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Identification and Behavioral Evaluation of Sex Pheromone in *Xanthopimpla pedator* (Fabricius)—A Serious Pupal Parasitoid of Tropical Tasar Silkworm *Anthereae mylitta* Drury

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ABSTRACT

Xanthopimpla is a major parasitoid of silk worm cocoons. The female *Xanthopimpla pedator* (Fabricius) lays the eggs in male cocoons. Control of this infestation with pesticides is not recommended because of its concealed behavior. Various control methods were found to be inefficient. Ecofriendly management is the best strategy that can be applied. We have studied the sex communication in *Xanthopimpla pedator* (Fabricius), which helps to develop management strategy. Bioassays were done in the laboratory by using olfactometer and pheromone extraction chambers. It was found that female *Xanthopimpla* produces sex pheromones. The results show a strong attraction of male by female *Xanthopimpla*. Present results with male and female volatiles also show that female volatiles attract male *Xanthopimpla*. Fractionation of female volatiles by column chromatography has proven that 20% fraction has highest attraction of males by females.

1. Introduction

The tasar silk is produced by *Anthereae mylitta* Drury (*Lepidoptera: Saturnidae*), a wild polyphagous tropical sericigenous insect distributed over central India. The species has wide distribution over diverse ecological niche as high as 44 ecoraces, but only a few are semidomesticated and applied commercially for seed (egg) and silk production (Suryanarayana and Srivastava 2005). Rearing of tasar silkworm, *Anthereae myliia* Drury, on forest grown plantation like *Terminalia arjuna*, *Terminalia tomentosa*, *and Shorea robusta* results in 80–90% crop loss because of parasites, pedators, and vagaries of nature (Mathur and Shukla 1998). It has been estimated that in hibernating stock about 20–30% loss of seed cocoons was because of pupal mortality and unseasonal emergence, which in turn reduces the multiplication rate of tasar cocoons. *Ichneumon fly*, *Canthecona bug*, *reduviid bug*, *Hicrodulla bipapilla* (*praying mantis*), and others are natural enemies in the rearing field, which cause maximum crop loss (Singh *et al.* 1992). *Ichneumons* like *Xanthopimpla* (*Hymenoptera*) and *Blepharipa* (*Diptera*) are important endoparasitoids of insect hosts, mainly larvae and pupae of order *Lepidoptera* (Singh *et al.* 2010). The cumulative effect of these pathogens results in 30–40% of Tasar crop loss. A pupal parasitoid, *Xanthopimpla stemmator*, was recorded from Maharashtra and Andhra Pradesh (Duale and Nwanze 1999). It was also recorded that *Xanthopimpla pedator* has sexual preference for male cocoons in parasitism (Lakshmi and Bhagavanulu 2012).

Xanthopimpla is one of the largest genera of Ichneumonidae, and the species of the genus are endoparasitoids of the Lepidopterans (Gomez *et al.* 2009), which show greater degree of biological adaptations (Gauld and Bolton 1988). The Pimplinae subfamily has become taxonomically one of the best-known ichneumonid taxa (Gauld 1991). Many *Xanthopimpla* species are abundant in tropical areas and are lemon yellow in coloration and has very stout bodies. *X pedator* begins its life cycle in 5th instar spinning larva of *Anthereae mylitta* by the time of hammock formation to early cocooning stage and uses its long ovipositor to drill through silky envelope of spinning larva and deposits single egg in the abdominal segment (Aruna *et al.* 2014). In the course of development, silkworm transforms into pupa, and after hatching, ichneumon larva feeds the entire content of pupa. Parasitoid's larva pupates there and transforms into adult and pierces its way out by making a circular characteristic hole at the anterior region of the cocoon near peduncle (Singh *et al.* 2010). Pimpla instigator detects host-mediated vibrational echoes for locating their hosts in microhabitats (Henaut and Guerdoux 1982).

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Wackers *et al.* (1998) also established that ichneumon wasps transmit their self-produced vibrations via the antennae onto the substrate covering the host and analyze the reflected signals to detect the position of host for accurate oviposition.

