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Effect of *Silybum marianum* methanolic extract on nutritional indices, crustacean cardioactive peptide, α-amylase and protease activities of *Helicoverpa armigera* (Lep.: Noctuidae)

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Abstract
The efficacy of milk thistle, *Silybum marianum* methanolic extract was investigated on the third instar larvae of cotton bollworm, *Helicoverpa armigera* (Hübner). The experiments were done at 25±1 °C, 65±5 RH and photoperiod of 16:8 (L: D) h. The LC_{50} and LC_{20} values were estimated 10449 and 5654 ppm, respectively. One hundred microliters of the plant extract at 5654 ppm was added to 0.9 grs of artificial diet. Nutritional indices, crustacean cardioactive peptide (CCAP) content and digestive enzymatic (α-amylase and protease) activities were measured after 72 h. Plant extract decreased nutritional indices including approximate digestibility (AD), relative growth rate (RGR), relative consumption rate (RCR), efficiency of conversion of ingested food (ECI) and efficiency of digested food (ECD). Methanolic extract of *S. marianum* decreased α-amylase and protease activities in the midgut of *H. armigera*. The activity of α-amylase from 194 mU in control decreased to 86.8 mU in treatment. It also decreased protease activity from 108.2 mU in control to 60.6 mU in treatment. Incubation of dissected midgut with CCAP increased α-amylase and protease activities in *H. armigera* whereas the buffer alone had no effect. Feeding on artificial diet containing methanolic extract of *S. marianum* caused CCAP level in the midgut of the insect to decrease. Feeding on artificial diet containing methanolic extract of *S. marianum* inhibits release of CCAP in the midgut and leads to reduction of α-amylase and protease activities.
Key words: *Silybum marianum*, *Helicoverpa armigera*, crustacean cardioactive peptide, digestive enzymes, Nutritional indices

اثر عصاره متانولی ماریتیغال بر شاخص‌های تغذیه‌ای، نوروپپتید سی سی ا پی و فعالیت آلفا آمیلاز و پروتئازکرم غوزه پنبه

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چکیده
اثرات عصاره متانولی بذر ماریتیغال Silybum marianum روی لارو سن سه کرم غوزه پنبه *Helicoverpa armigera* (Hübner) در قالب رژیم غذایی مصنوعی در دمای ۱±۲۵ درجه، رطوبت نسبی ۵ ±۶۵ درصد و دوره نوری ۸ :۱۶ مورد بررسی قرار گرفت. LC50 و LC20 عصاره ماریتیغال به ترتیب برابر ۱۰۴۴۹ و ۵٦٥٤ پی‌پی‌ام بود. صد میکرولیتر از عصاره گیاهی به غلظت ۵٦٥٤ پی پی ام (LC20) به نه دهم گرم غذای مصنوعی اضافه شده و پس از گذشت ۷۲ ساعت، شاخص‌های تغذیه‌ای، مقدار نوروپپتید crustacean cardioactive peptide (CCAP) و میزان فعالیت آلفا آمیلاز و پروتئاز مورد بررسی قرار گرفت. عصاره گیاهی شاخص‌های تغذیه‌ای از جمله شاخص تقریبی هضم شوندگی غذا، نرخ رشد نسبی، نرخ مصرف نسبی ، کارایی تبدیل غذای خورده شده و کارایی تبدیل غذای هضم شده را کاهش داد. عصاره گیاهی میزان فعالیت آنزیم آلفا آمیلاز و پروتئاز را در معده میانی کرم غوزه پنبه کاهش داد. فعالیت آنزیم آلفا آمیلاز در شاهد ۱۹٤ mU بوده در حالی‌که در تیمار ۸٦/۸ mU اندازه گیری شد. میزان فعالیت آنزیم پروتئاز نیز از ۱۰۸/۲ mU در شاهد به ٦۰/٦ mU در تیمار رسید. . قرار دادن معده میانی در بافر حاوی CCAP منجر به افزایش فعالیت آلفا آمیلاز و پروتئاز شد در حالی‌که بافر به تنهایی هیچ تاثیری نداشت. تغذیه از غذای مصنوعی حاوی عصاره گیاهی میزان نوروپپتید CCAP را در معده میانی لارو کاهش می‌دهد. در کل می‌توان نتیجه گرفت که تغذیه از غذای مصنوعی حاوی عصاره

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ماریتیغال باعث کاهش آزاد سازی نوروپپتید CCAP در معده میانی شده که این کاهش به نوبه خود منجر به کاهش فعالیت آلفا آمیلاز و پروتئاز می شود.

واژگان کلیدی: ماریتیغال، کرم غوزه پنبه، crustacean cardioactive peptide، آنزیم‌های گوارشی، شاخص‌های تغذیه‌ای

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Introduction

Helicoverpa armigera (Hübner), commonly known as cotton bollworm, is an important polyphagous pest, that infests more than 500 plant species in tropical and subtropical regions leading to heavy damage to agricultural crops (Talekar *et al.*, 2006; Muthusamy *et al.*, 2015). It has developed resistance against many common synthetic insecticides including pyrethroids, organophosphorus and carbamates in many countries in the world (Bues *et al*., 2005). Moreover, environmental pollution from insecticide use and its impacts on non-target organisms and hazards for human led us to find an alternative way to control this insect pest. Over the last three decades, greater attention has been paid to the bioactivity of botanical products for their effect on phytophagous insects (Hasheminia *et al*., 2011).The plant families Asteraceae, Euphorbiaceae, Fabaceae and Meliaceae contain most of the insecticidal plant species (Charleston *et al.*, 2005). Plant extracts derived from different plant species have been proved to have insecticidal activity against different pest species (Feng *et al.*, 2012). Muthusamy *et al*. (2015) showed that methanol extract of *Caesalpinia bonducella* L. could be an antifeedant, oviposition deterrent and larvicidal agent against *H. armigera*. Baskar *et al*. (2010) observed the antifeedant and larvicidal activities of crude extract of *Couroupita guianens* against *H. armigera.* It was also noted that methanolic extract of *Terminalia arjuna, C. bonducella* and *Trachyspermum roxburghianum* have antifeedant activity against the fourth instar larvae of *H. armigera* (Thushimenan *et al*., 2016).The methanol extract of *Melia dubia* caused growth inhibitory against the larvae of this insect (Koul *et al.*, 2000). Kamaraj *et al.* (2008) showed that methanol extract of *Citrus sinensis* L., acetone extract of *Ocimum sanctum* L. and acetate extracts of *Ocimum canum* L. were highly effective against the larvae of *H. armigera*. Other authors indicated that the effect of crude and partially purified extracts from ultraviolet-B irradiated leaves of *Oryza sativa* L. demonstrated antifeedant and growth inhibitory against *H. armigera* (Caasi-Lit 2005).The *Capsicum annum* leaf extracts has the potential in inhibiting *H. armigera* larval growth and fertility (Tamhane *et al*.,2005).

