



Can heteronomous hyperparasitoids recognize host abundance and adjust offspring ratio?

Xiao-Ming Man¹, Li-Ying Sun¹, Frédéric Francis², Nian-Wan Yang^{1,3,*}, Wan-Xue Liu¹

¹ State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China

² University of Liege, Gembloux Agro-Bio Tech, Functional & Evolutionary Entomology, 5030 Gembloux, Belgium

³ Western Agricultural Research Center, Chinese Academy of Agricultural Sciences, Xinjiang 831100, China

* Corresponding author: yangnianwan@caas.cn

With 4 figures and 1 table

Abstract: Sex ratio is crucial in the reproductive dynamics of bisexual insects. In the Aphelinidae family, heteronomous hyperparasitoids like *Encarsia sophia* show distinct behaviors where females (from fertilized eggs) target primary hosts, and males (from unfertilized eggs) parasitize secondary hosts. This sex determination pattern means that host resource abundance significantly impacts sex ratio, affecting population dynamics. However, the sex distribution of these parasitoids remains a topic of debate. This study examined *E. sophia* targeting *Bemisia tabaci*, adjusting host densities (30, 50, 70 hosts/9.6 cm²) and secondary host ratios (0.2, 0.5, 0.8). Females were observed for recognition of varying host conditions and adjustments in offspring sex ratio and behavior. When ratio of secondary hosts surpassed that of primary hosts or in cases of low host density (host limitation), *E. sophia*'s offspring sex ratio adapted according to the relative abundance of primary and secondary hosts. Conversely, with low secondary host ratios (< 0.5) and higher host density, the sex ratio approached 1:1. Observations showed that females quickly perceived host density, increasing oviposition on secondary hosts with higher density, resulting in more males, and increasing feeding on primary hosts, reducing female offspring. Importantly, by examining oviposition and feeding under different host resource conditions, we identified the optimal rearing strategy: a secondary host ratio of 0.2 and a host density of 30 hosts/9.6 cm². This study not only introduces the sex ratio theory for heteronomous hyperparasitoids but also provides a framework for more accurately assessing their environmental adaptability and for large-scale production.

Keywords: *Encarsia sophia*; reproductive dynamics; sex allocation; parasitoid behavior

1 Introduction

Heteronomous hyperparasitoids in the Aphelinidae family are a special type of parasitoid wasps, primarily including genera such as *Coccophagus*, *Coccobius*, *Coccophagoides*, and *Encarsia* (Hunter & Woolley 2001). They play a significant role in the biological control of many major pests (Tize et al. 2023; Zhang et al. 2023; Shahbazvar et al. 2022). Similar to other haplodiploid insects, autoparasitic wasps evolve from unfertilized haploid eggs into males and fertilized diploid eggs into females. The distinction lies in the fact that female wasps originate from fertilized eggs, developing as primary parasitoids of Hemiptera nymphs, while male wasps stem from unfertilized eggs, evolving into secondary parasitoids (hyperparasitoids) on wasp larvae or pupae within Hemiptera nymphs (Walter 1983; Godfray & Hunter 1992; Hu et al. 2010). Heteronomous hyperparasitoids have the

potential to induce mortality in conspecific or heterospecific primary parasitoid larvae, thus giving rise to lethal interference competition. This phenomenon has spurred controversy in biological control and attracted significant scientific attention (Xu et al. 2018; Kidane et al. 2020; Zhao et al. 2022). Nevertheless, based on the reproductive characteristics of heteronomous hyperparasitoids, lethal interference competition primarily manifests during the reproduction process of male offspring. Consequently, the sex ratio distribution of heteronomous hyperparasitoids emerges as a pivotal factor influencing their biological control effectiveness.

For heteronomous hyperparasitoids, they reproduce male offspring using larvae of conspecific or heterospecific primary parasitoids. The abundance of host resources and the diversity of related parasitoids directly influence the regulation of offspring sex ratios (Colgan & Taylor 1981; Godfray & Waage 1990; Bon et al. 2022). Therefore, does the sex ratio

