Influence of mating and oviposition behavior on progeny production of the ichneumonid parasitoid, *Campoletis chlorideae*

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Running title: Mating and oviposition behavior of *Campoletis chlorideae*

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Abstract

The *Campoletis chlorideae* Uchida is an important natural enemy of the noctuids, being the most important larval parasitoid of *Helicoverpa armigera* (Hubner) in different agro-ecosystem. Regulation of sex ratio in the hymenopterans has an important bearing on the use of natural enemies for the management of insect pests. There is no clear concept of sex ratio manipulation by *C. chlorideae*, therefore the present studies were conducted to understand the mechanism of sex ratio in *C. chlorideae* in 4 different mating schemes. Mating and parasitization regimes had a significant influence on the fecundity of *C. chlorideae* females, while no significant differences were observed for cocoon formation, adult emergence, and larval and pupal periods across mating regimes. There was a positive correlation (r = 0.68) between fecundity and longevity of *C. chlorideae* females. Attraction of more males to copulating pair resulted in mating of a female with two or three males simultaneously, suggested increased release of sex pheromone by the females during copulation and the competition among the males for mating. However, mating with multiple males simultaneously is not an additive advantage in progeny production, since only 20% of the *C. chlorideae* females (mated with two males) were able to parasitize the *H. armigera* larvae successfully. There was no consistent trend in sex ratio across treatments and parasitization days, except in case of unmated females. Studies on sexual and ovipositional behaviors suggested that extrachromosomal factors could be responsible for sex-determination in *C. chlorideae*, which needs to be tested systematically.

Key words: *Campoletis chlorideae*, *Helicoverpa armigera*, courtship behavior, oviposition behavior, progeny production

Introduction

The endoparasitoid, *Campoletis chlorideae* Uchida (Hymenoptera: Ichneumonidae) is one of the most important natural enemies of the noctuid, *Helicoverpa armigera* (Hubner) and other lepidopteran insects in different agro-ecosystem (Bhatnagar et al., 1982; Pawar et al., 1986; Romeis and Shanower, 1996; Yan and Wang, 2006). It prefers to parasitize late second-instar (4 to 5 days old) larvae of *H. armigera* (Nikam and Gaikwad, 1991; Dhembare, 1999; You et al., 2002). After parasitization, the egg hatch in about 1.0 to 1.5 days inside the host larva, and the larval development is completed in about 6 to 8 days (Sharma et al., 2006). During this period, the larva feeds on the contents of the host larva. When the larval development is completed, the larva emerges from the host larva, killing the host later in the process. It then weaves a cocoon around itself, and the pupal period lasts for about 5 to 7 days. Total development period of *C. chlorideae* takes about 12 to 16 days (Nandihalli and Lee, 1995; Sharma et al., 2006). The female is generally bigger than the male.

There is significant correlation between the age of host larvae and percentage parasitism (Gunasena et al., 1989). Parasitization potential of *C. chlorideae* varies across climatic conditions, seasons, and crop and insect hosts. Mating between males and females of the same age (48 h after emergence) has been reported to result in male-biased sex ratio, while mating of males and females of different ages or mating 24 h old males and females produce equal number of females and males (Venkatesan et al., 1999). The sex ratio is generally female-biased at fixed host densities, but shows a linear decrease with an increase in parasitoid density (Kumar et al., 2000). However, with an increase in more host availability, the parasitoid emergence also increases, but stabilizing at a density of 32 hosts per parasitoid (Kumar et al., 2000). Sex determination in hymenopterans is well studied, but the regulation of sex ratio in *C. chlorideae* is unclear. In many hymenopterans, sex is determined at a single polymorphic sex locus. The individuals that are heterozygous at this locus develop as females, whereas homozygotes develop as diploid, and hemizygotes as haploid males (Heimpel et al., 1999). Based on single-locus complimentary sex determination (CSD), diploid males are either developmentally inviable or sterile (Whiting, 1939, 1943), but such an assumption does explain sex determination in *C. chlorideae*. Similarly, multilocus CSD theory also fails to sex determination in this parasitoid (Crozier, 1971). Information on regulation of sex ratio in *C. chlorideae* may have important bearing on implementing control strategies using this koinophytic parasitoid against several

lepidopterous insect pests. Keeping in view the importance of sex ratio in natural enemies, and the unclear concept of sex ratio manipulation by *C. chlorideae*, the present studies were conducted to understand the mechanism of sex ratio in *C. chlorideae* under different host densities, duration of male-female companionship, and under the mated - unmated condition of the parasitoids. Courtship and oviposition behaviors of *C. chlorideae* adults have also been discussed.