Milk thistle (*Silybum marianum* L.) belonging to Asteraceae family is a popular herbal product used for chemoprevention (Brantley *et al*., 2010). It has toxic, deterrent, and feeding inhibitory effects on *Pieris rapae* L. (Hasheminia *et al*., 2013).

Insect neuropeptides are involved in most physiological processes such as feeding (Maestro & Bellés, 2006). Crustacean cardioactive peptide (CCAP) is a neuropeptide that was originally isolated as a cardio accelerator in the pericardial organs of the shore crab, *Carcinus maenas* L. (Stangier *et al*., 1987). It was identified in the nervous system of many insects (Park *et al*., 2003). Later, Sakai *et al.* (2006) stated that CCAP up-regulated digestive enzyme activities in the American cockroach, *Periplaneta americana* L.

In the present investigation the effect of methanolic extract of milk thistle has been considered on toxicity, nutritional indices and enzymatic activities of *H. armigera*..

Material and Methods

Insect rearing

H. armigera were collected in cotton Fields in Golestan province, Iran. The culture of *H. armigera* was maintained in the laboratory on an artificial diet (Shorey & Hale., 1965) in a growth chamber at 25 ± 1 °C, 65 ± 5% RH, and a photoperiod of 16:8 L: D. Third instar larvae were used in all experiments.

Methanolic extract preparation

Seeds of *S. marianum* were obtained from Institute of Medicinal Plants in Isfahan, Iran in July, 2015. They were dried at room temperature in the shade after washing with distilled water. Dried seeds were powdered using an electronic grinder. The powder was used for methanolic extraction and used according to the procedure described by Warthen *et al*. (1984). Briefly, in a 1000-mL flask, 30 g of seed powder was stirred for 1 hour with 300 mL of 85% methanol. The solution was incubated at 4 °C for 48. Later it was stirred for one hour more and filtered through Whatman No. 4 filter paper. The solvent was removed at 40 °C by vacuum in a rotary evaporator. Finally, the residue was dissolved in 10 mL of methanol which was used as a stock solution. For preparing different concentrations, further dilutions with methanol were used.

Bioassays and treatment

Toxicity tests

Five concentrations of the plant extract were prepared to evaluate LC_{50} along with a control treated with methanol. Each concentration was mixed with artificial diet. 24 hour-aged third instars were used for all the experiments. In each experiment 30 insects were tested with 4 replicates for each concentration. Mortality was recorded after 48 hours and the LC_{50} values were estimated using SAS 6.12 software (Finney, 1971).

Nutritional indices assay

One hundred microlitesr of the plant extract at 5654 ppm was added to 0.9 gr of artificial diet and transferred into plastic containers (diameter: 15 cm, depth: 7 cm). 24

hour- aged third instar larvae were divided into four replicates (15 larvae per replicate). They were transferred into plastic containers (diameter: 15 cm, depth: 7 cm) which had a hole covered by a fine mesh net. The experiments were done in 25 ± 1°C, 65 ± 5% RH, and a photoperiod of 16:8 (L:D) hours. After 72 h, nutritional indices were measured. The nutritional indices were calculated according to the following formula (Waldbauer, 1968):

Relative growth rate (RGR) =P / (T × A)

Where: P is dry weight gain of larvae (mg), A is dry weight of the insect over unit time (mg), T is the duration of the experimental period (day).

Relative consumption rate (RCR) = E / (A × T)

Where: E is dry weight of food consumed (mg).

Efficiency of conversion of ingested food (ECI) = (P / E) × 100

Efficiency of conversion of digested food (ECD) =[P / (E - F)] × 100

Where: F is dry weight of feces produced (mg).

Approximate digestibility (AD) = [(E - F) / E] × 100

Measurement of α-amylase and protease activities

α-Amylase activity was observed as had been described by Sakai *et al*. (2006). For measuring α-amylase activity, α-amylase measuring kit (Kikkoman Corp., Chiba, Japan) was used. The midgut of third instar larva was dissected in 50 mM Tris–HCl (pH 7.4) and food particles were removed. After the incubation of the midgut in 50 mM Tris–HCl (pH 7.4) for 30 min at room temperature, the enzyme activity released into the medium. The sample (0.1 mL) was incubated at 37 °C for 10 min with 0.5 mL of substrate buffer which contained 2-chloro-4-nitrophenyl 65-azido-beta-maltopentaoside (N3-G5-CNP) and 0.5 mL of co-working enzyme solution that contained glucoamylase and β-glucosidase. Finally, by adding 2.0 mL of stop solution which contained sodium carbonate, the reaction was stopped. One unit (U) of enzyme activity was defined as the amount of enzyme that produces 1 μmol 2-chloro-4-nitrophenol (CNP) from N3-G5-CNP for 1 min. The absorbance of CNP was measured by microplate reader (Biotek, U.S.A) at 400 nm.

Protease activity was measured by digestion of azocasein according to the method of Elpidina *et al*. (2001). Briefly, the midgut of third instar larva was dissected in 50 mM Tris–HCl (pH 7.4). After incubation of the midgut in 50 mM Tris–HCl (pH 7.4) for 20 min at room temperature and releasing of protease activity into the supernatant, 300 μL of the sample were incubated with 300 μL of 0.5% (w/v) azocasein solution in Tris–HCl (pH 7.4) at 37 °C for 30 min. The 800 μL amount of 20% trichloroacetic acid on ice for 10 min were added to stop the reaction. Sample was centrifuged (4000 g at 4 °C, 15 min) and the precipitated azocasein was removed. The absorbance of the supernatant was measured by microplate reader at 335 nm. One unit (U) of hydrolytic activity of the protease was determined as the amount of enzyme required to cause an increase of 0.01 A335 units per minute in 1 mL of reaction mixture.