Because of the concealed feeding behavior of pedator, application of insecticides for its management is restricted. However, applied insecticides also kill nontarget arthropods, typically insects involved in pollination and pedators. Insecticide residues find their way into water courses and affect the water we drink and food we eat. Furthermore, quite often the indiscriminate and unscientific use of pesticides has led to many problems, such as pests developing resistance and resurgence of once minor pest into a major problem besides environmental and food safety hazard (Cork *et al.* 2005). Hence, ecofriendly pest management would be the best strategy for managing this key pedator of tasar cocoons. Pheromones can provide a means of monitoring and controlling insects that are nontoxic to animals and plants and specific for the target pest (Cork and Hall 1998).

In parasitoid insects, most of the studies on mate finding have focused on the role of sex pheromone. Sex pheromones in like manner are widely and successfully used against several crop pests, particularly forests (Srivastava and Dhaliwal 2012). Female sex pheromones can be involved in mate location from a distance when mating takes place after dispersal, as it does in most parasitoid species (Hardy 1994). In parasitic wasps, learning of odors in association with oviposition in their victims has also been shown to be highly important (Vinson 1984). In numerous species, females attract males by emitting volatile sex pheromones that are detectable from a distance at specific times (Fauvergue *et al.* 2007). This is occasionally associated with calling behavior such as wing fanning while the ovipositor is exposed to the atmosphere (Jurenka 2003). Mate finding in parasitoids is mainly based on pheromones released by females, although on some occasions, the roles are reversed, with the males also releasing sex pheromones (Quike 1997; Ruther 2013). After mating, females switch from releasing pheromones or searching for males to search for hosts (Jang 1995; Kugimiya *et al.* 2010).

There is an urgent need for a sensitive means of monitoring and control of *Xanthopimpla* infestation using mating disruption. The existing literature on pheromone communication in this pedator revealed that no published work is available on this aspect. To provide this needed tool, the present research was carried out to know the sexual communication in this species so as to develop a management strategy (Table 1).

2. Materials and Methods

2.1. Collection and rearing of *X pedator* (Fabricius)

X pedators were collected from the *Terminalia arjuna* field immediately after emerging from the infested cocoons (Figure 1).

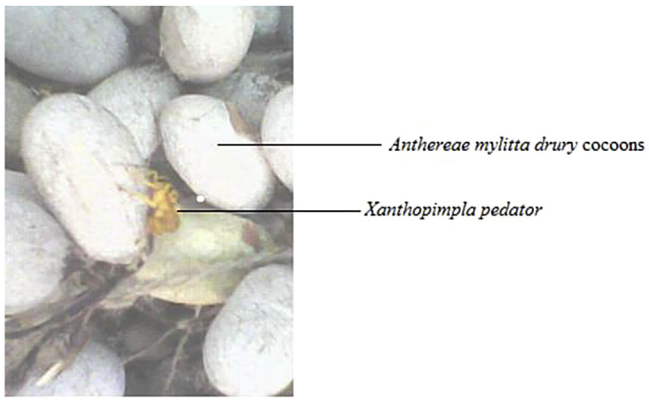

Figure 1. *Xanthopimpla pedator* attacking *Anthereae mylitta* Drury cocoons.

They were reared in the laboratory under a photoperiod of 12 hours light/12 hours dark cycle, at 28 ± 2°C temperature, and 75–80% humidity.

2.2. Behavioral assay

2.2.1. Olfactometer

Olfactometer is used to identify the sex having pheromonal attraction. Behavioral assay was carried by the olfactometer according to the method of Willem *et al.* (1999) (Figure 2). It consists of a central tube (13.5 cm long and 24 mm diameter) and two lateral arms (5.75 cm long and 24 mm diameter) (14.5 cm long and 19 mm diameter) that are fitted to broad tubes serving as a test chamber (B) in which the materials to be tested and compared were kept. The middle portion of the y-tube is fitted with a broad conical chamber called as release chamber (C), where the insects to be tested for responsiveness were released. There is a sieve inlayed in the extending glass tube 5.25 cm away from the connection to prevent escape of insects. Humidified and purified air was passed from an end of y-tube (A) at a rate of 200 mL/min. Airflow was regulated by a valve situated in the release chamber. Using the olfactometer, selecting the arm containing either the live adults or their volatile extract is possible. The entire olfactometer was washed thoroughly using soap solution and oven dried before each experiment.