Materials and methods

Insect culture

The ichneumonid parasitoid, *C. chlorideae* culture was maintained on the *H. armigera* larvae reared on chickpea based semi-synthetic artificial diet (Armes et al., 1992) at 27 ± 2 °C, and 65 to 85% relative humidity in the insectary at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), for conducting the experiments.

Mating scheme of the parasitoid, *Campoletis chlorideae*

The mating behavior and biology of *C. chlorideae* fed on *H. armigera* larvae was studied under four mating schemes. Before being used for parasitization, the *C. chlorideae* females were allowed to mate for 72 h inside a cage (30 x 30 x 30 cm), except for one treatment where the unmated females were used in the experiments. The mating schemes were: i) mated males and females throughout the lifespan, and twenty *H. armigera* larvae were exposed to the females for parasitization daily; ii) after 72 h of mating the males were removed, and twenty *H. armigera* larvae were exposed to the females daily; iii) mated males and females were kept together throughout the lifespan, and *H. armigera* larvae were offered to the females up to maximum parasitization capacity daily; and iv) the 72 h old unmated females were used to parasitize *H. armigera* larvae to their maximum parasitization capacity daily. Parasitization process was terminated when a female took more than 10 minutes between two parasitization attempts. After parasitization, the *H. armigera* larvae were transferred to the artificial diet. There were five replications for each experiment, in a completely randomized design. Observations were recorded on cocoon formation, days to cocoon formation (egg + larval development period),

pupal period, adult emergence, sex ratio, and fecundity of the *C. chlorideae* females in different treatments.

Influence of courtship behavior on oviposition and progeny production in *Campoletis chlorideae*

The *C. chlorideae* adults were observed for courtship and oviposition behavior visually. The *C. chlorideae* females found in courtship with two males simultaneously were studied for their oviposition behavior, parasitization potential, cocoon formation, adult emergence, and sex ratio. A total of 15 such females having courtship with two males simultaneously were used in these studies.

Statistical analysis

Data were subjected to analysis of variance, and the significance of differences between the treatments was tested by F-test, while the treatment means were compared using least significant difference (LSD) at P = 0.05. The data recorded from different mating schemes on cocoon formation and adult emergence across days of parasitization was converted to three day moving means.

Results and discussion

Courtship behavior in *Campoletis chlorideae*

In insects, mates may be located by visual, auditory, or olfactory stimuli, or a combination of these. The occurrence of sex pheromones has received a great deal of attention and has been demonstrated in several insect species, including *C. sonorensis* (Cameron) (Guillot and Vinson, 1972; Vinson, 1972). Within a few minutes of emergence, the males of *C. chlorideae* start a continuous moment in several directions with antennae in forward direction, and the wings held over the abdomen, while females have a normal moment. When both males and females were released together in 2 L plastic cages, the males raised the anterior part of the body within seconds of release inside the cage along with the females. The antennae moved up and down, and the wing vibration increased until mounting, prior to copulation. The wing vibration was repeated until the female to which the male oriented was contacted with antennae. In this process, often a male would orient towards another male, and attempt copulation. All elements of

