *P. solenopsis*, followed by adult and 2nd instar host stages.

Table 1: Morphometric measurements of mummies from different stages of *P. solenopsis*

Host stage at parasitization[a]	**Measurements (mm; *n*=50)**	
	Mummies length	**Mummies width**
2nd instar	1.80±0.01c	0.87±0.01c
3rd instar	2.48±0.02b	1.54±0.01b
Adult	3.49±0.01a	1.88±0.01a

Note: [a]No successful parasitism occurred on 1st instar nymphs of *P. solenopsis*; Mean (±SE) within the same column followed by different lowercase letters are statistically different at *P*<0.05 level according to ANOVA: Tukey HSD test.

Table 2: Morphometric parameters of *A. arizonensis* adults (male) that emerged from different stages of *P. solenopsis*

Host stage at parasitization[a]	**Measurements (mm)**					
	Body length	**Body width**	**Antenna length**	**Head width**	**Fore-wing length**	**Fore-wing width**
2nd instar	0.70±0.01c	0.35±0.01c	0.50±0.01c	0.40±0.01b	0.67±0.00c	0.31±0.00c
3rd instar	0.94±0.01b	0.41±0.01b	0.55±0.01b	0.43±0.01b	0.95±0.00b	0.50±0.00b
Adult	1.28±0.01a	0.47±0.00a	0.59±0.00a	0.51±0.00a	1.19±0.01a	0.54±0.01a

Host stage at parasitization[a]	Measurements (mm)				
	Hind-wing length	Hind-wing width	Fore-leg length	Mid-leg length	Hind-leg Length
2nd instar	0.39±0.01c	0.15±0.00c	0.37±0.00c	0.54±0.01c	0.62±0.00c
3rd instar	0.47±0.01b	0.23±0.00b	0.42±0.00b	0.63±0.00b	0.73±0.00b
Adult	0.55±0.01a	0.29±0.00a	0.50±0.01a	0.82±0.00a	1.03±0.01a

Note: [a]No successful parasitism occurred on 1st instar nymphs of *P. solenopsis*; Mean (±SE) within the same column followed by different lowercase letters are statistically different at *P*<0.05 level according to ANOVA: Turkey HSD test.

Table 3: Morphometric parameters of *A. arizonensis* adults (female) that emerged from different stages of *P. solenopsis*

Host stage at parasitization[a]	**Measurements (mm)**					
	Body length	**Body width**	**Antenna length**	**Head width**	**Fore-wing length**	**Fore-wing width**
3rd instar	1.47±0.00b	0.80±0.00b	0.59±0.00b	0.78±0.01b	1.18±0.01b	0.60±0.01b
Adult	1.77±0.01a	0.88±0.01a	0.67±0.01a	0.89±0.01a	1.36±0.01a	0.66±0.01a

Host stage at parasitization[a]	Measurements (mm)				
	Hind-wing length	Hind-wing width	Fore-leg length	Mid-leg length	Hind-leg Length
3rd instar	0.61±0.01b	0.26±0.01b	1.15±0.00b	1.32±0.01b	1.47±0.01b
Adult	0.68±0.01a	0.49±0.01a	1.31±0.01a	1.39±0.01a	1.61±0.01a

Note: [a]No successful parasitism occurred on 1st instar nymphs of *P. solenopsis*; only male adults emerged from mummies of 2nd instar *P. solenopsis*; Mean (±SE) within the same column followed by different lowercase letters are statistically different at *P*<0.05 level: student's t-test.