Effect of CCAP on α-amylase and protease activities

The midgut was dissected and incubated for 30 min at room temperature in 50 mM Tris–HCl (pH 7.4) in the absence or presence of CCAP (PFCNAFTGCamide, Genemed Synthesis Inc. South San Francisco, Canada) in different concentrations. Enzyme activity released into the medium was measured.

Competitive ELISA

Competitive ELISA followed Sakai *et al.* (2006). The midgut was dissected in Tris buffered saline (TBS; 135 mM NaCl, 2.6 mM KCl, 25 mM Tris–HCl, pH 7.6) and food particles were removed. After homogenization, it was centrifuged (4000 × g, 4 °C, 15 min) and the supernatant was used for assay. A synthetic CCAP and the antiserum against CCAP were used to quantify CCAP in the samples. By coupling CCAP to BSA with dimethyl suberimidate (Sigma–Aldrich, U.S.A) a CCAP-BSA conjugate was prepared. The plates were coated with CCAP–BSA (0.6 μg/ml per well) in 0.05 M sodium carbonate–bicarbonate buffer (pH 9.0) and kept at room temperature for 3 h, followed by adding 250 μL of 2% skimmed milk to each well and incubated for 1 h. Standard peptides (0.01–100 nmol/well) or supernatant of midgut in a volume of 50 μL per well. Later, 50 μl of the diluted antiserum against CCAP (1:11,000 concentrations in TBS with 2% skimmed milk) were added to each well. The plate was incubated overnight at 4 °C. The plate was rinsed three times with TBS containing 0.5% Tween-20 (TBS-Tw) followed by incubation with 100 μL of secondary antibody solution in TBS (1:1000) for 1 h at room temperature. The plate was washed three times with TBS-Tw, followed by adding 100 μL of substrate solution [1 mg/mL ρ-nitrophenylphosphate disodium salt hexahydrate (Sigma–Aldrich, U.S.A) in 10 mM diethanolamine buffer (Sigma–Aldrich, U.S.A), pH 9.5] to each well and incubation in room temperature for 1 h. The reaction was stopped by adding 50 μL 4 M NaOH to each well. Finally, the absorbance was read at 405 nm using a microplate reader.

Statistical analysis

The results are shown as mean ± SEM and p values of < 0.05 was used as the level of significant difference between means using one-way ANOVA (Fishers LSD).

Result

Toxicity test

The LC_{50} value, confidence limit (95%) and regression slope at 72 h exposure to artificial diet which contained fifty microliter of the plant extract are shown in Table 1 and Fig. 1. The LC_{50} for third instar larva was estimated as 10449 ppm.

Table 1. Toxicity of methanolic extract of *Silybum marianum* to third instar larvae of *Helicoverpa armigera*, 72 hours after eating artificial diet containing plant extract.

Extraction	N	χ^2 (df)	P-value	Slope ± SE	LC_{20} (ppm) 95% confidence limits (ppm)	LC_{50} (ppm) 95% confidence limits (ppm)	LC_{80} (ppm) 95% confidence limits (ppm)
Silybum marianum	30	0.977(3)	0.807	3.156±0.390	5654 (4514-6587)	10449 (9325-11756)	19310 (16417-24620)

Effect of methanolic extract of *S. marianum* on nutritional indices

The Approximate digestibility (AD) of 3rd instar *H. armigera* larva, feeding on artificial diet containing methanolic extract of *S. marianum* at 5654 ppm (LC_{20}), significantly reduced. Plant extract decreased relative growth rate (RGR) from 0.75 ±0.01 in control to 0.24± 0.02 mg/mg/day in the treatment and relative consumption rate (RCR) from 1.07 ±0.07 in control decreased to 0.22± 0.01 mg/mg/day in treatment. Efficiency of conversion of ingested food (ECI) and efficiency of digested food (ECD) from 38± 0.2% and 82.8 ±0.42% in control reduced to 14.8 ±0.009% and 30.2 ±0.1% in treatment respectively (Table 2).

Table 2. Nutritional indices of third instar larvae of *Helicoverpa armigera*, 72 h after eating artificial diet containing 5654 ppm (LC_{20}) of *Silybum marianum*.

Treatment	PGR	PCR	ECI	ECD	AD
Control	0.75±0.01	1.07±0.07	38±0.2	82.8±0.42	92±0.05
Silybum marianum	0.24±0.02*	0.22±0.01*	14.8±0.009*	30.2±0.1*	63±0.06*

RGR : relative growth rate; RCR: relative consumption rate; ECI: efficiency of conversion of ingested food; ECD: efficiency of conversion of digested food; AD: approximate digestibility. An asterisk indicates a significant difference relative to the control treatment.

Effects of methanolic extract of *S. marianum* on digestive enzyme activities

The result indicated that fifty microliters of the plant extract at 5654 ppm (LC_{20}) sharply decreased α-amylase activity in *H. armigera* using oral ingestion treatment. The activity from 194 mU in control decreased to 86.8 mU in treatment (Fig 2A). It also significantly decreased the activity of protease in third instar larvae of the insect, from 108.2 mU in control to 60.6 mU in treatment (Fig. 2B).

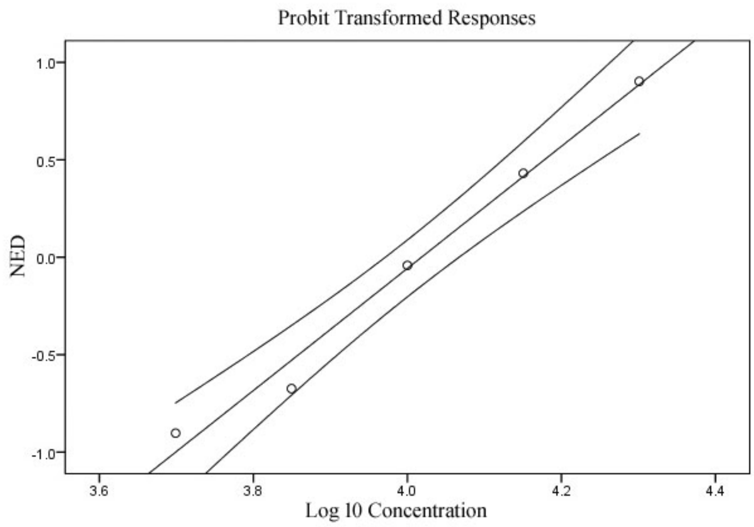

Fig. 1. Probit analysis of mortality in third instar larvae of *Helicoverpa armigera*, 72 h after eating artificial diet containing 5654 ppm (LC_{20}) of *Silybum marianum*. NED is referred to normalized equivalent deviation.