courtship behavior and attempted copulation occur in the presence of female pheromone, and male is able to orientate to this odor source with the aid of wing vibration (Vinson, 1972). When the male comes in contact with a female, the male palpitates the female with his antennae to orientate his head and her head. The male then mounts the female, ceases wing vibration, and moves slightly backward while curving his abdomen to make genital contact. This is followed by the male moving farther behind the female and taking up a copulatory position with his abdomen bent at perpendicular to the thorax. The male remains in the characteristic copulatory position with abdomen bent at 90° and does so for 5 to 30 minutes, if forcibly separated from the females. If female wants to terminate mating, she moves forward dragging the male behind in copulatory position. But, this type of female behavior is not enough to terminate mating until and unless the male intends to terminate it. This suggests that there is a locking mechanism in male genitalia supported by male copulatory position. Following copulation, the female walk off, while the male usually flies a short distance and preens. On several occasions, two males appeared to copulate with the same female simultaneously. Similar courtship behavior has earlier been reported in case of *C. sonorensis* (Vinson, 1972). At times, three males were also observed to copulate with one female, indicating competition among the males for mating. Male were also observed to mate with dead females, suggesting that males are quite active sexually. The males tempted to copulate when they came across a copulating pair, and almost every male passing through the courting pair tried to copulate with the same female at the same time. Such a mating system indicates increased release of sex pheromone by the females during copulation. This kind of courtship behavior was common in fresh adults (1 to 5 days after emergence) in a big group of about 18 to 20 pairs as compared to that in old adults. Several pairs were observed in courtship during collection of males and females in the aspirator for use in oviposition behavior studies.

Oviposition behavior of *Campoletis chlorideae* females

The *C. chlorideae* females are very active parasitoids of *H. armigera* larvae. The orientation of a female parasitoid to a habitat by host odor provides an adaptive advantage to it. When the female perceives the host odor, it moves faster till it reaches the host larvae. Olfactory stimuli help the adult females to orientate to the host larvae. Studies involving intact host larvae, saliva of host larva, larval faeces, and extracts of host larvae suggested that host finding by *C. chlorideae* is olfactory rather than visual, as no stabbings were observed on pigeonpea plants (Sathe and

Santhakumar, 1990). After release of females in the parasitizing glass vials, they start moving around the walls in search of its host. When a single second-instar *H. armigera* larva was offered for parasitization, the *C. chlorideae* female moved towards the larva, and started moving the antennae on and around the larva. It attempts this behavior several times until it finds the host to be suitable for parasitization. Once it locates the host, it parasitizes the larva, and then moves away from the host larva or the host larva moves away and tries to hide himself from the parasitoid female. If there is no another host larva in the immediate periphery, the parasitoid female comes back, and parasitizes the same larva again. Up to 7 stabbings were observed on the same larva after an interval of 20 to 60 seconds. However, only one cocoon was recovered from the larva. This may be because of false stabbing (without egg laying), or competition among the parasitoid larvae for food in host larvae, resulting in survival of only one parasitoid larva. During the course of multiple stabbings by one or more females, the ratio of survival of host larvae was very low. Sometimes the host larva died during the parasitizing process. There may also be some symbiotic virus associated with *C. chlorideae*, as in case of *C. sonorensis* (Kroemer and Webb, 2003), which are injected with the egg and ovarian proteins into a the host during the parasitization. These virions interact with the prothoracic glands to reduce ecdysone levels, and thus, reduce the growth of parasitized host insect, and enable the survival of wasp progeny (Dover et al., 1988; Gunasena et al., 1989; Kroemer and Webb, 2003). During the process of multiple stabbings, more number of virions might have accumulated in the host insect, and hence the host larva could not sustain for longer because of lack of enough antibodies against that virus. Low host haemolymph protein content results in the delayed and abnormal development of parasitoid larvae (Ren et al., 2004), and hence one host larva cannot sustain more number of parasitoid larvae.

Although, on several occasions the adult female copulate with 2 or 3 males simultaneously, but it is not an additive advantage for the females in progeny production. During the course of copulation with several males simultaneously, the ovipositor of the females got curved upwards, which even after having stabbing intentions could not parasitize the host larvae. This situation may be limited only to the laboratory culture, because of heavy density of insects with a high probability to come in contact with each other. We studied the oviposition behavior of 15 females copulated with 2 males simultaneously, where 20% of such mated females were able to parasitize the host, *H. armigera* larvae. Numbers of stabbings on *H. armigera* larvae by

females mated with two males (205.3 stabbings female^{-1}) was comparatively lower than the females mated with one male (262.7 stabbings female^{-1}), but the differences were nonsignificant. Similarly, there were no significant differences in cocoon formation and adult emergence among the females mated with either one or two males (Fig. 1), suggesting that there is no additive advantage to *C. chlorideae* females mating with two males over those mating with one male. The sex ratio of the *C. chlorideae* progenies obtained from females mated with two males was 1: 0.6, and for females mated with one male was 1: 0.9, but the result were statistically nonsignificant.

