

Mass Rearing Optimization of *Cotesia vestalis* (Hymenoptera: Braconidae) Based on the Host and Parasitoid Densities

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Abstract

The present study aimed to explore some simple ways to optimize the mass rearing of *Cotesia vestalis* (Haliday) (Hymenoptera: Braconidae), one of the most important biocontrol agents of the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae). The effects of host and parasitoid initial densities on the quantity and quality of produced wasps were investigated. In particular, a factorial experiment was established using three different host densities (20, 50, and 100 *P. xylostella* larvae per cage) in presence of *C. vestalis* at three different densities (2, 5, and 7 females, with a 24-h exposure) in well-ventilated cages. There was a significant effect of wasp's initial density on the percentage parasitism (measured based on the cocoon and emerged adult wasps); comparatively the greatest percentage parasitism was obtained with five wasps per cage. Initial host density had no significant effects on the percentage parasitism, sex ratio, and larval period, but it influenced the survival rate (measured based on the cocoon and adult emerged) and pupal period of produced wasps, and also percentage host mortality. The density of 20 host larvae was the best in this regard. These findings were discussed in the contexts of optimum mass rearing, life history, and potential of *C. vestalis* as a biological control agent.

Introduction

The general requirements for the insect rearing system include pure and contaminant-free culture, healthy production of arthropods with a broad genetic base in a reliable and predictable manner, and development of a time- and cost-efficient method of production (Wei *et al* 2003, Vacari *et al* 2012, Rezaei *et al* 2018). Moreover, many other factors must be accounted for a mass rearing program of entomophagous insects, such as providing sufficient numbers for release, various materials for colonization of different ecological zones, sufficient production throughout the year and enough materials for large-scale experiments (Singh 1982, Yang *et al* 2006, Saleh *et al* 2010, Díaz *et al* 2019). For most parasitoids there

is only limited information on the optimization of mass rearing conditions (Dindo & Grenier 2014).

Cotesia vestalis (Haliday) (Hymenoptera: Braconidae) is one of the most efficient natural enemies of the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), the key pests of cruciferous plants worldwide (Furlong *et al* 2013, Rezaei *et al* 2018). This solitary, koinobiont, larval endoparasitoid has been reared and released for *P. xylostella* management in many parts of the world (Sarfranz *et al* 2005, Rezaei *et al* 2014). In addition, this parasitoid has been the subject of more than 20 classical biological control introductions, of which many have been successful (Sarfranz *et al* 2005, Furlong *et al* 2013). For instance, Anuradha (2009) reported that a maximum of 81.77% parasitization of *P. xylostella* larvae by the *C. vestalis*

was observed in parasitoid release block in comparison with 35.93% in control block. In addition, it has reported that the release ratios of 1:6.4, 1:11, and 1:22 of *C. vestalis* females to *P. xylostella* larvae would be sufficient to successfully suppress the population growth of *P. xylostella* after two successive generations (Abe et al 2007). In order to have an efficient and economic biocontrol program, the biocontrol agent must be reared and produced in large numbers on a short-time period. There is however little information on the mass-rearing techniques of *C. vestalis*.

One of the paramount factors in mass rearing programs of parasitoids is providing a suitable diet or food supply for adults, which can influence life-history parameters of the parasitoids (Dindo et al 2019). For example, an artificial food supply, as a source of carbohydrate to feed adult parasitoids, strongly increased the longevity and parasitism of *C. vestalis* (Mitsunaga et al 2012). In addition, the color and height of artificial feeding site altered some biological characteristics of *C. vestalis*, such as parasitism rate and survival rate. Another study showed that *C. vestalis* adults were attracted to honey bee bread more than honey, indicating that bee bread could be incorporated into the feeding program of *C. vestalis* (Soyelu 2013, 2014). In some mass-rearing studies, instead of the targeted host an alternative host of the parasitoid has been successfully used. For instance, *Antheraea pernyi* Guerin-Meneville (Lepidoptera: Saturniidae) has been used for mass rearing of *Chouioia cunea* Yang (Hymenoptera: Eulophidae), a parasitoid of the fall webworm, *Hyphantria cunea* Drury (Lepidoptera: Arctiidae) (Yang et al 2006). *Cotesia vestalis* however is a highly specific parasitoid to its host, *P. xylostella* (Karimzadeh & Wright 2008), and there is no chance of finding an alternative host for such a parasitoid. The studies on mass-rearing of *C. vestalis* must be then focused on host plants, or other ecological behavior of the parasitoid or the host-parasitoid interactions. The effect of the host plant on the parasitism success of *C. vestalis* has been already reported by many researchers (e.g., Liu & Jiang 2003, Karimzadeh et al 2013, Heidary & Karimzadeh 2014). Chinese cabbage (*Brassica pekinensis*) is among *P. xylostella*'s most highly preferred host plants (Verkerk & Wright 1996; Daniazadeh et al 2014). It has been indicated that Chinese cabbage is the most suitable host plant for *P. xylostella* (in terms of preference, performance, and fitness) compared with common cabbages, cauliflower, rapeseed, turnip, and radish (Liu & Jiang 2003, Daniazadeh et al 2014, Karimzadeh & Wright 2008, Karimzadeh et al 2013). In addition, it has been shown that *C. vestalis* females prefer *P. xylostella* larvae fed on Chinese cabbage over *P. xylostella* larvae fed on common cabbages (Liu & Jiang 2003, Karimzadeh et al 2013). Furthermore, the highest percentage parasitism by *C. vestalis* was found on *P. xylostella* larvae fed on Chinese cabbage compared with *P. xylostella* larvae fed on common cabbages or cauliflower (Liu & Jiang 2003,

