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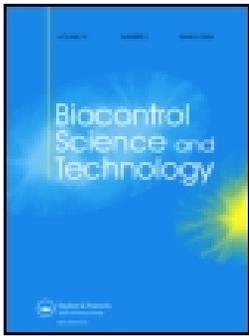
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## **Host stage preference and parasitism behaviour of *Aenasius bambawalei* an encyrtid parasitoid of *Phenacoccus solenopsis***

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### **Abstract**

In Pakistan, the cotton mealybug, *Phenacoccus solenopsis* Tinsley (Sternorrhyncha (Homoptera): Pseudococcidae), is a serious pest of many cultivated plants. A parasitoid, *Aenasius bambawalei* Hayat (Hymenoptera: Encyrtidae), is associated with *P. solenopsis*. In order to mass rear *A. bambawalei* for a biological control program, it is important to investigate the parasitoid's host stage preference and its parasitism behavior for *P. solenopsis* in order to optimize production. The present study showed that under both choice and no choice conditions, the parasitoid preferred 3<sup>rd</sup> instar and pre-reproductive host stage mealybugs for parasitism. Parasitoid larva developing inside the host exhibited a greater longevity, shorter developmental period and longer body size in these preferred host stages. Our study also confirmed that *A. bambawalei* showed no attraction to male mealybugs and no host feeding on any host stage was recorded. The ability of the parasitoid to effectively discriminate between suitable and non-suitable stages means that it is feasible to rear it on a mixed population.

**Keywords:** Cotton mealybug; nymph; antennation; host feeding

## Introduction

Since its accidental introduction into Pakistan in 2005, *Phenacoccus solenopsis* Tinsley (Sternorrhyncha: Pseudococcidae) commonly known as the cotton mealybug has been recorded as a serious pest of cultivated cotton (*Gossypium hirsutum* L. [Malvaceae]) (Muhammad 2007; Hodgson et al. 2008). It spread quickly and is now present in all provinces of Pakistan including vegetable farms in Federally Administered Tribal Areas. In Pakistan, it has been reported from more than 154 host plants including cotton (Arif et al. 2009).

Farmers mostly rely on insecticides for its control and the cost of these insecticides has thus increased the cost of production, which has made cotton production unprofitable for poor growers (Abdullah et al. 2007). Moreover, these insecticides can affect non-target organisms, can cause environmental degradation and health problems to farmers and also may result in the development of resistance in pests (Asogwa et al. 2010).

Therefore biological control appeared to be one of the most sustainable methods to use against this invasive and destructive pest. For this purpose the presence of indigenous predators and parasitoids were surveyed in the field and some natural enemies mostly belonging to Coleoptera, Neuroptera and Hymenoptera, particularly encyrtid parasitoids, were recorded (Bodlah et al. 2010). In Indian cotton growing areas a hymenopterous parasitoid *Aenasius bambawalei* Hayat (Hymenoptera: Encyrtidae) was discovered in 2008 (Hayat 2009). The same parasitoid was earlier reported as *Aenasius* nov. sp. *longiscapus* (Hymenoptera: Encyrtidae) in Pakistan in 2007 (CCRI 2007). Later it was confirmed to be *Aenasius bambawalei* Hayat in 2008 (Bodlah et al. 2010). This parasitoid was reported to be an efficient natural enemy and potentially effective for controlling *P. solenopsis* associated with cotton, fruits and other vegetables in India and Pakistan since 2008 (Fand et al. 2011; Bodlah et al. 2010; Gautam et al. 2009; Mohindru et al. 2009; Ram et al. 2009). It is believed that this parasitoid was introduced alongside the mealybug and is moving with the mealybug into newly invaded countries (Muniappan 2009; NCIPM 2010). Besides India and Pakistan it has also been observed in the Guangdong and Hainan Provinces of China (Chen et al. 2010).