2.2.2. Volatile-collecting apparatus

Pheromone collection was done in the dark room during early hours of scotophase or calling behavior according to Golub and Weatherson (1984). After confirmation of the sex that releases pheromone, it is confined to volatile-collecting apparatus for collection of volatiles (Figure 3). It consists of an air-loading chamber (B) of size (46 and 32 cm) through which air was blown

Table 1. Behavioral assay of live males, females, and volatiles

Experiment	Adults in release chamber			Percentage attracted	Test chamber live sex/volatile	Combination	χ^2	Notes
	Sex	Number	Number responded					
1	Female	30	01	3.3	40 M	M vs. F	22.0 NS	Females are not attracted by males
2	Male	30	30	100	40 F	F vs. M	0.0*	Males are attracted by females
3	Female	25	—	—	FV	FV vs. F	22.0 NS	Females are not attracted by female volatiles
4	Male	25	25	100	FV	FV vs. M	0.0*	Males are attracted by female volatiles
5	Female	25	—	—	MV	MV vs. F	22.0 NS	Females are not attracted by male volatiles
6	Female	25	—	—	FV, MV	FV vs. F	22.0 NS	Females are not attracted by females
						MV vs. F	22.0 NS	Females are not attracted by male volatiles
7	Male	25	25	100	FV, MV	FV vs. M	0.0*	Males are attracted by female volatiles
						MV vs. F		

M = male; F = female; NS = nonsignificant; FV = female volatile; MV = male volatile.
* Significant.

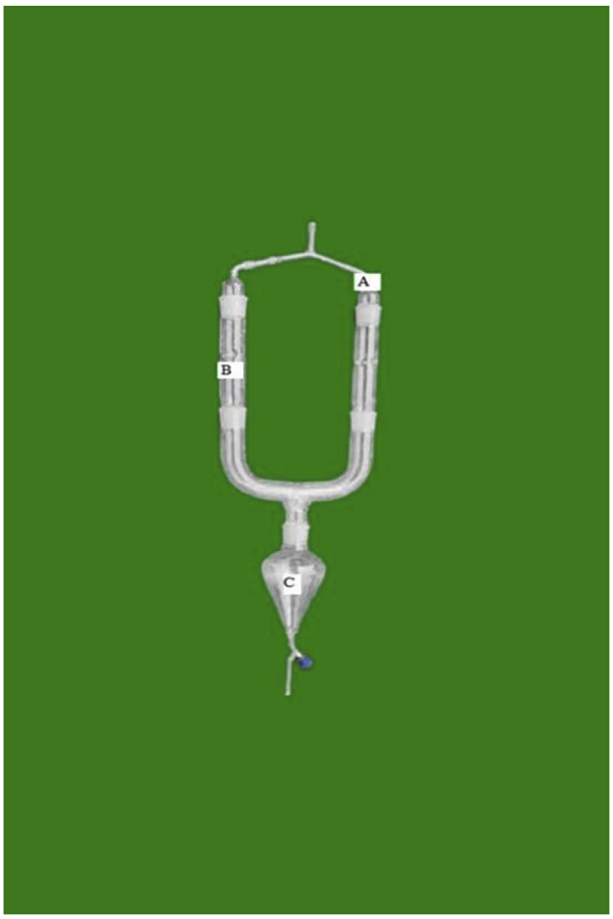

Figure 2. Olfactometer. (A) Y-tube. (B) Test chamber. (C) Release chamber.

Figure 3. (A) Insect confinement chamber. (B) Air-loading chamber.

and opened at five exits. The exits were closed with wide-mouthed small chambers for confining the adults, with long capillary tubes of 30 cm (A). Air was pumped using a motor. Air from the motor was filtered by passing through activated charcoal and then onto the air-loading chamber. From this chamber, air was passed through multiresting chambers in which the adults were confined. The air loaded with pheromones finally came out through the capillary tubes of 30 cm length where these volatiles are collected by washing several times with n-hexane. Opposite sex volatiles were also collected by using same volatile-collecting apparatus. The collected volatile extract was preserved at −20°C in 5-mL glass vials with Teflon liners amber bottles for further experiment. Live insects and collected pheromone were used for bioassay. The response of each sex to opposite sex, same sex, and collected pheromone was observed by the number of insects entering into the arm. n-Hexane was used as control for pheromones, and empty arm was used as control for live insects.