Influence of different mating and parasitization regimes on *Campoletis chlorideae* progeny production

Mating and parasitization regimes have a significant influence on the fecundity of *C. chlorideae* females (P = 0.05) (Table 1). The mean fecundity per female varied between 259.1 to 427.6 stabbings. Greater fecundity was observed when females and males were kept together life long, and when the females were allowed to parasitize *H. armigera* larvae to maximum daily capacity, than when unmated females were used for parasitization. The total period of parasitization for the *C. chlorideae* females in lifespan companionship with males, parasitizing *H. armigera* larvae to the maximum daily capacity, was higher (20 days) than in other mating regimes. There were no significant differences in fecundity of *C. chlorideae* females having life long companionship with males and parasitizing 20 *H. armigera* larvae daily as compared to unmated *C. chlorideae* females parasitizing *H. armigera* larvae up to daily maximum parasitization potential.

Companionship periods and extent of parasitization influenced the longevity of *C. chlorideae* females (Table 1). Females lived longer when they were kept in company of males for only 72 h as compared to those kept under other mating regimes (Fig. 2). There was a positive correlation (r = 0.68) between fecundity and longevity of *C. chlorideae* females.

No significant differences were observed for cocoon formation, adult emergence, and larval and pupal periods across treatments (Table 1). Although, the differences for cocoon formation and adult emergence under different mating and parasitization regimes across days of parasitization were significant (P = 0.05), but the fluctuation (increase/decrease) in these parameters was consistent, except in a few cases (Fig. 3). Under life long companionship, the *C. chlorideae* females maintained a parasitization rate of 20 *H. armigera* larvae per day till half of the lifespan, which started declining thereafter. However, under the 72 h companionship regime,

the females could maintain the targeted limit throughout it's life, except on 1st, 15th, 16th, and 17th day of parasitization. Such females survived for 4 extra days. The *C. chlorideae* females of lifelong companionship with males and parasitization of *H. armigera* to maximum daily capacity could maintain higher parasitization potential, percent cocoon formation, and adult emergence till first half of the lifespan than the second half. The virgin females laid maximum eggs (≈ 80% of the total) between 2nd to 10th day. Like many hymenopterans, the ichneumonid parasitoid, *C. chlorideae* produces haploid (males obtained from unmated females) and diploid (males obtained from mated females) males. The studies indicated that both haploid and diploid males were fertile. These findings are dissimilar to other hymenopteran parasitoids such as braconids, where haploid males were found to be sterile.

The sex ratio under life long companionship with daily 20 larval parasitizations, 72 h companionship with daily 20 larval parasitization, and life long companionship and maximum daily larval parasitization had female biased sex ratio, while arrhenotoky (unmated females produces only males) was observed under unmated conditions (Table 1). Sex ratio was statistically under life long companionship with daily 20 larval parasitizations, 72 h companionship with daily 20 larval parasitization, and life long companionship and maximum daily larval parasitization conditions was on par with each other (Fig. 4). Several factors have been reported to influence sex ratio in insects including extrachromosomal factors, sperm availability, population size, lack of genetic variation, and behavioral constraints (King, 1993). During the present studies, enough genetic diversity was expected as the initial culture of *C. chlorideae* was started with natural field population from different crops at different times, and the culture was maintained by parasitizing *H. armigera* larvae with at least 15 to 20 females on a particular day. Population size in the experiment and the number of progenies obtained from them were enough to workout an unbiased sex ratio. Polygamy and polyandry sexual behavior was also observed in *C. chlorideae*, and hence, there is remote possibility of sperm nonavailability. Since, sexual and ovipositional behaviors were extensively studied during the investigation, the only potential sex-manipulating factor in *C. chlorideae* seems to be the extrachromosomal, which needs to be tested systematically.