Effects of CCAP on digestive enzyme activities

Incubation of the dissected midgut with CCAP increased α-amylase and protease activities (Fig. 3 A, B).

Effect of methanolic extract of *S. marianum* on CCAP content in the midgut

Competitive Elisa result showed that CCAP titer in the midgut extract of larvae was significantly lower after 3 days of starvation. The titer sharply increased after 3 h of refeeding. Subsequently, we evaluated the effects of oral ingestion of fifty microliters of the plant extract at 5654 ppm (LC_{20}) which was added to 1gr of artificial diet. The result indicated that at 72 h post-feeding, midgut CCAP was clearly lower than in the control (Fig. 4).

Discussion

Secondary metabolites synthesized by plants play an important role in their defense against insects and act as toxicants or anti-feedants (Shekari *et al*., 2008). Our results show that methanolic extract of *S. marianum* functions as an insecticide at high concentrations (Table 1) and anti-feedant at low concentrations (Table 2) against *H. armigera* . Kamaraj *et al*. (2008) stated that chloroform extract of *C. sinensis* flower and methanol extract of *O. canum* had much more larvicidal activity against the larvae of *H.armigera* (LC50 = 65.10 and 51.78 ppm respectively). The toxic property of *S. marianum* against *Pieris rapae* was mentioned by Hasheminia *et al.* (2013).

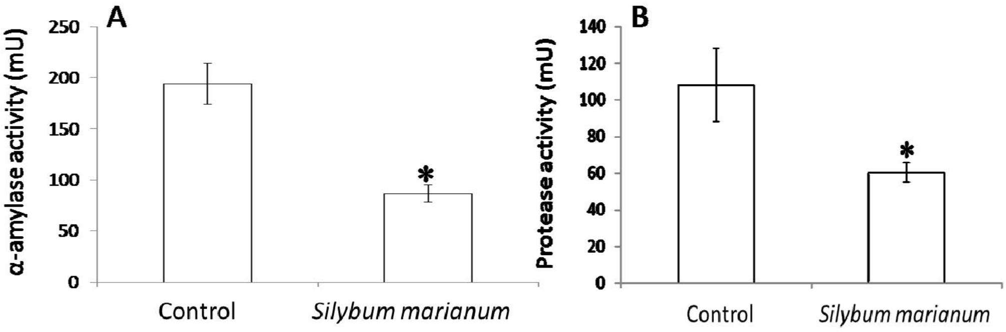

Fig. 2. α-Amylase (A) and protease (B) activities in third instar larvae of *Helicoverpa armigera*, 72 h after eating artificial diet containing 5654 ppm (LC_{20}) of *Silybum marianum* methanolic extract. Each point represents the mean±SEM. *p < 0.05, significantly different from control (Student's t-test).

The evaluation of the feeding indices at 5654 ppm (LC_{20}) of methanolic plant extract showed that AD in larval feeding on treated artificial diet decreased (Table 2) which disagrees with the earlier report (Hasheminia *et al*., 2013) on *P. rapae* larva, with unchanged AD after the treatment with the same plant extract. In this experiment, AD and ECI reduction prevented the insect from gaining weight resulted from detoxificating diet (Shekari et al., 2008).

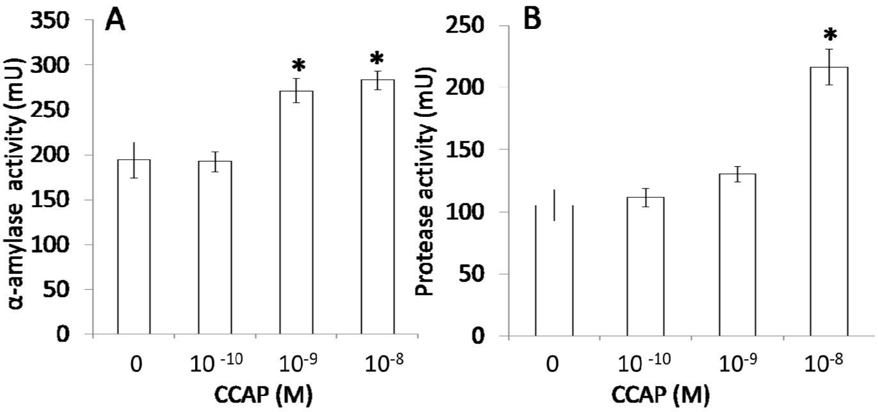

Fig. 3. Effect of CCAP on the midgut α-amylase (A) and protease (B) activities in third instar larvae of *Helicoverpa armigera*. Each point represents the mean ± S.E.M. of 8 preparations. *p < 0.05, compared with α-amylase or protease activity in the absence of CCAP (LSD test).

The results also showed that RCR and RGR were significantly lower in larvae feeding on a diet which contained plant extract (Table 2). These results are in agreement with the findings of Hasheminia *et al*., whose findings suggested reduced PCR and PGR in *P.rapae* using the same plant extract.

α-Amylase and protease are very important in digesting polysaccharides and protein respectively. The treatment with *S. marianum* extract led to α-amylase and protease activities decline (Fig. 2 A, B) which is consistent with previous researches that showed lower activity of these enzyme following treatment with the same plant extract *(*Hasheminia *et al*., 2013). Other reports also indicated decrease of α-amylase and protease activities after various plants extract treatments (Shekari *et al*., 2008; Hasheminia *et al*., 2011).