This parasitoid has been reported as a solitary endoparasitoid which lays eggs in the host body and the hatched larvae are entirely dependent on the host for its development, as it feeds internally in the host until a single adult emerges (NCIPM 2010). Generally, for a parasitoid, the choice of the host stage is important for the fitness of the parasitoid's progeny (Zhang et al. 2016; Hagvar and Hofsvang 1991), and the fitness of the parasitoid larvae is

positively correlated with the host size and quality, and thus with the host selection of the ovipositing females (Charnov 1982; King 1993).

Knowledge of host acceptance behavior and parasitism success of *A. bambawalei* are important prerequisites to optimize the mass production of this parasitoid species for its use in biological control programs. Although some information is available for the biology of *A. bambawalei* (Zhang et al. 2016; Abdin et al. 2013; Fand et al. 2011; Vijaya and Saini 2011; Bodlah et al. 2010 and Hayat 2009) little information is available on the host preference by *A. bambawalei* when it has a choice of host developmental stages, and the influence of the host stage on the development of this parasitoid species (Fand et al. 2011).

The present experiments were conducted to investigate the host stage selection of *A. bambawalei* during host parasitism and the effect of host stage on the parasitoid's fitness. The host handling behavior of *A. bambawalei* during parasitism for each host stage was also studied to establish whether or not any host feeding occurs in this species.

## **Materials and Methods**

### **Insect culture**

Mealybug *P. solenopsis* were reared in the laboratory on sprouted *Solanum tuberosum* (potato) according to the procedures of Meyerdirk et al. (2002) and Vennilla et al. (2010). The sprouts were placed in plastic cages (70 cm length x 40 cm width x 40 cm height) and then *Hibiscus rosa-sinensis* (China rose) infested plant shoots (collected from Agricultural Research Institute ARI, Tarnab Peshawar-Pakistan) containing female mealybugs with nymphs sacs [previously confirmed to be *P. solenopsis* through the taxonomic keys of Hodgson et al. (2008)] were put in the middle of the cage, to facilitate movement of the hatched and active crawlers on to the uninfested sprouts. After 30-40 minutes, the *Hibiscus* shoots were removed and the cage was closed. The cage was observed/checked regularly and after 30 days the culture of the mealybug containing all developmental stages was ready for use.

For parasitoid rearing wooden cages (40 cm length x 40 cm width x 30 cm height) were used having arm entrance holes with a closable sleeve cloth in front and covered with 0.02 mm mesh screen from all sides except the bottom for proper aeration and to maintain proper humidity. Mature parasitoid pupae from an infested *P. solenopsis* population were collected and enclosed in a small vial. When adults emerged they were confirmed as *A. bambawalei* through the key of Bodlah et al. (2010). Mature parasitoids were released onto mealybugs of different developmental stages, already reared on potato sprouts. Pure honey droplets were sprayed daily onto the inner side of the cage and also onto a cotton swab in a

small Petri dish placed inside. To get a pure culture the insects were reared for at least three generations prior to testing. For all experiments a colony of newly emerged *A. bambawalei* adult males and females, previously fed on pure honey solution for 24 hrs, according to the procedures of Joyce et al. (2001) and Sagarra et al. (2001), were used. The insects were kept at 25-32°C, 55-65% R.H. and 12:12 light: dark photoperiod.

### **Host stage preference tests**

For choice tests, one male and female pair of newly emerged *A. bambawalei* was added to a 15 ml glass vial containing *P. solenopsis* mealybugs, four each of each different developmental stage (1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup> instar, pre-reproductive female, reproductive females and males making a total of 24 individuals per vial) that had already settled on an *Abelmoschus esculentus* (okra) fruit. Host preference of the female *A. bambawalei* for each host stage was observed under a binocular microscope for at least one and a half hours. For the no choice test a 10 ml vial containing 10 mealybugs at the same developmental stage was used

For both tests, the following behavioural parameters were recorded: percent host antennation (i.e. how many hosts were touched by the female parasitoid's antennae), the duration of antennation on each individual antennated (in seconds), the ovipositor probing (i.e. insertion of the ovipositor in the mealybug body) and the duration of probing (seconds) in each individual probed for each parasitoid wasp. Thereafter the duration of host feeding before and after parasitism was also observed and recorded. All probed individuals from each experimental vial were separated, kept in the incubator and after 48 hrs dissected in a drop of saline water under a binocular microscope to check if egg laying had occurred, for the presence of any larvae to confirm the parasitism and for the number of parasitoid larvae within each dissected mealybug. These experiments were replicated ten times (n=10).