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Table 1. Influence of mating behavior on fecundity, survival, and development of *Campoletis chlorideae* (ICRISAT, Patancheru, India)

Treatments	Fecundity (stabbings female^{-1} day^{-1})	Cocoon formation (%)	Adult emergence (%)	Larval period (days)	Pupal period (days)	Sex ratio (male: female)	Female longevity (days)
T1. Mated females (exposed to 20 *Helicoverpa* larvae daily)	16.9 (259.1)*	79.7	56.7	7.4	5.8	1: 1.9	14.8 (11-19)**
T2. Males removed after 72 h of mating (exposed to 20 *Helicoverpa* daily)	19.0 (366.9)	80.0	53.8	7.3	5.9	1: 1.4	18.8 (18-20)
T3. Mated females (exposed to as many *Helicoverpa* larvae as they could parasitize)	22.3 (427.6)	76.2	55.2	7.3	5.9	1: 1.5	16.0 (13-20)
T4. Unmated females (exposed to as many *Helicoverpa* larvae as they could parasitize)	15.6 (251.9)	84.2	56.5	7.4	6.0	1: 0.0	13.3 (10-17)
Fp	<0.001	0.163	0.777	0.185	0.174	<0.001	0.057
SE ±	0.55	2.23	2.25	0.04	0.07	0.19	1.22
LSD (P = 0.05)	1.53	7.13	7.21	0.13	0.21	0.60	3.90
CV (%)	24.5	5.6	8.1	1.1	2.2	30.6	15.6

* = Figures in the parentheses are average number of stabbings per female across replications. ** = Figures in the parentheses indicates female longevity across replications.

Fig. 1. Cocoon formation and adult emergence in *Campoletis chlorideae* obtained from the females mated with two males simultanously. The bars for a parameter following the same letter are statistically nonsignificant at $P = 0.05$.

Fig. 2. Relationship between fecundity and longevity of *Campoletis chlorideae* females on *Helicoverpa armigera* larvae across different treatments.

Fig. 3. Cocoon formation and adult emergence of *Campoletis chlorideae* females on *Helicoverpa armigera* larvae across parasitization days in different treatments (T1, T2, T3, T4). For treatment details please see Table 1.

Fig. 4. Proportionate females to one male of *Campoletis chlorideae* on *Helicoverpa armigera* larvae across different treatments. The bars following the same letter are statistically nonsignificant at $P = 0.05$

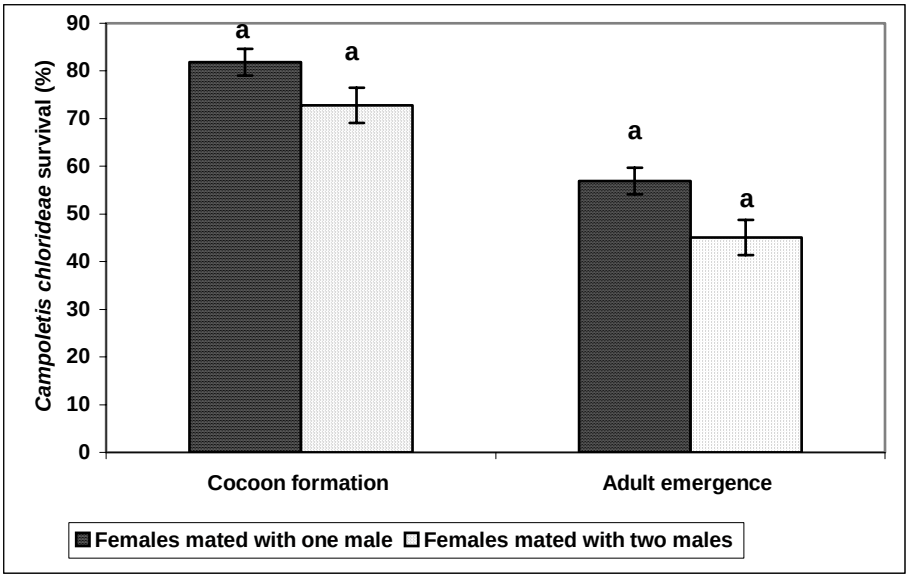
90
80
70
60
50
40
30
20
10
0
Campoletis chlorideae survival (%)
a
a
a
a
Cocoon formation
Adult emergence
Females mated with one male
Females mated with two males