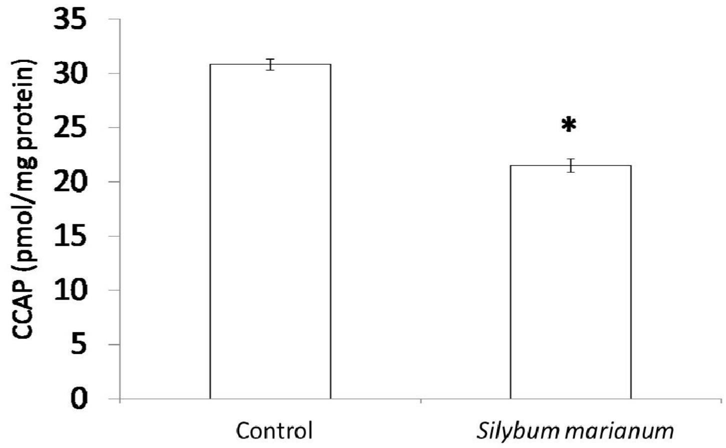

Fig. 4. A competitive ELISA detected CCAP titer in the midgut of third instar larvae of *Helicoverpa armigera*, 72 h after eating artificial diet containing 5654 ppm (LC_{20}) of *Silybum marianum* methanolic extract. Each point represents the mean ± SEM. *$p < 0.05$, significantly different from control (Student's t test).

Neuropeptides are small molecules which are used by neurons to communicate with each other and other tissues. Different neuropeptides are involved in a wide range of functions, including reproduction, social behaviors, food intake, memory and learning (Fouda & Takeda., 2015). The midgut produces and releases several neuropeptides and shows immunoreactivity to many peptides (Fuse *et al.*, 1999). For example short neuropeptide F, tachykinins, and diuretic hormone were all identified in the midgut of *Drosophila melanogaster* (Reiher *et al.*, 2011). Some peptides affect digestive enzyme activity levels in the midgut. Short neuropeptide F and allatostatin-A show inhibitory and stimulatory effect on α-amylase activity in *Periplaneta americana* respectively (Sakai *et al*., 2006., Mikani *et al.*, 2012). Sakai *et al.* (2006) mentioned that midgut itself regulates enzyme secretion. CCAP mRNA was detected in the endocrine cells of the midgut in *P. americana,* and administration of CCAP to the midgut caused α-amylase and protease activity level to elevate. Our experiment, CCAP increased α-amylase and protease activities in the midgut of *H. armigera* (Fig. 3) while CCAP level decreased after the pest fed on artificial diet containing methanolic extract of *S. marianum* (Fig. 4). The plant extract reduced α-amylase and protease activities (Fig. 2) and eating artificial diet containing methanolic extract of *S. marianum* inhibits release of CCAP (Fig. 4), which down-regulates α-amylase and protease activities.

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Reproductive performance of *Chouioia cunea* Yang (Hym.: Eulophidae) parasitizing fall webworm, *Hyphantria cunea* Drury (Lep.: Arctiidae)

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Abstract
The fall webworm, *Hyphantria cunea* Drury (Lep.: Arctiidae), is an important pest of forest and cultivated plants in Guilan Province, Iran. The reproductive performance of *Chouioia cunea* Yang (Hym.: Eulophidae), a gregarious pupal parasitoid of *H. cunea* was studied at 24±1°C, 70±5% (RH), and a photoperiod of 14:10 (L:D) hours. The pupal hosts were exposed to 1, 2, 4, 8, 12, 16 newly emerged adult parasitoids. The parasitoids remained in contact with host pupae for 24 hours in Petri-dishes (10x1 cm) until the death of all parasitoids. The results showed that parasitoid density influenced offspring production, as the higher parasitoid densities resulted in the lowest mean number of offspring per female (179.06± 6.29). The sex ratio was not influenced by parasitoid density, but the age of parasitoid affected sex ratio as a higher sex ratio (0.92±0.001♀) was observed in the progeny produced by younger parents. Rate of parasitism was higher at density of 4 wasps (33.33%). The mean percent parasitism by 1, 2 and 3 day-old female parasitoids were 21, 13 and 9, respectively ($P<0.05$). Maximum number of offspring produced per female was obtained at host/parasitoid ratio of 15 to 4. The female parasitoids survived 1-3 days after oviposition. The searching efficiency of the parasitoid decreased from 0.18 to 0.009h^{-1} with increasing its density. The survival rate for *C. cunea* was not significantly different at all densities of male or females, but a statistical difference was observed with increasing parasitoid age. It was concluded that the performance of *C. cunea* was mainly affected by its density and age.
Key words: foraging behavior, parasitism, reproduction, *Hyphantria cunea*, *Chouioia cunea*

عملکرد تولیدمثلی *Chouioia cunea* Yang (Hym.: Eulophidae) زنبور پارازیتویید پروانه ابریشم‌باف پاییزی *Hyphantria cunea* Drury (Lep.: Arctiidae)

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چکیده
پروانه ابریشم‌باف پاییزی *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae) آفت پروانه آفت مهم گیاهان جنگلی باغی و محصولات زراعی در استان گیلان است. در این پژوهش، عملکرد تولید مثلی زنبور *Chouioia cunea* Yang (Hym.: Eulophidae)، پارازیتوئید تجمعی شفیره *H. cunea* در دمای ۱±۲٤ °C، رطوبت ٪۵±۷۰ و دوره نوری ۱٤:۱۰ ساعت روشنایی، به تاریکی بررسی شد. شفیره‌های میزبان در معرض تراکم‌های ۱، ۲، ٤، ۸، ۱۲ و ۱٦ حشرات کامل پارازیتویید تازه خارج شده قرار داده شدند. زنبورهای پارازیتویید داخل ظروف پتری (۱x ۱۰ سانتی‌متر) به مدت ۲٤ ساعت در تماس با شفیره‌های میزبان باقی ماندند. این آزمایش تا پایان عمر تمام زنبورهای پارازیتویید ادامه یافت. نتایج نشان داد که تراکم پارازیتویید در تولید نتاج موثر بود، به‌طوری‌که بالاترین تراکم منجر به تولید کم‌ترین نتاج شد (٦/۲۹±۱۷۹/۰٦). تراکم پارازیتویید روی نسبت جنسی تاثیری نداشت، اما سن پارازیتویید بر نسبت جنسی تاثیر داشت و بالاترین نسبت جنسی در نتاج تولید شده توسط زنبورهای یک روزه مشاهده شد (۰/۹۲±۰/۰۰۱♀). بالاترین نرخ پارازیتیسم در تراکم ٤ عدد زنبور پارازیتویید به‌دست آمد (٪۳۳/۳۳). متوسط درصد پارازیتیسم زنبورهای ماده با طول عمر ۱، ۲ و ۳ روزه به ترتیب ٪۲۱، ٪۱۳ و ٪۹ تعیین شد (p <۰/۰۵). بیشینه تعداد نتاج تولید شده به ازای هر ماده در نسبت ٤ عدد پارازیتویید به ۱۵ شفیره میزبان به‌دست آمد. پارازیتوییدهای ماده ۱ تا ۳ روز پس از تخم‌ریزی زنده ماندند. قدرت جستجوی پارازیتویید با افزایش تراکم آن، از ۰/۱۸ به ۰/۰۰۹ بر ساعت کاهش یافت. نرخ بقای نتاج زنبور پارازیتویید