of heteronomous hyperparasitoid offspring depend solely on the type of host? Fisher (1930) introduced the foundational theory of sex ratio regulation in species. He posited that, in a large population with random mating between sexes, parents should invest equivalent resources in both male and female offspring, resulting in an offspring sex ratio of 1:1. However, the applicability of this theory to heteronomous hyperparasitoids and the mechanisms governing sex ratio adjustment remain contentious (Fisher 1930; Harvey et al. 2013). Godfray extended Fisher's sex ratio regulation theory to heteronomous hyperparasitoids, proposing a sex ratio regulation mechanism under conditions of host and egg limitation. In cases of abundant host resources (egg limitation), Godfray argued that the offspring sex ratio of heteronomous hyperparasitoids is 1:1. Conversely, in situations with a lack of host resources (host limitation), the offspring sex ratio is determined by the relative abundance of primary and secondary hosts (Godfray & Waage 1990; Godfray & Hunter 1992, 1994). Diverging from Godfray's perspective, Walter and Donaldson disputed the applicability of Fisher's theory to sex adjustment in heteronomous hyperparasitoids. They contended that the sex ratio of heteronomous hyperparasitoid offspring is not 1:1 when host resources are sufficient. According to their viewpoint, female wasps adopt a fixed reproductive strategy, producing offspring of the corresponding sex based on the type of host, irrespective of host resource abundance. Then, the offspring ratio of heteronomous hyperparasitoids, whether in resource-rich or resource-limited conditions, is linked to individual reproductive behavior and the relative abundance of the two hosts (Walter & Donaldson 1994). The central focus of the debate between Godfray and Walter regarding the theory of sex ratio regulation revolves around whether, as the host abundance increases, the offspring sex ratio of heteronomous hyperparasitoids is dictated by the ratio of primary hosts to secondary hosts or tends towards 1:1.

In the mass rearing of Hymenoptera parasitoids, the overproduction of male offspring is common and can lead to unnecessarily high costs (Ode & Heinz 2002; Riccardo et al. 2018). Moreover, the reproduction of male offspring by heteronomous hyperparasitoids utilizes primary parasitoids, with each male offspring produced at the cost of a female parasitoid larva. This exacerbates the difficulties and costs of the rearing process. To control male production and increase female production, thereby enhancing the efficacy of biological control programs, it is essential to understand the factors influencing sex ratios.

Therefore, to address the controversy over sex ratio allocation in heteronomous hyperparasitoids and to determine if manipulating sex allocation can reduce interspecific competition and maximize the production of female parasitoids, we designed an experiment using an important heteronomous hyperparasitoid, *E. sophia*, a key biological control agent for the "super pest" *B. tabaci*. The experiment aims to:

1. Determine whether *E. sophia* can adjust the sex ratio of its offspring under different host resource conditions (varying host densities and host ratios). 2. If the females have the ability to regulate offspring sex ratios, identify the specific behaviors they employ to make these adjustments.

2 Materials and methods

2.1 Insect culture and host plant

The *B. tabaci* MEAM1 laboratory colony was obtained from the greenhouses at the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (CAAS) in Beijing. This population has been continuously grown under greenhouse conditions for 4 years, with annual supplementation from wild populations to rejuvenate it, and has never been exposed to pesticides. Laboratory colonies of *E. sophia* were generously provided by the Vegetable Integrated Pest Management Laboratory at the Texas Agricultural Experiment Station in Weslaco, TX, USA. To establish colonies of *E. sophia*, *B. tabaci* served as the host insect, and cotton plants (cv. xinke no.8, Hebei Zhongchuang Seed Technology Co. Ltd., China) were used as host plants in the laboratory experiments. Cotton plants, approximately 20 cm in height with 4–5 fully expanded leaves, were selected for this purpose. All host plants and insect colonies were meticulously maintained at Langfang Experimental Station (39°30'N, 116°36'E), Langfang, Hebei Province, China, under controlled conditions of $26 \pm 2^\circ\text{C}$, $65\% \pm 5\% \text{RH}$, and a 14 L: 10 D regime.

To prepare the culture medium, leaf discs containing primary hosts (third instar nymphs of *B. tabaci*) and secondary hosts (nymphs of *B. tabaci*, which had been parasitized by *E. sophia* and the parasitoid larvae developed into the third instar to the pre-pupal stage), 50 pairs of whitefly adults were introduced into microscopic insect cages (specifications: 3 cm in diameter, 1 cm in height, covered with 120-mesh gauze) placed on cotton leaves. The adults were removed 24 hours after laying eggs. After 6 days, the same micro-insect cage was utilized to introduce 30 pairs of adult whiteflies, and the adults were removed 24 hours after egg-laying. Following the removal of the second batch of adults after 6–8 days, 15–20 mating *E. sophia* females were introduced into the micro-insect cage when the eggs laid by the first batch of whitefly adults developed into third instar nymphs. After 24 hours of egg-laying, the females were removed. A mesh bag (10 × 10 cm, 120-mesh gauze) was placed on the leaves to prevent contamination. After 6 days, appropriately aged primary and secondary hosts were distributed on the leaves. A 1% agar solution was poured into 2/3 of the Petri dish (d = 3.5 cm, S = 9.6 cm²), and circular shapes (d = 3.5 cm, S = 9.6 cm²) of cotton leaves with primary and secondary hosts were cut. When the agar solution was about to solidify, small tweezers were used to place the leaves into the Petri dish, ensuring they adhered tightly to the agar for preservation. After the agar solidified, the leaf disc was examined

under a dissecting microscope. According to the experimental requirements, a corresponding number of primary and secondary hosts were retained, and any excess nymphs and pupae of the whitefly that did not meet the experimental conditions were removed.