### **Parasitism and development**

Choice test: the percentage parasitism and different developmental parameters of the parasitoid in the host were studied under choice tests. Ten mealybugs of each developmental stage were added to a 15 ml glass vial containing an okra fruit and allowed to forage for at least two hours. Then a pair of newly emerged *A. bambawalei* adults was released into the tube for 24 hrs. After 24 hrs the parasitoid pair was removed and all host individuals were separated into 10 ml glass tubes containing fresh okra fruit and kept in an incubator.

For no choice tests, 10 mealybugs of a given developmental stage were added to a 10 ml glass vial containing okra fruit and allowed to forage for at least two hours before *A. bambawalei* adults were added. For both of these tests daily observations were made until pupae formation (mealybug mummies) and adult emergence of the parasitoid. When the

adult parasitoids emerged, honey water solution was provided as food. The adults were observed daily until death. Dead males and females were separated for sex ratio estimation and hind tibial length through a dissection microscope. For each developmental stage data on percent parasitism, percent parasitoid emergence, adult developmental time and adult longevity were recorded. These tests were replicated ten times (n=10).

The data were subjected to analysis of variance (ANOVA) in CRD (Completely Randomized Design). For the percent emergence data a square root transformation was undertaken due to the high percentage values obtained. Untransformed data however are presented in the tables. All means were separated by LSD (Least Significance Difference) at the 5% level. Statistical tests were performed using the STATISTIX 8.1 package.

## Results

### Choice tests

When mealybugs of all developmental stages were offered together to *A. bambawalei*, parasitoids preferred to antennate on third instar nymphs, and pre-reproductive and reproductive females of *P. solenopsis* (percent antennation:  $F_{5,54}=317.00$ ;  $p<0.0001$ ) (Table 1). Furthermore, the duration of antennation was significantly lower on these developmental stages compared to the others (duration of antennation:  $F_{5,54}=386.00$ ;  $p<0.0001$ ). In addition, *A. bambawalei* preferred to probe on third instar nymphs followed by pre-reproductive and reproductive females (for percent host probed:  $F_{5,54}=52.00$ ;  $p<0.0001$ ; for duration of probing:  $F_{5,54}=60.8$ ;  $p<0.0001$ ). The highest mean number of parasitoid progeny were obtained in 3<sup>rd</sup> instar and pre-reproductive individuals followed by reproductive female ( $F_{5,54}=6.26$ ;  $p<0.002$ ). The parasitoid did not parasitize 1<sup>st</sup> instar nymphs or males (Table 1). No host feeding by the parasitoid was observed for any stage of the host, before or after parasitism. There was no significant difference in percentage emergence ( $F_{5,54}=0.93$ ;  $p<0.4359$ ) between any of the parasitized stages. However, the amount of time a the parasitoid took to develop in the various stages varied significantly, developing most rapidly in the reproductive female and slowest in the 2<sup>nd</sup> instar nymph (Table 2). For each host stage, female wasps took longer to emerge than males. A similar proportion of males to females emerged from the three most developed host stages, whereas a higher proportion of males emerged from the 2<sup>nd</sup> instar stage ( $F_{3,36}=20.9.00$ ;  $p<0.0001$ ) and Fig. 1. Those parasitoids that emerged from 3<sup>rd</sup> instar nymphs or pre-reproductive females lived longer than those that emerged from the reproductive female or from the 2<sup>nd</sup> instar nymphs. The same pattern was observed for parasitoid size, hind tibial length was longer for wasps that emerged from 3<sup>rd</sup> instar nymphs or pre-reproductive females.