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C. cunea از لحاظ آماری در همه تراکم‌ها در هر دو جنس نر و ماده دارای تفاوت معنی‌دار نبود، اما با افزایش سن پارازیتویید تفاوت آماری مشاهده شد. نتیجه‌گیری شد که تراکم و سن زنبور پارازیتویید *C. cunea* به‌طور اساسی عملکرد تولیدمثلی آن را تحت تاثیر قرار می‌دهد.
واژگان کلیدی: رفتار کاوشگری، پارازیتیسم، تولید مثل، *Hyphantria cunea*، *Chouioia cunea*

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Introduction

The Fall webworm, *Hyphantria cunea* Drury (Lepidoptera: Arctiidae), is a polyphagous defoliating pest native to the US, Canada (Warren and Tadic, 1970) and New Zealand (Kean and Kumarasinghe, 2007). It is presently invaded many world areas such as Europe and Asia (Li *et al*., 2001). It was first spotted in Iran in 2002. The eggs of the pest hatch within one to two weeks, and emerging larvae immediately begin spinning their silk tent. Full-grown larvae leave the web to pupate in leaf litter or bark crevices. It overwinters in the pupal stage. Pupation takes place in a thin cocoon. There are two generations per year in Guilan Province (Rezaei *et al*., 2003).

A native pupal endoparasitoid, *Chouioia cunea* Yang (Hym.: Eulophidae, Tetrastichinae) causes considerable mortality on *H. cunea* pupae in some areas of China (Yang, 1989). *C. cunea* was found to be the dominant parasitoid of fall webworm pupae in Sangachin and Lashtenesha in Guilan Province during 2004-2005 as the rate of pupal parasitism was observed to be higher in the second generation (Ejlali, 2005). It completes its egg, larval, pupal and pre-egg-laying adult stages in the host pupae. The emerging adults also mate inside the host pupa where serves as an empty shell after the content was eaten by the parasitoid's larvae. Female, then bites a hole to come out, and all the other wasps usually follow her way through the hole. The females lay eggs soon after emergence by pricking host pupa with the ovipositor. The parasitoid larvae feed on haemolymph and organs in the host pupa. Once the larvae mature, the materials inside the host pupa are all consumed (Yang and Xie, 1998).

The reproductive potential of a parasitoid is one of the factors to be considered in evaluating its performance as biological control agent. It determines the population growth, and efficiency of the parasitoid. The reproductive potential of the both parasitoid and the insect pest are essential in evaluating the parasitoid’s capability as a viable control agent (van Lenteren, 1986).

The mass rearing represents an important stage of control programs (Parra *et al*., 2002; Pastori *et al*., 2008; Pereira *et al*., 2009), and the nutritional quality, size, age, mechanical resistance and capacity of immunological response of parasitoids should be considered to select alternative hosts (Godfray, 1994).

The parasitoids density per host affects the parasitoid offspring (Thomazini and BertiFilho, 2000; Matos Neto *et al*., 2004), the sex ratio (Choi *et al*., 2001), the parasitism capacity (Sampaio *et al*., 2001), the duration of the life cycle, the body size, and the longevity of the adults (Silva-Torres and Matthews, 2003).

To maximize the mass rearing of parasitoids, it is necessary to study the relation of parasitoids density to the host (Sagarra *et al*., 2000).

The aim of this study was to investigate reproductive performance of the parasitoid *C. cunea* on the fall webworm *H. cunea* Drury in laboratory conditions.

Material and methods

Host culture

Overwintering pupae of fall webworm were collected from various locations in Guilan Province, especially Shaft and Somae-Sara areas, under barks of old or dead forest trees, among leaf litters, ornamental trees, shrubs and hedgerows to establish a rearing stock of *C. cunea* wasps. The larvae of the pest were collected from the infested trees and transferred to transparent plastic trays (15×10×8 cm) for pupation in a growth chamber at 24±1°C, 70±5% of related humidity (RH), and a photoperiod of 14:10 (L:D) hours.

Parasitoid culture

Once adult parasitoids of *C. cunea* emerged from hosts' pupae, they were kept in glass petri dishes (10×2 cm) and fed with honey. The 48 to 72 hour-old pupae of *H. cunea*, after removal from their cocoons, were exposed to the parasitoid females for 24 hours in a growth chamber with identical condition.

Effect of parasitoid density on reproduction

To study the effect of parasitoid, different densities of progeny produced per female. A total of 15 pupae of the host were presented to a one day-old female parasitoid in a Petri-dish (10 x1cm) for 24 hours. The parasitoid densities of 1, 2, 4, 8, 12, 16 were used and each density level was replicated 15 times. Host pupae were replaced daily until the death of adult parasitoids. Parasitized pupae were kept under the same conditions until progeny emerged. The Percentage of parasitized host, mean number of progeny in each pupa and sex ratio of the parasitoid were recorded.

Effect of parasitoid age on offspring production and survival

The effect of parasitoid age on the rate of parasitism, offspring production, and sex ratio, female parasitoids aged 1, 2, and 3 days were individually exposed to 15 host pupae in a Petri-dish (10 x1cm) for 24 hours at the same condition. Host pupae were replaced daily until the death of adult parasitoids.

Effect of parasitoid density on searching efficiency

The data obtained from the the section 3 were used to find out the effect of parasitoid density on its searching efficiency. For data analysis, the method used by Hassell and Varley's (1969) was applied. The related equation is:

$$\log \alpha = \log Q - m \log Pt$$

α is the searching efficiency, Q is the quest constant, P_t is the parasitoid density and m is the coefficient of mutual interference.