Karimzadeh et al 2013, Heidary & Karimzadeh 2014). These findings have shown sufficient documentation for using Chinese cabbage as a suitable food plant in mass rearing of the diamondback moth and its specific parasitoid, *C. vestalis*.

Superparasitism has been considered a key factor that may affect the quality of mass-reared parasitoids with a significant impact on biological features such as sex ratio, adult emergence, survival rate, flight ability, offspring body size, and fecundity (González et al 2007, Montoya et al 2012, Krüger et al 2019). Other studies indicated that host densities may influence the quality parameters (including the sex ratio) of a parasitoid, as shown for *Cotesia flavipes* (Cameron), being mass reared on its host, *Diatraea saccharalis* (Fabricious) (Vacari et al 2012).

Among the factors that possibly influence parasitoid phenotype characteristics (such as body weight, body part size, or wing presence), both host and parasitoid densities are directly related to mass rearing productivity and thus should be optimized for commercial purposes. Excessive densities can affect the quality and performance of the mass-produced natural enemies. The most direct effects are the reduction in size and emergence rate, and male-biased sex ratio (Zboralski et al 2016, Chardonnet et al 2019). Ghimire & Phillips (2010) showed that host and parasitoid density can affect adult production and sex ratio of *Bracon hebetor* Say (Hymenoptera: Braconidae).

In recent years, there has been a dramatic increase in the number of studies which have focused on mass rearing of beneficial insects (Wei et al 2003, Saleh et al 2010, Vacari et al 2012, Dindo et al 2016); the most important factors influencing the mass rearing of the parasitoids have been host-parasitoid ratio (Wei et al 2003, Lou et al 2013), temperature and photoperiod (Sagarra et al 2000, Zhang et al 2016, Moore et al 2019), which may affect life history parameters (e.g., fecundity and longevity) of captive parasitoids.

The results reported in the literature, such as those mentioned above, may help researchers to find their path for mass rearing of beneficial insects. There is, however, no general rule to optimize the conditions of cage-size, environment, feeding and illumination for rearing such natural enemies. The specific techniques must be developed based on the species and required number of individuals. Other factors, such as cost and scale of production, would come next (Singh 1982, Vacari et al 2012, Chardonnet et al 2019).

The present study aimed to determine the optimum initial densities of a host (*P. xylostella*) and its parasitoid (*C. vestalis*) for mass rearing of the natural enemy with the highest efficiency. The specific objectives were whether and to what extent increasing host density and parasitoid initial density would influence (increase or decrease) the quality/quantity of produced parasitoids (measured using *C. vestalis* percentage parasitism, survival rate, sex ratio and developmental period, and *P. xylostella* mortality).