### No-choice tests

When different stages of the mealybug were offered to the parasitoids under no-choice conditions, *A. bambawalei* preferred to antennate third instar nymphs, pre-reproductive and reproductive females of *P. solenopsis* ( $F_{5,54}=43.7$ ;  $p<0.0001$ ) (Table 3) and as with the choice test the duration of antennation was significantly lower on these developmental stages ( $F_{5,54}=39.1$ ;  $p<0.0001$ ). In contrast to the choice tests the parasitoid did antennate males and also probed the 1<sup>st</sup> instar mealybugs. The female parasitoid spent significantly more time probing the 3<sup>rd</sup> instar nymphs, pre-reproductive and reproductive females than the others ( $F_{5,54}=175.00$ ;  $p<0.0001$ ) and more progeny were obtained from these stages. Although *A. bambawalei* probed 1<sup>st</sup> instar nymphs the parasitoid was unable to oviposit properly and no larvae developed in this host stage. As with the choice test no host feeding was recorded before or after parasitism in the no choice tests (Table 3). Once a host mealybug had been probed the pattern of percent emergence, emergence time, longevity, proportion of males and hind tibia length was the same as in the choice test (Table 4).

### Discussion

In order to define the optimum conditions to rear natural enemies, it is important to determine the host stage preference since many encyrtids, and particularly parasitoids of mealybugs, show a strong preference for the host's stage and size (Chong and Oetting 2006a). Similar to other encyrtids (Godfray 1994), *A. bambawalei* fully examined the host body by antennal drumming on all host stages except males under choice conditions while under no choice conditions some males were also examined. Antennal drumming is known to play an essential role in the location, recognition and acceptance of hosts by parasitoids (Joyce et al. 2001; Vinson 1998, 1980). Through antennation *A. bambawalei* quickly identified the preferred host stages (3<sup>rd</sup> instar, pre-reproductive and reproductive females). This rapid identification could help it avoid the defensive behavior displayed by the host (Fand et al. 2011; Bertschy et al. 2000).

Third instar nymphs and pre-reproductive females are the preferred host stages also of other encyrtid parasitoid species for parasitism. For example, the encyrtid *Coccidoxenoides perminutus* Girault parasitizing all developmental stages of the citrus pseudococcid mealybug, *Planococcus citri* (Risso), showed a strong preference for second and third-instar nymphs (Chong and Oetting 2006; Ceballo and Walter 2004). Another citrus mealybug parasitoid, *Leptomastix dactylopii* Howard, preferred the pre-ovipositing adults and rejected the first and second-instar nymphs for parasitism (de Jong and van Alphen 1989; Chong and Oetting 2006). Moreover, another encyrtid parasitoid *Anagyrus mangicola* Noyes, probed

and oviposited more frequently in 2<sup>nd</sup> and 3<sup>rd</sup> stages of *Rastrococcus invadens* Williams (Homoptera: Pseudococcidae) than in 1<sup>st</sup> and 4<sup>th</sup> stages (Bokonon-Ganta et al. 1995).

In our study under both choice and no-choice conditions the parasitoid took around 10 seconds to probe the mealybug. The duration of probing is generally considered as a good indication of effective parasitism (i.e. egg laying), a minimum of time is necessary for efficient parasitism and for *A. bambawalei*, 7 seconds was the minimum time observed for successful parasitism. The duration of host probing also depends on the parasitoid species. For example, *Anagyrus mangicola* attacking *R. invadens* takes 5 seconds to oviposit, while the encyrtid *Leptomastix abnormis* attacking *P. citri* takes more than 141 seconds (Cadee et al. 1997). In our study the duration of probing depended on which host stage the parasitoid was probing. Regardless of the experimental setup (choice or no-choice), the wasp took the longest time on the full adult mealybug and the shortest on 1<sup>st</sup> and 2<sup>nd</sup> instars. This was probably due to the physical properties of the body surface of the host, the adults having more waxy components and thus a harder integument than the younger stages.