Fig. 1

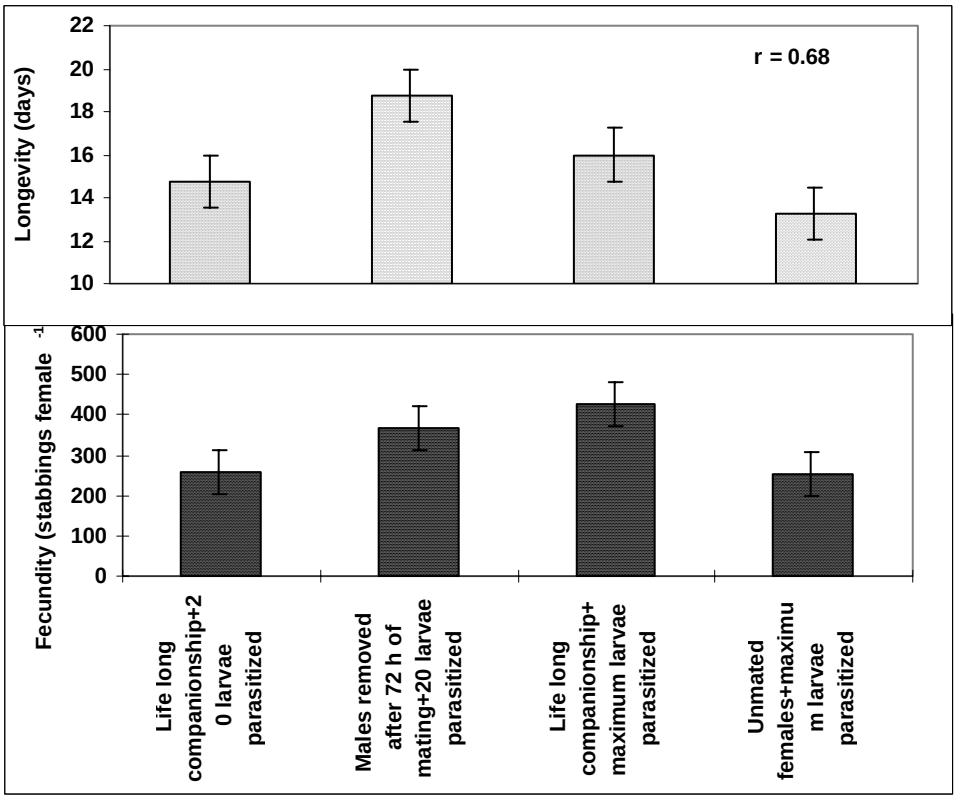
Longevity (days)
22
20
18
16
14
12
10
r = 0.68
Fecundity (stabbings female -1
600
500
400
300
200
100
0
Life long companionship+20 larvae parasitized
Males removed after 72 h of mating+20 larvae parasitized
Life long companionship+ maximum larvae parasitized
Unmated females+maximum larvae parasitized