Materials and Methods

Plant and insect rearing protocol

Chinese cabbage, *Brassica pekinensis* cv. Hero, was grown organically in plastic pots (10 cm diameter) containing sterilized soil, peat moss, perlite, and cocopeat in an appropriate ratio under greenhouse condition ($25 \pm 5^\circ\text{C}$, $70 \pm 5\%$ RH and L:D 16:8 h) without the application of any pesticide or chemical fertilizer. These plants were used to rear *P. xylostella*. To start the colony of *P. xylostella*, its pupae and larvae were collected from common cabbage and cauliflower fields in Pirbakran region ($32^\circ 28' 8''\text{N}$ and $51^\circ 33' 28''\text{E}$, at 1610 m altitude) of Isfahan province (central Iran). A *C. vestalis* culture was started with adults emerged from parasitized *P. xylostella* larvae collected from the same fields. Populations of *P. xylostella* were kept on 5-week-old Chinese cabbage in ventilated cages ($40 \times 40 \times 40$ cm; Karimzadeh *et al* 2004). Similarly, the cultures of *C. vestalis* were in turn maintained on *P. xylostella* larvae in ventilated oviposition cages ($40 \times 40 \times 40$ cm; Karimzadeh *et al* 2013). In each cage, 90–150 s instar larvae of *P. xylostella* were established on three 5-week-old Chinese cabbage, which were then exposed to 10 pairs of the parasitoids for 24 h. Both cultures were reared at standard constant environmental condition ($25 \pm 2^\circ\text{C}$, $70 \pm 5\%$ RH and L:D 16:8 h). Aqueous honey solutions (40% honey) were placed in each cage as a source of carbohydrates for adults of both *P. xylostella* and *C. vestalis*. The adult food supply (honey solution) was replaced every 48 h (Rezaei *et al* 2014).

Experimental set-up and measurements

The influences of host and parasitoid densities on *C. vestalis* biological key characteristics were investigated in a factorial experiment with two factors, (1) the initial host density (at three levels: 20, 50, and 100 s instar larvae of *P. xylostella* per cage) and (2) the initial parasitoid density (at three levels: 2, 5, and 7 mated, 3-day-old females of *C. vestalis* per cage). These host and parasitoid densities were chosen based on the previous studies conducted in our laboratory (Heidary & Karimzadeh 2014, Jafary *et al* 2016, Karimzadeh & Besharatnejad 2017) and according to the provided resources including space (i.e., cage size) and food (i.e., host-plant species and age) and parasitoid ability for host searching and parasitism. The parasitoid females were released in each cage (the well-ventilated cages described above) for 24 h. The experiment was conducted in the randomized complete block design (RCBD) with four replications. To start the experiments, the defined numbers of second instar larvae of *P. xylostella*,

which is the preferred host stage by *C. vestalis* (Alizadeh *et al* 2011), were placed on 5-week-old Chinese cabbages in each cage. After 24 h, the defined numbers of *C. vestalis* were released in cages and fed with honey solution (as above). The wasps were then removed after 24 h, while host larvae were maintained on the host plants (with no food limitation for host larvae) until the hosts were pupated or the wasp cocoons were formed. Each treatment was daily monitored, and the numbers of pupated hosts (P_h) and formed parasitoid cocoons (P_p), and the time of parasitoid cocoon formation were recorded. The parasitoid cocoons were further monitored for adult emergence. The newly emerged adults (A_p) were sexed based on the female individuals, which have a distinct ovipositor. The experiment was conducted under constant environmental condition ($25 \pm 2^\circ\text{C}$, $70 \pm 5\%$ and L:D 16:8 h). Since *C. vestalis* is a solitary, larval endoparasitoid (Karimzadeh & Wright 2008), its egg and larval stages develop inside host and cannot be monitored separately; given this fact, developmental period of egg and larval stage summed up and was considered the “Egg-Larva” stage, which was monitored from parasitization to cocoon formation. In addition, the developmental period of pupae was calculated from the cocoon formation to adult emergence. Parasitoids sex ratio was calculated as a ratio of female offspring to total offspring. Percentage parasitism and parasitoid survival rate were calculated at two different times, cocoon formation and adult emergence, such that the number of formed cocoons of *C. vestalis* (called “cocoon-based”) or the number of emerged adults of *C. vestalis* (called “adult-based”) was used for calculations. It is notable that *C. vestalis* is a solitary parasitoid, and only one parasitoid can develop from each host larvae. The number of *C. vestalis* cocoons, therefore, can be considered parasitism success (i.e., the number of parasitized hosts that can be developed as parasitoid), which does not include host mortality before pupation (mortality from unknown factor). Parasitism success can be considered until pupation (as a biocontrol agent for current host generation) or until adult emergence (as a biocontrol agents for upcoming host generation; Godfray 1994). In particular, percentage parasitism was calculated as a ratio of the number of produced parasitoid to the sum number of produced hosts and parasitoid even “cocoon-based” ($100 \times \frac{P_p}{P_p + P_h}$) or “adult-based” ($100 \times \frac{A_p}{A_p + P_h}$). Similarly, survival rate was calculated as a ratio of the number of produced parasitoid to the initial number of exposed hosts (H) even “cocoon-based” ($100 \times \frac{P_p}{H}$) or “adult-based” ($100 \times \frac{A_p}{H}$). The proportion of exposed hosts that neither pupate as *P. xylostella* nor formed as *C. vestalis* cocoon was considered host mortality due to unknown factors (Karimzadeh and Wright 2008).