The value of α at each parasitoid density was estimated utilizing the following formula, which derived from Nicholson's model by Hassell (1978):

$$\alpha = \frac{1}{Pt} \log_e \frac{Nt}{Nt - N\alpha}$$

N_t is the initial host density and N_a is the number of parasitized hosts.

Experiments were done in a completely randomized design and the means were separated by Tukey's test at the 5% level. The data analyses were performed through SAS software and figures drawn by Excel.

Results

Effect of parasitoid density on reproduction

The density of female *C. cunea* affected percentage of parasitism significantly (df=5, 89, F=7.03, P<0.0001) (Table 1).

Table 1. Effect of different densities of *Chouioia cunea* on parasitism of *Hyphantria cunea*.

Parasitoid density	No. of hosts	No. of hosts parasitized	Mean No. of pupae parasitized (Mean±SE)	Percent parasitism
1	225	27	1.8±0.03	12 c
2	225	51	3.4±0.07	22.66 abc
4	225	75	5±0.10	33.33 a
8	225	56	3.73±0.17	24.88 ab
12	225	48	3.2±0.11	21.33 bc
16	225	32	2.13±0.76	14.22 bc

Means within a column followed by the same letter do not differ significantly (Tukey's test, P < 0.05).

The parasitoid density also influenced the number of progeny produced per *H. cunea* pupa significantly (df=5, 89, F=47.76, P<0.0001). The quantity of offspring per pupa ranged from 0 to 996 (Table 2). There was no significant difference in sex ratios of progeny produced by the parasitoid in all different densities tested (df = 5, 89, F = 2.18; P=0.063).

This study also showed that there was no significant difference in survival rate of male and female offspring in all parasitoid densities (Table 3).

Table 2. Effect of the parasitoid density on offspring produced by *Chouioia cunea*, reared on *Hyphantria cunea*.

Parasitoid density	Mean No. offspring (Mean±SE)	Range	
		Min	Max
1	268.5± 2.58 cd	139	289
2	545.75± 3.52 b	339	634
4	828.62± 9.52 a	425	996
8	373.86± 12.81 c	72	701
12	256.66± 10.80 cd	9	548
16	179.06± 6.29 d	0	365

Means within a column followed by the same letter do not differ significantly (Tukey's test, P < 0.05).

Effect of parasitoid age on offspring production and survival

The mean percentage of parasitism by 1, 2 and 3 day-old females were significantly different (df=2,44, F=26.30, P<0.05). The age of *C. cunea* affected its sex ratio significantly (df =2, 44, F = 27.30; P < 0.0001). The mean sex ratios (female proportion) of 1 to 3 day-old females decreased with age from 0.92 to 0.53, respectively (Table 4). The number of offspring per host pupa decreased with increasing parasitoid age (Figure 1). The parasitoid offspring survival decreased significantly with increasing parasitoid age. It was also shown that the age of ovipositing females influenced female (df=2, 44, F=17.52, P<0.0001), and male (df=2, 44, F=10.66, P<0.0001) survival rate significantly (Table 5).

Table 3. Effect of *Chouioia cunea* density on its progeny survival rate when reared on *Hyphantria cunea*.

Parasitoid density	Mean female survival rate (Mean±SE)	Mean male survival rate (Mean±SE)
1	0.75±0.006 a	0.24±0.001 a
2	0.72±0.004 a	0.27±0.0005 a
4	0.63±0.007 a	0.36±0.0003 a
8	0.69±0.008 a	0.30±0.001 a
12	0.67±0.014 a	0.32±0.004 a
16	0.66±1.65 a	0.40±1.66 a

Means within a column followed by the same letter do not differ significantly (Tukey's test, P < 0.05).

Effect of parasitoid density on searching efficiency

Figure 2 shows the relationship between the searching efficiency (α) and the parasitoid density (P_t). The results indicated that with increasing parasitoid density, the searching efficiency of individual parasitoids reduced. This suggested that there was a mutual interference among the searching female parasitoids. The coefficient of mutual interference (m) and the model were estimated as -0.9268 and log a = −0.9268 log P_t− 0.7044.

Table 4. Effect of *Chouioia cunea* age on the percentage of parasitism and sex ratio on *Hyphantria cunea*.

Parasitoid age (day)	**Parasitism percentage (%) (Mean±SE)**	**Sex ratio (Mean±SE)**
1	21±0.007 a	0.92±0.001 a
2	13±0.005 b	0.70±0.004 b
3	9±0.005 b	0.53±0.005 c

Means within a column followed by the same letter do not differ significantly (Tukey's test, P < 0.05).

Interaction between age and parasitoid density

The number of progeny per female *C. cunea* in densities of 1, 2 and 4 parasitoids increased, and decreased at densities of 8, 12, 16 parasitoids (Figure 3). The rate of parasitism increased up to density of 4 parasitoids and later decreased. However, the number of progeny decreased as parasitoids grew older (Figure 4).

Table 5. Effect of *Chouioia cunea* age on its adult survival rates reared on *Hyphantria cunea*.

Parasitoid age	**Mean female survival rate (Mean±SE)**	**Mean male survival rate (Mean±SE)**
1	0.85±0.002a	0.30±0.001a
2	0.79±0.002 b	0.20±0.002 a
3	0.69±0.001c	0.14±0.002 b

Means within a column followed by the same letter do not differ significantly (Tukey's test, P < 0.05).

Discussion

Data analysis showed that the mean number of offspring and percentage of parasitism significantly decreased with parasitoid density. Higher densities of adult parasitoids produced lower number of offspring, probably because of the fixed number of hosts. Lu (1992) also found that an increase in parasitoid density reduced the number of parasitized eggs per female.

Statistical analysis showed that parasitoid densities did not affect the sex ratio in all densities. This relationship was also stated by other researchers (e.g., Murdoch *et al*., 2003; Ode and Hardy, 2008; Irvin and Hoddle, 2006).