2.3. Experiments conducted with *X pedator* (Fabricius)

To identify the response of sex in live adults, two sets of experiments were conducted. In the first set of experiment, 40 males and 40 females were kept in two arms of test chamber (Figure 2A), and 30 males were released in the release chamber (Figure 2B), on which all the 30 males entered into the arm of test chamber containing females. This indicates that the females attracted the males. In the second set of experiment, 40 males and 40 females were kept in two arms of test chamber (Figure 2A), and 30 females were released into the release chamber (Figure 2B) on which a single female entered into the arm containing male. This indicates that the females attracted neither to the females nor to the males. Each set of experiment was carried for about 2 hours. The results obtained from both the experiments indicate that females are the attractants. So, the volatiles were collected from females, and responses of males and females to female volatiles were estimated.

2.4. Experiments conducted on the effect of volatile extracts of female against live females

In the first experiment, female volatiles were kept in one arm of test chamber and n-hexane in other arm. Five groups of females of five each were released in the release chamber (Figure 2C). But no single female get attracted to volatile showing that female volatile does not attract females.

2.5. Experiments conducted on the effect of volatile extracts of female against live males

In the second experiment, female volatiles were kept in one arm of test chamber (Figure 2B) and n-hexane in other arm. Five groups of males of five each were released in the release chamber (Figure 2C). All the males entered into female volatile-containing arm showing 100% attraction of males by females.

2.6. Experiments conducted on the effect of volatile extracts of male against live females

In the third experiment, male volatiles were kept in one arm of test chamber (Figure 2B) and n-hexane in other arm. Now five groups of females of five each were released in the release chamber (Figure 2C), but no single female entered into test chamber showing that males do not attract females.

2.7. Experiments conducted on the effect of volatile extracts of both males and females against live females

In the fourth experiment, both male and female volatiles were kept in arms of test chamber (Figure 2B) simultaneously, and five groups of females of five each were released in release chamber (Figure 2C), but not a single female entered into arms containing male and female volatiles indicating that both males and females do not attract females.

2.8. Experiments conducted on the effect of volatile extracts of both male and female against live males

In the fifth experiment, both male and female volatiles were kept in the arms of test chamber (Figure 2B) simultaneously, and five groups of males of five each were released in release chamber (Figure 2C). All the males entered into arm containing female volatiles clearly indicate that females attract males.

2.9. Column chromatography for fractionation of female volatiles

Fractionation of the female volatile compound was done with column chromatography. Hexane solution was applied to a chromatography column (30 × 10 mm) packed with 30 g of Florisil (100–200 mesh; Floridin Co., Hancock, MI, USA) in hexane. After sample loading, column flow rate was adjusted to 2.5 mL/min. An elution solvent composed of 10% hexane in ether, 15% hexane in ether, 20% hexane in ether, 25% hexane in ether, and ether alone was applied to the column. Resultant five fractions were bioassayed in the olfactometer using the respective solvent as control.

2.10. Statistical Analysis

The data collected were analyzed by (IBM) SPSS, version 19, statistical software for χ^2 goodness-of-fit test for significance of response. Each experiment was repeated three times, and the results mentioned are an average of three replications. The data collected from the readings were transferred to an electronic format and converted into a spreadsheet layout (Microsoft Excel, 2007). Graphs were generated from the spreadsheets. Attractive index (AI) was calculated according to the following formula:

$$\mathrm{AI} = \frac{\#\ \text{responded to test material} - \#\ \text{responded to control}}{\#\ \text{released} - \#\ \text{responded to control}}$$

3. Results

In this experiment, 30 live male *Xanthopimpla* were released in the release chamber (Figure 2C). All the 30 *Xanthopimpla* remained stationary in the release chamber for 15 minutes. Later, they started moving toward the arm of test chamber containing females. After 30 minutes from the start point, 10 *Xanthopimpla* entered into arm of test chamber (Figure 2B) containing female *Xanthopimpla*, and one entered into arm of test chamber (Figure 2B) containing male *Xanthopimpla.* But immediately after 5 minutes, this male *Xanthopimpla* returned back into test chamber (Figure 2B) containing female. After 1 hour from the movement, all the leftover entered into test chamber (Figure 2B) containing female. This shows attraction toward the arm of test chamber (Figure 2B) containing female *Xanthopimpla* and shows a strong attraction of male by female *Xanthopimpla*.