Statistical analyses

The proportional data such as percentage parasitism, parasitoid survival rate, parasitoid sex ratio, and host mortality were analyzed using logistic analysis of deviance (binomial error distribution; Karimzadeh et al 2013, Jafary et al 2016). In case of overdispersion, the model was refitted using quasi-binomial rather than binomial errors. To achieve the minimal adequate model, non-significant terms were removed through model simplification, in which, the original and simplified models were compared by an F test instead of a Chi-square test. The statistical significance of minimal adequate model was then expressed as a standard normal deviate (z -value) or t -value (in case of overdispersion; Crawley 2013, Saadat et al 2014). Data on sex ratio was further analyzed using exact binomial test for a comparison with a sex ratio of 1:1 (Crawley 2013, Karimzadeh et al 2013; Jafary et al 2016). Data on developmental periods were analyzed using nested ANOVA, where individual host larvae were nested within cages, and cages were nested within treatments. For these data, a cage (containing 20, 50 or 100 larvae) was considered a replication; the treatments (3×3 , a factorial experiment) were replicated four times (Crawley 2013, Jafary et al 2016). Pairwise comparisons were performed using Tukey's honestly significant difference. All statistical analyses were conducted by in R 3.5.3 (R Development Core Team).

Results

Percentage parasitism of *Plutella xylostella* larvae by *Cotesia vestalis*

When percentage parasitism was calculated based on the parasitoid cocoons, there was a significant ($t_2 = 3.283$, $P < 0.005$) difference between the densities of the parasitoid (Table 1). Percentage parasitism was higher when five (75.9%) or seven (65.9%) *C. vestalis* females were released compared with two females (50.0%). On the contrary, the host density did not significantly ($t_2 = -1.185$, $P = 0.236$) affect percentage parasitism (Tables 1 and 2). In addition there was no significant interaction ($t_2 = 0.753$, $P = 0.452$) effect of the host-parasitoid densities on percentage parasitism (Table 1).

When percentage parasitism was evaluated based on the emerged parasitoid adult, there also was a significant ($t_2 = 3.545$, $P < 0.005$) difference between the densities of the parasitoid. Percentage parasitism was again higher with five (74.5%) or seven (63.2%) *C. vestalis* females release compared with two females (46.0%). On the contrary, the host density did not significantly ($t_2 = -0.155$, $P = 0.878$) affect the percentage parasitism (Tables 1 and 2). In addition there was

no significant ($t_2 = -0.798$, $P = 0.435$) effects of the host-parasitoid densities interaction on percentage parasitism (Table 1).

Survival rate of *Cotesia vestalis*

When survival rate was assessed based on the parasitoid cocoons, there was no significant ($t_2 = -1.381$, $P = 0.181$) difference between the initial densities of the parasitoid (Table 1). On the contrary, the host density significantly ($t_2 = -2.655$, $P < 0.05$) influenced survival rate (Table 1). The higher survival rate occurred when the initial number of *P. xylostella* larvae was 20 (68.9%) compared with host densities of 50 (42.0%) or 100 (39.3%) (Table 3). In addition there was no significant ($t_2 = -0.140$, $P = 0.890$) effects of the host-parasitoid densities interaction on parasitoid survival rate (Table 1).

When survival rate was analyzed based on the emerged adult wasps, there was no significant ($t_2 = 1.583$, $P = 0.128$) difference between the initial densities of the parasitoid (Table 1). On the other hand, the host density significantly ($t_2 = -2.468$, $P < 0.05$) influenced survival rate (Table 1). The higher survival rates were obtained when the initial number of *P. xylostella* larvae was 20 (60.6%) compared with host densities of 100 (35.1%) (Table 3). In addition there were no significant ($t_2 = -0.428$, $P = 0.674$) effects of the host-parasitoid densities interaction on parasitoid survival rate (Table 1).