In our study no host feeding was recorded for *A. bambawalei* before or after probing/parasitism. Heimpel and Collier (1996) reported that adult females of many species of hymenopteran parasitoid can also exploit hosts for feeding as well as for parasitism. Host-feeding can be a key source of nutrition, especially protein, for female parasitoids (Heimpel and Collier 1996; Jervis et al. 1996). Host-feeding can occur when the female injures their host by frequent ovipositor probing, thus making them unfit for oviposition (Ueno 1999; Jervis and Kidd 1986).

Regardless of the experimental setup (choice or no-choice), *A. bambawalei* could not develop inside 1<sup>st</sup> instar nymphs or adult males. It developed best in 3<sup>rd</sup> instar nymphs and in pre-reproductive females and reproductive females. Among the developmental stages tested, 1<sup>st</sup> instar nymphs and males have the smallest body size. Although koinobiont parasitoids have a wide range of suitable host stages, there are costs involved in the parasitism of small hosts: developmental time is often lengthened and survival reduced (Godfray 1994). Moreover selection of the most profitable host stage can influence the sex ratio. A higher proportion of females can be produced from larger hosts because of the greater nutritional requirement and reproductive benefits for the female progeny (Charnov et al. 1981; Chong and Oetting 2006; Fand et al. 2011). In our study, *A. bambawalei* progeny's sex ratio was affected by the host stage – a higher number of parasitoid females emerged from 3<sup>rd</sup> instar, pre-reproductive females and reproductive females compared to the smaller 2<sup>nd</sup> instars.

Moreover, similar to other parasitoids (Fand et al. 2011; Abdin et al., 2013), the *A. bambawalei* females lived longer than the males.

It is also well known that host stage influences parasitoid size and further that female fitness may be more influenced by body size than the male fitness (Charnov 1982; King 1993; Fand et al. 2011). In our study an increase in the size of *A. bambawalei*, as measured by hind tibial length, was observed with an increase in the host size. Milonas (2005); Chong and Oetting (2006) reported that many parasitoids select a specific age and size of host that will have sufficient resources to produce progeny with good physical vigor. They also reported that hosts of a more advanced developmental stage could provide sufficient resources for parasitoid development without significant detriment to themselves.

In conclusion, the current study revealed that although *A. bambawalei* was able to parasitize all developmental stages of *P. solenopsis*, except 1<sup>st</sup> instar and males, they preferred to parasitize, and developed better, in 3<sup>rd</sup> instars and pre-reproductive adults. This preference was observed under both choice and no choice conditions. The parasitoid also developed well in reproductive females although the progeny were slightly smaller in body size than those that developed in the two younger stages. In contrast it did not develop well in 2<sup>nd</sup> instar nymphs. The ability of *A. bambawalei* to discriminate efficiently between mealybug stages means that it would be feasible to rear the parasitoid on a mixed population of mealybugs without the costly procedure of having to separate out different developmental stages. There would be a slight gain in efficiency if 2<sup>nd</sup> instar nymphs were not included in the rearing procedure, however this may not justify the additional effort involved.