2.2 Offspring sex ratio of *E. sophia* under varied host resources

Building upon our previous investigations into the host parasitization behavior of *E. sophia* females, we established three distinct parasitism scenarios characterized by varying host abundance in relation to the number of eggs the female wasp could lay: insufficient, moderate, and sufficient. The ratio of secondary hosts to the total number of hosts was set at 0.2, with host densities configured at 30, 50, and 70 hosts per dish ($S = 9.6 \text{ cm}^2$) (Sun 2014). Specifically, for a host density of 30 hosts per dish ($S = 9.6 \text{ cm}^2$), the secondary host proportions were set to 0.5 and 0.2, and for a host density of 50 hosts per dish ($S = 9.6 \text{ cm}^2$), the secondary host proportions were set to 0.8 and 0.2.

Placing individual unmated males and single virgin females in Petri dishes ($d = 3.5 \text{ cm}$, $S = 9.6 \text{ cm}^2$), we removed males once mating behavior was observed. Subsequently, mated females were introduced into culture dish leaf discs containing primary and secondary hosts. The diameter of the leaf disc was 3.5 cm, with an area of 9.6 cm^2 . When investigating the impact of host density on offspring sex ratios, under a secondary host ratio of 0.2, host densities were set at 30, 50, and 70 individuals per leaf disc. When exploring the effect of host ratio on offspring sex ratios, at a host density of 30 individuals per leaf disc, the proportion of secondary hosts to the total host population was set at 0.5 and 0.2. Under a host density of 50 individuals per leaf disc, the proportion of secondary hosts to the total host population was set at 0.8 and 0.2. The culture dish leaf discs were covered with plastic wrap, pierced with insect pins, and the female wasps were transferred to leaf discs with the same oviposition environment every 24 hours for a continuous experiment over 5 days. After removing the female wasps for 5 days, the number of parasitized primary and secondary hosts was recorded under a dissecting microscope, representing the number of eggs laid by female wasps for female and male offspring, respectively. The quantity of primary and secondary hosts consumed by the wasps was also recorded as the host feeding amount. Each treatment was repeated 20 times.

2.3 Host processing behavior of *E. sophia* at different host resources

In Petri dishes ($d = 3.5 \text{ cm}$, $S = 9.6 \text{ cm}^2$), individual unmated males and single virgin females were placed. Once mating behavior was observed, the males were promptly removed. Mated females were then introduced into culture dish leaf discs containing primary and secondary hosts (host densities set at 30, 50, 70 individuals per leaf disc, with a secondary host ratio of 0.2). The leaf discs used in the experiment were

photographed and printed under a dissecting microscope, marking the positions and types of hosts and assigning each host a unique identifier. Upon entering the leaf discs, the females were covered with the lid of the culture dish. Under a dissecting microscope, the names, durations, and host identifiers for each behavior of the *E. sophia* females were recorded. After continuous observation for 1 hour, females were removed. Five days later, under the dissecting microscope, the number of parasitized primary and secondary hosts was recorded to determine whether *E. sophia* females laid eggs after ovipositor insertion into the hosts. This information was then matched with the previously assigned host identifiers, establishing the types of behaviors exhibited by females. Each treatment was repeated 20 times.

2.4 Data analysis

Data analysis was performed using SPSS 24.0 (SPSS Inc., Chicago, IL, USA). A two-way ANOVA test was employed to explore the factors influencing the offspring sex ratio, host parasitism and host feeding amount of *E. sophia* while maintaining a constant host ratio. Specifically, the analysis considered the effects of host density and female age. Similarly, under conditions where host density remained constant, a two-way ANOVA was used to investigate the impact of host ratio and female age on the offspring sex ratio and host parasitism. For assessing the significance of differences in normally distributed data, either in their original form or following transformation, an independent samples t-test was used for comparing two groups, and one-way ANOVA with Tukey's HSD was employed for comparing more than two groups. In cases where data, even after transformation, did not conform to normal distribution, the non-parametric Mann-Whitney test was used for comparing two groups, and the non-parametric Kruskal-Wallis test was employed for comparing more than two groups. In this study, the offspring sex ratio was defined as the proportion of male offspring relative to the total number of offspring, expressed as the ratio of offspring sex = number of male offspring / total number of offspring.