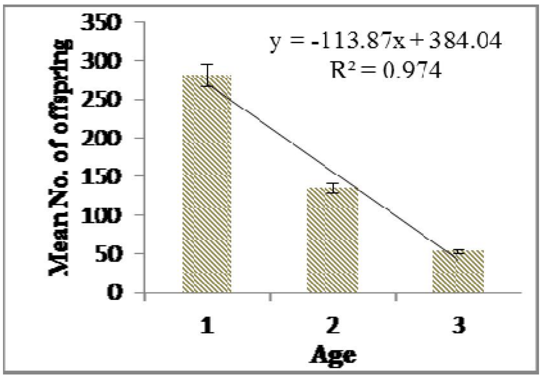

Fig. 1. Effect of *Chouioia cunea* age on offspring production.

It was found that the number of offspring and percentage of parasitism increased in the first days of parasitoid age in all densities and then reduced. Gonzalez-Zamora *et al*., (2015) obtained the same results when studied the influence of food source, host and parasitoid densities on the biology of *Aphytis melinus* DeBach to optimize its mass production.

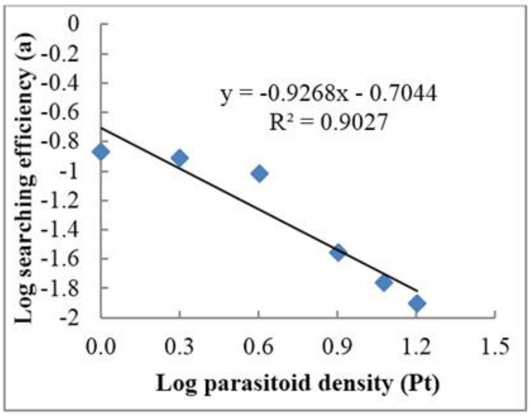

Fig. 2. Relationship between searching efficiency and searching parasitoid density (P_t) of *Chouioia cunea* parasitizing pupae of *Hyphantria cunea*.

The rate of progeny production, parasitism and sex ratio were effectively influenced by the parasitoid age, as the progeny production decreased the age of parasitoid increased. Amalin *et al.* (2005) demonstrated that the rate of parasitism in young females of

Ceratogramma etiennei was higher than their old counterparts. The rate of parasitism by *Glyptapanteles flavicoxis* also decreased as the parasitoid age increased (Hu *et al*., 1986). It has also been noted for *C. curvimaculatus* (Hentz, 1998) and *C. grandis* (Greenberg *et al*., 1995). The rate of parasitism by *C. cunea* is consistent with those documented by Hu *et al*. (1986) and Amalin *et al*. (2005). Kumar *et al*. (1990) and Singh *et al*. (1997) worked on *Nesolynx thymus* Girault and *Trichomelopsis apanteloctena*, respectively and recorded no significant effect of parasitoid age on the rate of parasitism.

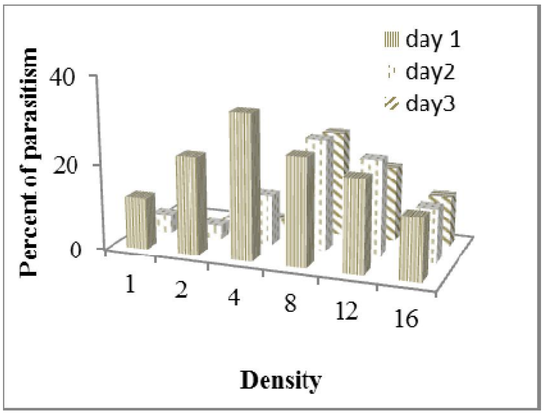

Fig. 3. The interaction between age and density of parasitoid on the percentage of parasitized host.

The rate of progeny production, parasitism and sex ratio were effectively influenced by the parasitoid age (Hirashima *et al*.; 1990; Greenberg *et al*.,1995; Leatemia *et al*., 1995; Hentz, 1998; Honda, 1998; Amalin *et al*., 2005). The sex ratio of *Chouioia cunea* is disproportionate to its age likely due to the production of relatively high number of female progeny by the younger female parasitoids. Significantly high sex ratio has been found in the progeny produced by younger parent females of *Trichogramma chilonis*, *T. ostriniae* (Hirashima *et al*., 1990) and *T. minutum* (Leatemia *et al*., 1995).

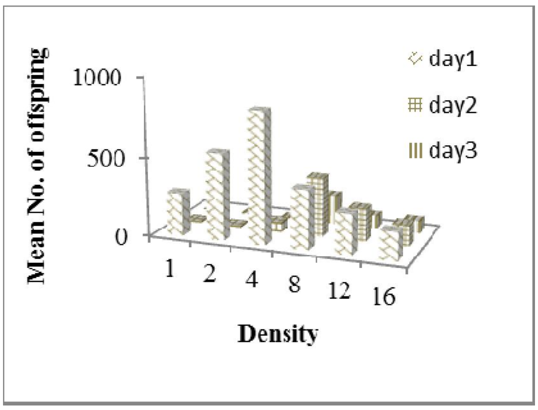

Fig. 4. The interaction between age and density of parasitoid on the offspring

Effectiveness of searching parasitoids decreases as parasitoid density increases due to "interference" of searching parasitoid (Farhad *et al*., 2011).

The negative value of coefficient of interference in regression line shows an inverse relationship between parasitoid density and its per capita searching efficiency. This relationship was also shown for *Diaeretiella rapae* (McIntosh) parasitizing *Lipaphis erysimi* Kaltenbach (Shukla *et al*., 1997) and for *D. rapae* on *B. brassicae* (Fathipour *et al*., 2004) that reflects intraspecific competition in parasitoids. In addition, high parasitoid density causes a higher proportion of male progeny, probably because females lay unfertilized eggs (Jones *et al*., 1999). The significant reduction of host parasitization per parasitoid with increasing parasitoid density suggests that interference amongst parasitoids also increases at higher parasitoid density. This is probably due to a closed experimental arena and limited time for parasitization and a high probability of mutual interference (Tahriri *et al*., 2007; Farhad *et al*., 2011). Results in this study showed that when parasitoid density increases from 1 to 16, the per capita searching efficiency decreases from 0.18 to $0.009h^{-1}$.

Our results indicated that age and density of a parasitoid significantly affect the progeny production, rate of parasitism and sex ratio of *C. cunea*. It was concluded that the performance of *C. cunea* caused by density and age.

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