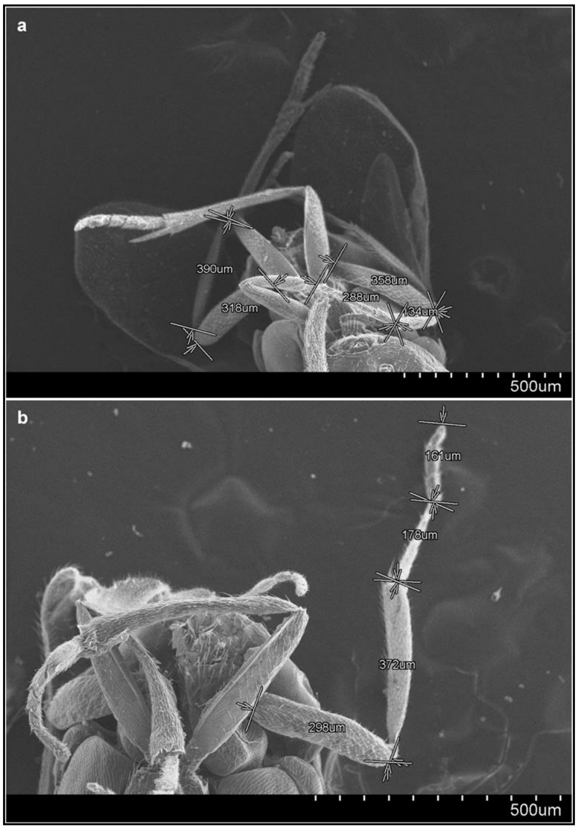

Fig 1: Scanning Electron Microscopy images with measurements for hind-leg of *Aenasius arizonensis* male adults that emerged from 3rd instar nymphal (a) and adult (b) stages of *Phenacoccus solenopsis*. Arrows are used to indicate the measurements of the different segments of hind leg.

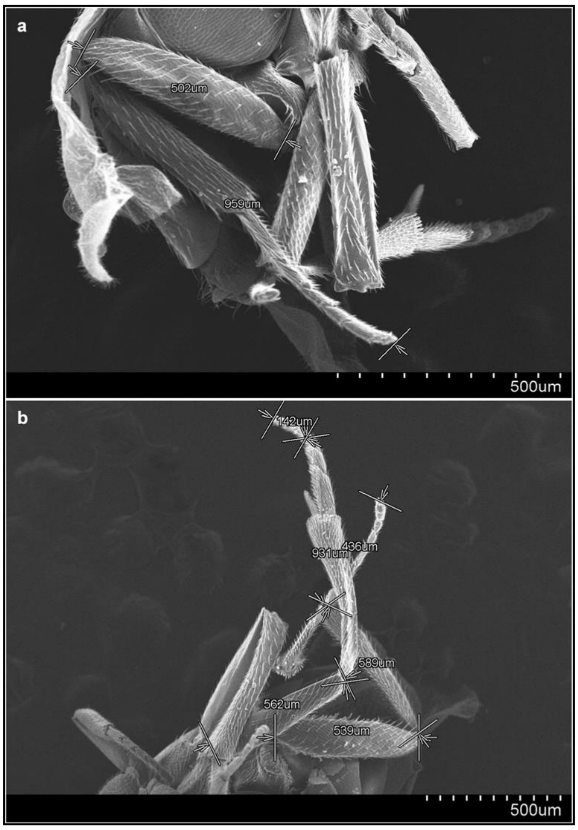

Fig 2: Scanning Electron Microscopy images with measurements for hind-leg of *Aenasius arizonensis* female adults that emerged from 3rd instar nymphal (a) and adult (b) stages of *Phenacoccus solenopsis*. Arrows are used to indicate the measurements of the different segments of hind leg.

4. Conclusions
The present findings indicated that parasitism of 3rd instar nymphs and adults of *P. solenopsis* yielded larger sized *A. arizonensis* adults in the progeny. Therefore, 3rd instar nymphal and adult stages of *P. solenopsis* may be considered as suitable for the mass production of *A. arizonensis* on the account of greater nutritional availability for the parasitoid. A better understanding of the parasitoid morphometry emerged from different host stages could be useful for monitoring the fitness or quality of *A. arizonensis* adults in the mass production system and thereby improving its field performance against *P. solenopsis* in biological control programmes. The authors further recommend future studies to assess the influence of a larger parasitoid size on the fitness of *A. arizonensis*.

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