Parasitoid sex ratio

Logistic analysis of deviance showed no significant effects of initial densities of the parasitoid ($t_2 = -1.674$, $P = 0.106$) and initial densities of host ($t_2 = -1.848$, $P = 0.076$) and their interaction ($t_4 = 0.777$, $P = 0.444$) on the parasitoid sex ratio (Tables 4 and 5). Exact binomial test, however, showed a significant difference between different host densities, and between different initial parasitoid densities. Between initial host densities, host density of 50 had a significantly higher sex ratio (0.59; female-biased) compared with a ratio of 1:1 (0.50) (Table 5). Between initial parasitoid densities, wasp density of 2 had a significantly higher sex ratio (0.61; female-biased) compared with a ratio of 1:1 (Table 5). Accordingly, the highest ratios of produced female wasps (0.67) were obtained when 50 host larvae were exposed to 2 female parasitoids.

Parasitoid developmental period

The developmental period of Egg-Larva stage of *C. vestalis* was not significantly influenced by host initial

Table 1 The effect of host and parasitoid densities on percentage parasitism and survival rate of *Cotesia vestalis*

Parameter measured	Source of variation									
	Percentage parasitism					Parasitoid survival rate				
	Cocoon-based ³		Adult-based ⁴			Cocoon-based		Adult-based		
	df	t value	P value	t value	P value	t value	P value	t value	P value	
H ¹	2	-1.185	0.236	-0.155	0.878	-2.655	< 0.05	-2.468	< 0.05	
P ²	2	3.283	< 0.005	3.545	< 0.005	1.381	0.181	1.583	0.128	
L × P	4	0.753	0.452	-0.798	0.435	-0.140	0.890	-0.428	0.674	

¹ Host density: the number of the 2nd instar larvae of *Plutella xylostella* per cage

² Parasitoid density: the number of mated 3-day-old females of *Cotesia vestalis* released per cage for 24 h

³ The number of formed cocoons of *Cotesia vestalis* was used for calculations

⁴ The number of emerged adults of *Cotesia vestalis* was used for calculations

densities ($F_{2,27} = 0.032, P = 0.969$), parasitoid initial densities ($F_{2,27} = 0.013, P = 0.987$) or their interaction ($F_{4,27} = 0.365, P = 0.832$) (Tables 4). The mean developmental period of Egg-Larva stage ranged from 7.52 to 8.24 days for different treatments (Table 6). However, pupal developmental period of *C. vestalis* showed a significant ($F_{2,27} = 5.369, P < 0.01$) difference between different host initial densities (Table 4). The parasitoid pupae developed faster (in 3.93 days) when initial host density was lowest (20), compared with higher initial density of 50 (parasitoid pupal period of 4.35 days) and 100 (parasitoid pupal period of 4.36 days). On the contrary, neither the parasitoid initial density ($F_{2,27} = 0.017, P = 0.983$) nor host and parasitoid density interaction ($F_{4,27} = 0.189, P < 0.942$) significantly affected *C. vestalis* pupal

developmental period. The mean developmental period of pupal stage varied between 3.85 and 4.45 days for different treatments (Table 6).

Host mortality

Host mortality from unknown factors was significantly ($t_2 = 2.233, P < 0.05$) affected by initial host density (Table 4). The lower host mortality was obtained when the number of *P. xylostella* larvae offered was 20 (4.4%) compared with 50 (34.7%) or 100 (35.7%) (Table 7). On the contrary, neither the parasitoid initial density ($t_2 = 1.179, P = 0.251$) nor host and parasitoid density interaction ($t_4 = 0.041, P = 0.968$) significantly affected *P. xylostella* larval mortality (Table 4).

Table 2 Percentage parasitism of *Plutella xylostella* larvae by *Cotesia vestalis* in different treatments of mass rearing

Percentage parasitism (mean ± SE)					
Parasitoid initial density ²					
	Host density ¹	2	5	7	Overall
Cocoon-based ³	20	54.5 ± 5.1	78.6 ± 4.0	73.3 ± 8.8	68.1 ± 4.3 a
	50	53.8 ± 3.5	66.2 ± 16.0	74.5 ± 2.0	64.3 ± 5.6 a
	100	46.1 ± 17.1	78.8 ± 1.4	59.3 ± 1.5	61.1 ± 6.3 a
	Overall	50.0 ± 5.4 A ⁵	75.9 ± 5.7 B	65.9 ± 3.4 B	
Adult-based ⁴	20	45.5 ± 6.9	78.2 ± 4.3	71.9 ± 9.3	65.3 ± 5.5 a
	50	50.5 ± 3.0	64.2 ± 15.6	72.4 ± 2.6	61.7 ± 5.6 a
	100	43.4 ± 16.4	77.3 ± 0.8	55.6 ± 2.6	58.4 ± 6.3 a
	Overall	46.0 ± 5.2 A	74.5 ± 5.8 B	63.2 ± 3.8 B	