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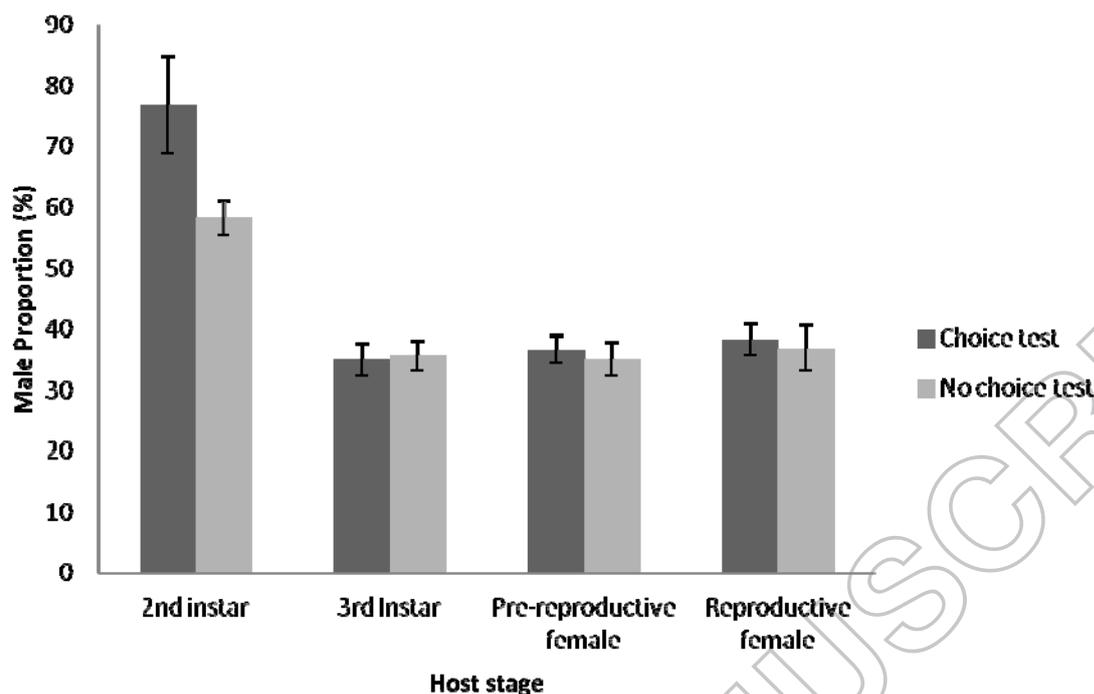
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**Fig 1, Male proportion (%) of parasitoid progeny when emerged from different host (mealybug) stages under both choice and no choice conditions**

**Table 1 Behavior of *Aenasius bambawalei* during parasitism on different stages of *P. solenopsis* under choice conditions (mean<sup>1</sup> ± SE, n=10).**

Host Stage	% Host antennated	Duration of antennation (seconds)	% Host Probed	Duration of probing (seconds)	Mean number of progeny per probed individual	% Host Feeding
1 <sup>st</sup> Instar	27.50 ± 2.5b	15.83 ± 0.72 a	0	---	---	0
2 <sup>nd</sup> Instar	37.50 ± 4.17b	9.03 ± 0.63 b	45.00 ± 11.67b	7.07 ± 1.80b	0.25 ± 0.083c	0
3 <sup>rd</sup> Instar	67.50 ± 5.34a	4.48 ± 0.40 c	67.50 ± 8.47a	10.93 ± 0.63a	0.68 ± 0.085a	0
Pre-reproductive female	62.50 ± 6.72a	4.85 ± 0.32c	65.83 ± 8.65a	10.71 ± 0.44a	0.66 ± 0.087a	0
Reproductive Female	57.50 ± 5.34a	5.05 ± 0.13c	56.67 ± 7.94ab	11.14 ± 0.63a	0.57 ± 0.080b	0
Male	0	---	0	---	---	0

<sup>1</sup>Means in each column followed by the same letters are non significant at 5% level, using LSD test following ANOVA.

Table 2 Development of *Aenasius bambawalei* on different stages of *P. solenopsis* under choice conditions (mean<sup>1</sup> ± SE, n=10).