When 30 live female *Xanthopimpla* were released in the release chamber (Figure 2C), all the 30 *Xanthopimpla* remained stationary in the release chamber for 60 minutes. Later, only one female *Xanthopimpla* started moving toward the arm of test chamber (Figure 2B) containing males, and till 2 hours, not a single female *Xanthopimpla* again entered into test chamber (Figure 2B). This indicates that female *Xanthopimpla* are attracted to neither the males nor the females.

Present results also show that on releasing 25 females in release chamber (Figure 2C) by keeping female volatiles in one arm of test chamber (Figure 2B) and n-hexane in other arm, no single female get attracted to volatile or n-hexane. This confirms that female *Xanthopimpla* are not attracted by female volatiles.

Experiments conducted by keeping female volatiles in one arm, n-hexane in other arm of chamber, and 25 males in release chamber have shown that all the males remained stationary in the release chamber for 10 minutes than 15 males that have started moving toward arm containing female volatile. After 45 minutes, all the males entered into arm containing female volatiles showed 100% attraction of males by females.

Results obtained by keeping male volatiles and n-hexane in the arms of test chamber (Figure 2B) simultaneously and 25 males in release chamber (Figure 2C) did not show any attraction of males by either males or n-hexane.

When 25 female *Xanthopimpla* were released in the release chamber (Figure 2C) with male volatiles in one arm and female volatiles in other arm of test chamber (Figure 2B), not a single female entered into arms containing male and female volatiles indicating that both males and females do not attract females.

Results obtained by keeping both male and female volatiles in the arms of test chamber (Figure 2B) simultaneously and 25 males in release chamber had shown 100% attraction of males by females. All the 25 males remained stationary in the release chamber for 10 minutes (Figure 2C). After 25 minutes from starting of experiment, 15 males entered into arm containing females. Finally, after 60 minutes, remaining 10 males entered into the same arm containing female volatiles clearly indicating that females attract males.

The five fractions of female volatiles eluted from column chromatography were bioassayed using the y-tube olfactometer (Figure 2A). It was already proven that female volatiles attract males. Further experiments with these fractions were done to identify the response of males. Fraction I when tested with 25 males attracted 12 males. This indicates that 48% males were attracted to this fraction. In fraction II, tested with the same number of males, 14 males were entered into test chamber indicating 56% males were attracted to this fraction. In further tests with fraction III with 25 males, 22 males entered into test chamber. It indicates that 88% of males were attracted to this fraction. In test with fraction IV with 25 males, only two entered into test chamber indicating that only 8% males were attracted. On repeating the same test with fraction V of female volatiles by releasing 25 males in release chamber, only three males entered into test chamber. It represents that 12% of males are attracted to this fraction. From the results, it was proven that fraction III (20% fraction) of female volatiles highly attracts males than all other fractions (Table 2).

Table 3 explains the responsiveness and AI between both male and female sex. A maximum value of 1 AI was identified in the males to live females and female volatiles. An AI value of 0.95 was identified with males against fraction III. This is almost similar to live female and female volatile.

4. Discussion

Our results constitute the first experimental evidence of release of sex pheromone by female *X pedator* (Fabricius). Behavioral assays conducted by using Y-Tube olfactometer with live *Xanthopimpla* have shown that female sex pheromone of *Xanthopimpla*, compared with that of male, was apparently strong and effective. Female moths store pheromone molecules within pheromone glands that may be dorsally located in the abdomen with pores

Table 2. Attractive efficiency of female volatile fractions I–V

Fractions	Adults in release chamber			Percentage of males attracted	χ^2
	Sex in release chamber	Number of males in release chamber	Responded		
I	Males	25	12	48	6.8
II	Males	25	14	56	3.6
III	Males	25	22	88	0.3*
IV	Males	25	2	8	12.5 NS
V	Males	25	3	12	10.6 NS

NS = nonsignificant.
* Significant.