¹ Host density: the number of the 2nd instar larvae of *Plutella xylostella* per cage

² Parasitoid density: the number of mated 3-day-old females of *Cotesia vestalis* released per cage for 24 h

³ The number of formed cocoons of *Cotesia vestalis* was used for calculations

⁴ The number of emerged adults of *Cotesia vestalis* was used for calculations

⁵ Different small letters within columns or different capital letters within rows show a significant ($P < 0.05$) difference

Table 3 Survival rate of *Cotesia vestalis* at different treatments of mass rearing

Parasitoid survival rate (%; mean ± SE)		Parasitoid initial density ²				
		Host density ¹	2	5	7	Overall
Cocoon-based ³	20		60.0 ± 2.5	73.3 ± 1.4	73.3 ± 8.8	68.9 ± 3.3 a
	50		42.0 ± 9.6	31.3 ± 14.2	52.7 ± 8.1	42.0 ± 6.1 b
	100		29.3 ± 10.2	48.3 ± 4.8	40.3 ± 7.8	39.3 ± 4.6 b
	Overall		36.7 ± 5.6 A ⁵	46.3 ± 6.8 A	47.8 ± 5.9 A	
Adult-based ⁴	20		41.7 ± 5.2	71.7 ± 2.9	68.3 ± 10.1	60.0 ± 5.3 a
	50		36.7 ± 8.1	28.7 ± 14.0	47.3 ± 5.8	37.6 ± 5.5 ab
	100		26.3 ± 8.9	44.3 ± 2.8	34.7 ± 6.8	35.1 ± 4.0 b
	Overall		31.2 ± 4.3 A	42.9 ± 6.9 A	42.4 ± 5.8 A	

¹ Host density: the number of the 2nd instar larvae of *Plutella xylostella* per cage

² Parasitoid density: the number of mated 3-day-old females of *Cotesia vestalis* released per cage for 24 h

³ The number of formed cocoons of *Cotesia vestalis* was used for calculations

⁴ The number of emerged adults of *Cotesia vestalis* was used for calculations

⁵ Different small letters within columns or different capital letters within rows show a significant ($P < 0.05$) difference

Discussion

The success of biological control programs depends upon the release of a huge number of the biocontrol agents in target areas, and subsequent reduction of the pest population to a level below the economic injury threshold. To achieve this aim, the production of insects in sufficient number and adequate quality is an essential step (Greenberg et al 1995, Rezaei et al 2018, Chardonnet et al 2019, Díaz et al 2019). Studies on insect rearing have different objectives, such that rearing is rarely an end itself, and, as a consequence, it does not require the optimization of insect production (Ghimire & Phillips 2010, Saleh et al 2010, Dindo & Grenier 2014). In mass rearing, however, the objective is different, as it must be optimized for many parameters, such as costs, time, and quality of the produced insects (Vacari et al 2012). *Cotesia vestalis* which has proven

successful in most biological control programs (Sarfranz et al 2005, Anuradha 2009, Furlong et al 2013, Afiunizadeh & Karimzadeh 2015). Although it is well known that *C. vestalis* has significant impact on diamondback moth populations (Karimzadeh et al 2004, Karimzadeh & Besharatnejad 2017), little is known about mass rearing techniques of this parasitoid. There are several examples of testing the effects of parasitoid or host density on reared insect colonies (Sagarra et al 2000, Ghimire & Phillips 2010, Silva et al 2014, González-Zamora et al 2015, Chardonnet et al 2019, Rezaei et al 2019), but they missed the simultaneous impacts of both the host and parasitoid density. In this study, on the contrary, the effects of host and parasitoid density were evaluated simultaneously.

The present study showed that the initial densities of *P. xylostella* and *C. vestalis* are paramount factors for mass rearing, influencing host mortality, parasitism, survival, and

Table 4 The effect of host and parasitoid densities on developmental period and sex ratio of *Cotesia vestalis* and mortality of *Plutella xylostella*

Parameter measured		Parasitoid developmental period				Parasitoid sex ratio		Host mortality	
Source of variation	df	Egg-Larva		Cocoon (Pupa)		t value	P value	t value	P value
		F value	P value	F value	P value				
		H ¹	2	0.032	0.969	5.369	< 0.01	-1.848	0.076
P ²	2	0.013	0.987	0.017	0.983	-1.674	0.106	1.179	0.251
L × P	4	0.365	0.832	0.189	0.942	0.777	0.444	0.041	0.968