Host Stage	% Host parasitized	% wasp emergence	Emergence time (days)		Longevity (days)		Hind tibial length (µm)	
			Male	Female	Male	Female	Male	Female
1 <sup>st</sup> Instar	0	0	---	---	---	---	---	---
2 <sup>nd</sup> Instar	22.0 ± 3.27b	80.83 ± 8.61a	14.85 ± 0.34a	16.80 ± 0.18a	10.05 ± 0.38c	12.80 ± 0.35d	380.75 ± 1.70c	470.29 ± 5.90d
3 <sup>rd</sup> Instar	33.0 ± 2.13a	91.67 ± 4.30a	12.4 ± 0.31b	13.98 ± 0.22b	11.10 ± 0.31a	17.35 ± 0.28a	470.45 ± 1.06a	580.63 ± 9.20a
Pre-reproductive female	32.0 ± 2.00a	89.17 ± 4.49a	12.1 ± 0.28b	13.75 ± 0.24b	10.90 ± 0.31ab	15.95 ± 0.52b	470.12 ± 0.98a	560.97 ± 4.30b
Reproductive Female	32.0 ± 2.00a	84.17 ± 5.33a	11.2 ± 0.25c	12.20 ± 0.17c	10.20 ± 0.20bc	14.90 ± 0.33c	440.16 ± 1.21b	510.34 ± 6.20c
Male	0	0	---	---	---	---	---	---

<sup>1</sup>Means in each column followed by the same letters are non significant at 5% level, using LSD test following ANOVA.

Table 3 Behavior of *Aenasius bambawalei* during parasitism on different stages of *P. solenopsis* under no-choice conditions (mean<sup>1</sup> ± SE, n=10).

Host Stage	% Host antennated	Duration of antennation (seconds)	% Host probed	Duration of probing (seconds)	Mean number of progeny per probed individual	% Host Feeding
1 <sup>st</sup> Instar	33.00 ± 3.67c	10.77 ± 0.50b	35.99 ± 3.53b	5.03 ± 1.03b	0	0
2 <sup>nd</sup> Instar	48.00 ± 4.16b	7.76 ± 0.22c	38.19 ± 2.29b	7.93 ± 0.22a	0.38 ± 0.023b	0
3 <sup>rd</sup> Instar	62.00 ± 2.49a	5.41 ± 0.31d	53.62 ± 7.15a	8.38 ± 0.53a	0.54 ± 0.072a	0
Pre-reproductive female	60.00 ± 2.18a	5.92 ± 0.61cd	53.43 ± 3.15a	8.68 ± 0.72a	0.53 ± 0.032a	0
Reproductive Female	59.00 ± 3.14a	6.02 ± 0.28cd	52.43 ± 6.38a	9.24 ± 0.61a	0.52 ± 0.064a	0
Male	16.00 ± 1.63d	15.49 ± 1.52a	0	---	---	0

<sup>1</sup>Means in each column followed by the same letters are non significant at 5% level, using LSD test following ANOVA.

Table 4 Development of *Aenasius bambawalei* on different stages of *P. solenopsis* under no-choice conditions (mean<sup>1</sup> ±SE, n=10).

Host Stage	% Host parasitized	% wasp emergence	Emergence time (days)		Longevity (days)		Hind tibial length (µm)	
			Male	Female	Male	Female	Male	Female
1 <sup>st</sup> Instar	0	0	---	---	---	---	---	---
2 <sup>nd</sup> Instar	35.00 ± 3.42b	85.67 ± 5.06a	14.25 ± 0.19 a	16.70 ± 0.29a	10.15 ± 0.18ab	12.75 ± 0.23d	390.48 ± 7.80b	480.11 ± 11.0c
3 <sup>rd</sup> Instar	59.00 ± 2.77a	92.62 ± 3.26a	12.22 ± 0.26b	13.62 ± 0.14b	10.67 ± 0.27a	17.18 ± 0.28a	450.69 ± 12.10a	560.44 ± 10.9a
Pre-reproductive female	58.00 ± 3.27a	91.81 ± 2.78a	11.95 ± 0.14b	13.21 ± 0.14b	10.40 ± 0.23ab	15.40 ± 0.25 b	440.76 ± 17.20a	540.89 ± 10.30a
Reproductive Female	58.00 ± 4.90a	86.19 ± 4.21 a	10.95 ± 0.14c	12.33 ± 0.13c	9.85 ± 0.27b	14.33 ± 0.11 c	430.95 ± 8.10a	520.50 ± 4.50b
Male	0	0	---	---	---	---	---	0

<sup>1</sup>Means in each column followed by the same letters are non significant at 5% level, using LSD test following ANOVA.