Fig. 2

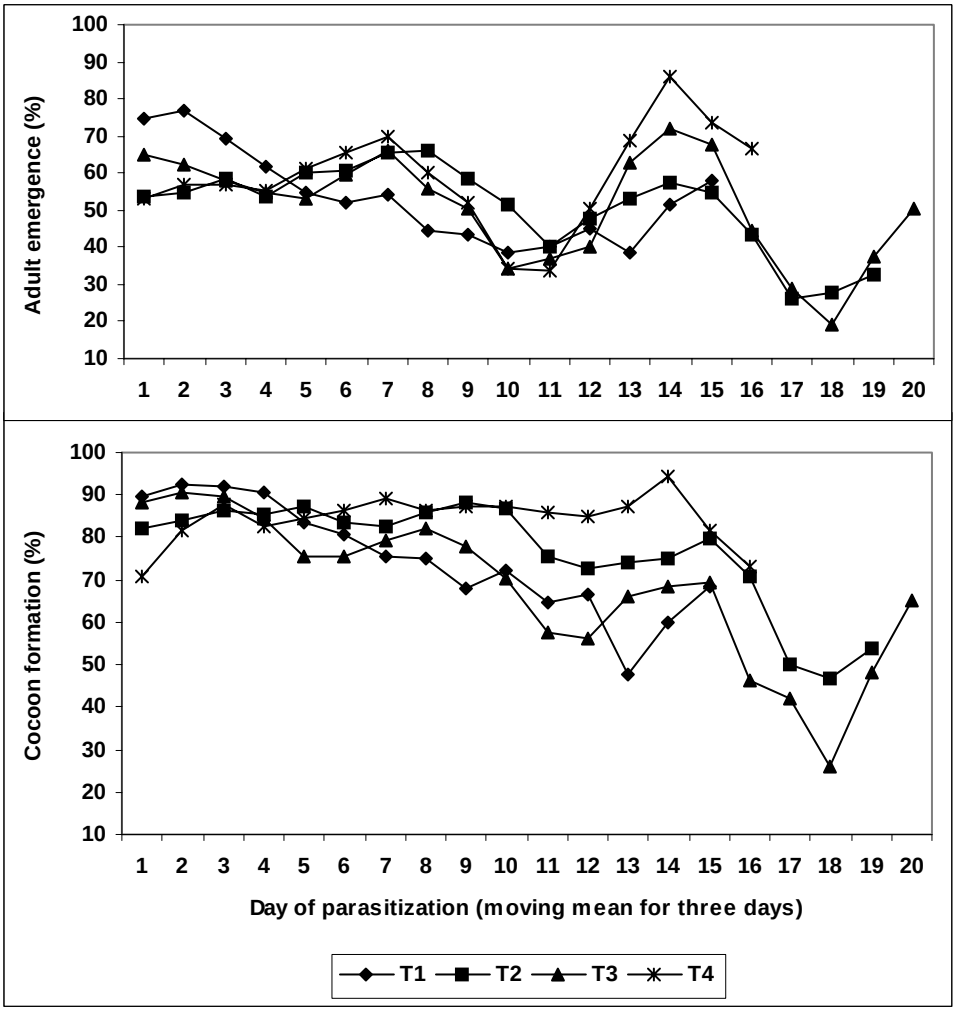
Adult emergence (%)
100
90
80
70
60
50
40
30
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Cocoon formation (%)
100
90
80
70
60
50
40
30
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Day of parasitization (moving mean for three days)
T1
T2
T3
T4

Fig. 3

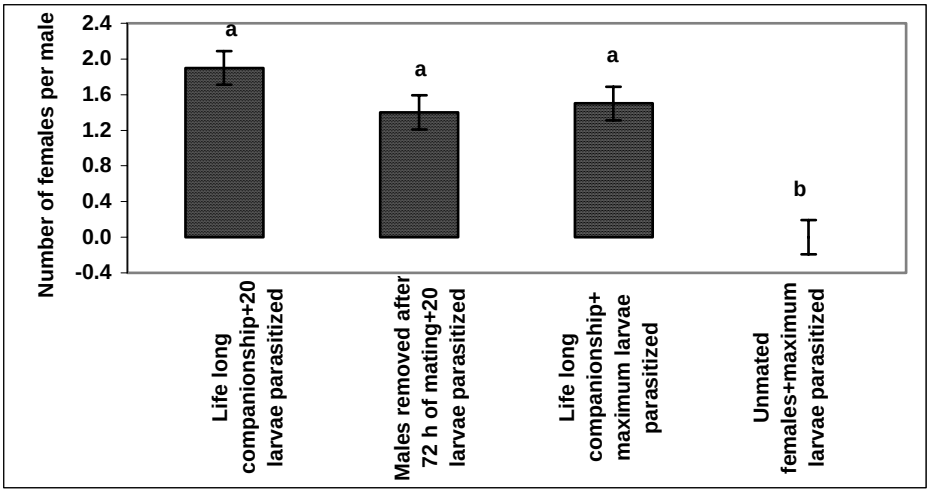
Number of females per male
2.4
2.0
1.6
1.2
0.8
0.4
0.0
-0.4
a
a
a
b
Life long companionship+20 larvae parasitized
Males removed after 72 h of mating+20 larvae parasitized
Life long companionship+ maximum larvae parasitized
Unmated females+maximum larvae parasitized

Fig. 4