¹ Host density: the number of the 2nd instar larvae of *Plutella xylostella* per cage

² Parasitoid density: the number of mated 3-day-old females of *Cotesia vestalis* released per cage for 24 h

Table 5 Sex ratio of *Cotesia vestalis* at different treatments of mass rearing

Parasitoid sex ratio (female proportion; mean ± SE)				
Parasitoid initial density ²				
Host density ¹	2	5	7	Overall
20	0.29 ± 0.14	0.58 ± 0.06	0.50 ± 0.15	0.49 ± 0.07 a
50	0.67 ± 0.09* ³	0.50 ± 0.24	0.58 ± 0.06	0.59 ± 0.09* a
100	0.65 ± 0.04*	0.47 ± 0.05	0.41 ± 0.10	0.50 ± 0.04 a
Overall	0.61 ± 0.07* A ⁴	0.50 ± 0.08 A	0.48 ± 0.06 A	

¹ Host density: the number of the 2nd instar larvae of *Plutella xylostella* per cage

² Parasitoid density: the number of mated 3-day-old females of *Cotesia vestalis* released per cage for 24 h

³ An asterisk show a significant ($P < 0.05$) difference compared with a ratio of 1:1

⁴ Different small letters within columns or different capital letters within rows show a significant ($P < 0.05$) difference

developmental period of the parasitoid. Parasitoid adult emergence and host mortality after exposure to parasitoid are the most important factors in the quality control of parasitoid wasps (Suarez *et al* 2012). In the current experiment, percentage parasitism was significantly affected by the initial parasitoid density but not by host density. When the number of parasitoids was increased (from 2 to 5 or 7 female wasps per cage), the percentage parasitism was first increased as well, but then it was leveled off. This might be due to negative influence of intraspecific interactions of the adult wasp individuals via interference (Soufba *et al* 2012, 2014, Rezaei *et al* 2019), in which, with increasing parasitoid density, female parasitoids will waste an increasing proportion of their searching time to encounter other conspecifics. A strong relationship between parasitoid densities and number of parasitized host has been determined in the literature in particular for the solitary parasitoids (e.g., Fathipour *et al* 2006, Yang *et al* 2015). As a result of interference, increasing the number of parasitoids to attack hosts does

not proportionally increase the number of parasitized hosts (Rezaei *et al* 2019). The initial number of *C. vestalis*, therefore, must be adjusted to the available space and host larval density. For the mass rearing of this parasitoid on *P. xylostella* in similar conditions explained here, initial parasitoid densities higher than 5 adult females per cage should be avoided, because using more female wasps will only result in adult parasitoid interference without any increase in percentage parasitism. Similar observations were performed for other parasitoids (e.g., Greenberg *et al* 1995, Luo *et al* 2013, González-Zamora *et al* 2015). Ghimire & Phillips (2010), however, noted that high density of parental *Habrobracon hebetor* (Say) in a mass rearing would result in a greater genetic variability. Therefore, quality of the progeny might be improved by use of a larger parental group of wasps in each container, due to promoting outbreeding and avoiding inbreeding. This hypothesis requires further investigation on *C. vestalis* and remains for our future works.

Table 6 The developmental period of *Cotesia vestalis* at different treatments of mass rearing

Developmental period (day; mean ± SE)					
Parasitoid initial density ²					
Host stage	Host density ¹	2	5	7	Overall
Egg-Larva	20	7.67 ± 0.44	7.75 ± 0.66	7.96 ± 0.65	7.80 ± 0.31 a
	50	7.59 ± 0.43	7.95 ± 0.57	8.19 ± 0.71	7.91 ± 0.31 a
	100	8.24 ± 0.43	7.76 ± 0.59	7.52 ± 0.63	7.84 ± 0.31 a
	Overall	7.83 ± 0.24 A ³	7.82 ± 0.32 A	7.89 ± 0.36 A	
Cocoon (pupa)	20	3.85 ± 0.30	3.91 ± 0.34	4.03 ± 0.12	3.93 ± 0.14 a
	50	4.31 ± 0.05	4.37 ± 0.05	4.36 ± 0.24	4.35 ± 0.07 b
	100	4.45 ± 0.18	4.33 ± 0.18	4.30 ± 0.07	4.36 ± 0.08 b
	Overall	4.20 ± 0.13 A	4.20 ± 0.13 A	4.23 ± 0.09 A	

¹ Host density: the number of the 2nd instar larvae of *Plutella xylostella* per cage