Table 3. Response and AI in *Xanthopimpla pedator*

Adults or volatiles in test chamber	Adults in release chamber	Response to test chamber	AI
Live male, live female	Male	Live female	1.0
Live male, live female	Female	Live male	0.05
Female volatile, n-hexane	Male	Female volatile	1.0
Female volatile, n-hexane	Female	Female volatile	0.0
Male volatile, n-hexane	Male	Male volatile	0.0
Male volatile, n-hexane	Female	Male volatile	0.0
Male volatile, female volatile	Male	Female volatile	1.0
Male volatile, female volatile	Female	Male volatile	0.06
Fraction I, n-hexane	Male	Fraction I of female volatile	0.46
Fraction II, n-hexane	Male	Fraction II of female volatile	0.63
Fraction III, n-hexane	Male	Fraction III of female volatile	0.95
Fraction IV, n-hexane	Male	Fraction IV of female volatile	0.02
Fraction V, n-hexane	Male	Fraction V of female volatile	0.04

AI = attractive index.

opening in glandular cells between the 8th and 9th abdominal segments (Ma and Ramaswamy 2003). Female-produced sex pheromones typically serve to attract males from long range to enable close range courtship behavior in several species of parasitoids (Ruther 2013). According to Xu *et al.* (2014), parasitoids like *Cotesia* males are strongly attracted to female pheromones than females to males. Similar results were obtained in *Trichogramma chilonis Ishii*, while experimenting with olfactometer and wind tunnel (Ranjith 2007). Nisha and Kennedy (2015) working on parasitoid *Acerophagus papayae Noyes* and *Schauff* using different olfactometers have reported the efficiency of female pheromones in attractiveness. Serotonin and octopamine modulate male sensitivity to sex pheromone when injected into males of several moths' species (Linn and Roelofs 1984, 1986; Linn 1997).

Volatiles collected by using n-hexane have proven that female volatiles attract males rather than females. Similar results obtained in volatile extraction of female *Earias vitelli* during scotophase have proven maximum attraction of males (Carde 1984). Purification of pheromonal gland extracts for volatile pheromone collection is best possible with a range of hexane to chloroform (Heath *et al.* 1988). Differences in the sensitivity of male and female wasps to odor stimuli arise from the sexual differences in the higher order processing of incoming peripheral olfactory information (Jyothi *et al.* 2002). However, female attraction to female volatiles and male volatiles was proven negative in this study. Male hamsters when exposed to female secretion connected to mating behavior containing both volatile and nonvolatile components showed increased responses to the volatile components and were no longer dependent on the nonvolatile components of the secretion for attraction and mating (Meredith 1986; Fewell and Meredith 2002).

The resultant five fractions of female *Xanthopimpla* volatiles obtained by column chromatography have shown that fraction III (20%) is highly effective in attracting the male *Xanthopimpla*. In *Diaphania indica*, fractionation of female volatiles by column chromatography resulted into five fractions of which 5% fraction of sex pheromones obtained found to be an active fraction (Miller and Roelofs 1978).

The maximum AI of females to males explains the release of sex pheromone by female in *X pedator* (Fabricius). According to Xu *et al.* (2014), parasitoids like *Cotesia* males are strongly attracted to female pheromones than females to males. Similar results were obtained in *Trichogramma chilonis Ishii*, while experimenting with olfactometer and wind tunnel (Ranjith 2007). Nisha and Kennedy (2015) working on parasitoid *Acerophagus papayae Noyes* and *Schauff* using different olfactometers have reported the efficiency of female pheromones in attractiveness.

In conclusion, *Xanthopimpla* is a major parasitoid of silk worm cocoons. Various control methods were found inefficient. Present study has proven that female *Xanthopimpla* releases sex pheromone that attracts males, and the study on this sexual communication helps to develop management strategy. Further research on this topic helps to isolate and synthesize pheromone and can be applied for *Xanthopimpla* trapping.

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Conflict of interest

Authors have declared that no competing interest exists.

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