² Parasitoid density: the number of mated 3-day-old females of *Cotesia vestalis* released per cage for 24 h

³ Different small letters within columns or different capital letters within rows show a significant ($P < 0.05$) difference

Table 7 The host (*Plutella xylostella*) mortality from unknown factors at different treatments of mass rearing

Host mortality (%; mean \pm SE)				
Parasitoid initial density ²				
Host density ¹	2	5	7	Overall
20	6.7 \pm 3.3	6.7 \pm 4.4	0.0 \pm 0.0	4.4 \pm 1.9 a
50	22.0 \pm 17.0	52.7 \pm 13.8	29.3 \pm 13.5	34.7 \pm 8.7b
100	36.3 \pm 7.8	38.7 \pm 5.8	32.0 \pm 14.6	35.7 \pm 5.2b
Overall	28.6 \pm 7.0 A ³	39.0 \pm 8.2 A	27.5 \pm 7.7 A	

¹ Host density: the number of the 2nd instar larvae of *Plutella xylostella* per cage

² Parasitoid density: the number of mated 3-day-old females of *Cotesia vestalis* released per cage for 24 h

³ Different small letters within columns or different capital letters within rows show a significant ($P < 0.05$) difference

Host mortality from unknown factors (i.e., the host percentage mortality before host pupation or parasitoid cocoon formation) was significantly affected by host density but not by parasitoid density. The lowest host density (20 larvae per cage) resulted in the least host mortality, which was probably due to intraspecific competition as a feedback of density dependence (Begon et al 2009), in particular for food or space. This phenomenon has been observed in mass rearing of many insects (Silva et al 2008, González-Zamora et al. 2015). It is important to clarify that no parasitized host larvae were dissected to monitor whether the parasitoid larvae were inside the host body or not. The purpose of experiments was to assess parasitism success (including host location, parasitization, and emergence of parasitoid offspring), and therefore, it was not the choice to dissect host larvae before host larval death. In addition, after host larval death, the host bodies were too wrinkled to be dissected. Further studies are required to determine unknown factors (pathogens, superparasitism or else) that caused host mortality.

The results obtained in our study, in which the least host mortality and the greatest parasitoid survival arose from the lowest host density (20 larvae per cage), suggested that, using an appropriate level of host density, a higher population of parasitoid wasps can be produced in mass rearing of *C. vestalis*. The host density seems to be more important than the parasitoid density. Also, the pupal period of the produced wasps was influenced by host larval density and the shortest duration was obtained under the least host density, possibly due to optimum host developmental conditions created by sufficient space and food resources. This has been also reported by other researchers (e.g., Asante & Danthanarayana 1993, Silva et al 2014). Furthermore, when a plant with 20 host larvae was used, one host plant was sufficient for the larvae to complete their development until

wasp cocoon formation. In comparison with other treatments (50 or 100 host larvae on a plant) that needed several times of food plant renewing, a plant with 20 host larvae required less labor work, and subsequently, less production costs. To our knowledge, there is no published article on the optimization of *C. vestalis* mass-rearing and its cost estimation. Further studies are thus necessary to estimate economic and applied aspects of *C. vestalis* productions using our proposed method compared with commercial rearing factories. In the current study, exposure time of parasitoid to host larvae was 24 h. According to effect of difference exposure time on superparasitism and many biological parameters of produced wasps (Montoya et al 2012, Suarez et al 2012, Zhang et al 2016), future studies must include different exposure time to parasitoid wasp.

In summary, *C. vestalis* showed high potential for use as a biological control agent of *P. xylostella*. Further research is needed to understand which factors influence the parasitoid performance under field conditions. The current experiments showed that the different densities of *P. xylostella* and *C. vestalis* influenced the life-history parameters of produced wasps, including percentage parasitism, survival rate, developmental period, and percentage host mortality. The best results were obtained when a host plant (5–6-week-old Chinese cabbage) with 20 larvae of the herbivore insect (2nd instar *P. xylostella* larvae) was exposed to 5 *C. vestalis* females in a standard-sized cage (40 \times 40 \times 40 cm) for 24 h. This optimized herbivore density reduced intraspecific competition for food and resulted in higher *P. xylostella* survival. In addition, in the 20-density due to low number of *P. xylostella* larvae on each plant there was no need for food plant renewal.

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Authors' Contributions MH performed experimental work and wrote the initial manuscript. JK planned and designed the experiment, analyzed the data, and reviewed the final manuscript. JSH helped in designing the experiment and reviewed the results.

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