3 Results

3.1 Offspring sex ratio of *Encarsia sophia* under varied host resources

3.1.1 Effects of host resources on offspring sex ratio

The offspring sex ratio of *E. sophia* demonstrated significant responsiveness to variations in host density and the proportion of primary to secondary hosts. The interaction among these factors, along with female age (1–5 days old), exhibited no noteworthy impact on the offspring sex ratio of *E. sophia* (Supplementary Table S1). For instances where the secondary host proportion was 0.2 and host density of 30, 50, 70 hosts/ 9.6 cm^2 , the daily average sex ratios of females

were 0.19 ± 0.01 , 0.31 ± 0.02 , and 0.40 ± 0.02 , respectively. A significant increase in sex ratio was observed with the rise in host density ($F_{2,37} = 34.81$, $P < 0.001$). Particularly, at host densities of 50 and 70 hosts/9.6 cm², the actual sex ratio was markedly higher than that observed at a host proportion of 0.2 ($t = 6.40$, $df = 19$, $P < 0.001$; $t = 10.52$, $df = 19$, $P < 0.001$) (Fig. 1).

In a habitat with a host density of 30 hosts/9.6 cm², the sex ratios of offspring when the secondary host proportions were 0.5 and 0.2 were 0.47 ± 0.04 and 0.19 ± 0.01 respectively, which were not significantly different from the corresponding secondary host proportions ($t = -0.80$, $df = 23$, $P = 0.432$; $t = -0.36$, $df = 19$, $P = 0.726$), while there is a significant difference in offspring sex ratio between the two host proportions ($t = 6.96$, $df = 30.02$, $P < 0.001$) (Fig. 2A). In a habitat with a host density of 50 hosts/9.6 cm², no significant difference was found in offspring ratio (0.76 ± 0.03) compared to the host ratio when the secondary host proportion was 0.8 ($t = -1.37$, $df = 19$, $P = 0.186$). Nevertheless, the offspring ratio (0.31 ± 0.02) was significantly higher when the secondary host ratio was 0.2 than the host proportion ($t = 6.50$, $df = 19$, $P < 0.001$). Furthermore, the offspring ratio at a secondary host proportion of 0.8 was significantly higher than that at a secondary host proportion of 0.2 ($t = 13.59$, $df = 38$, $P < 0.001$) (Fig. 2B).

3.1.2 Effects of host resources on the parasitism and host feeding

The variations in the ratio of primary hosts to secondary hosts and the age of females (1–5 days old) both have a significant impact on the parasitism level of females. However,

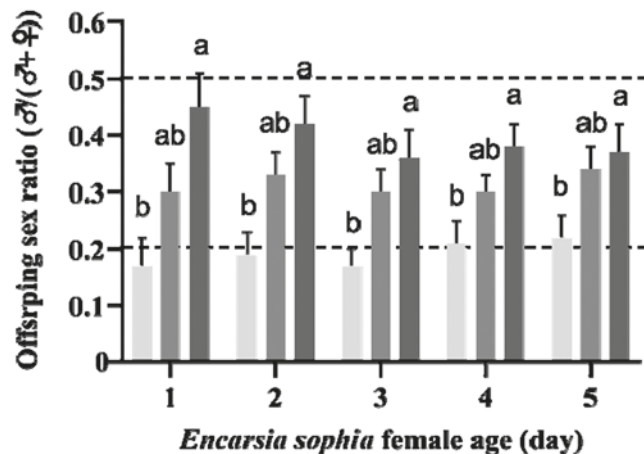


Fig. 1. Effect of different host density and female age (1–5 days old) on offspring sex ratio of *Encarsia sophia* at a secondary host proportion of 0.2. Bar heads with different lowercase letters indicate significant differences (HSD test; $P < 0.05$) in sex ratio of offspring produced by female in the same age among different host density. The line at 0.2 represents the expected values matching the ratio of sex allocation to the ratio of secondary hosts, while the line at 0.5 indicates a trend toward a 1:1 sex allocation ratio.

the interaction between these two factors does not show a significant influence on parasitism. Similarly, changes in host density and the age of females (1–5 days old) significantly influence the parasitism level of females, with the interaction only significantly affecting the parasitism level on primary hosts. Moreover, alterations in host density significantly influence the feeding amount on hosts by females. The age of females (1–5 days old) and the interaction between this factor and host density do not have a significant impact on the feeding amount on hosts by females (Supplementary Table S2). At a secondary host proportion of 0.2 and host densities of 30, 50, and 70 hosts/9.6 cm², the overall parasitism by females on the two hosts exhibited no significant difference with the increasing host density ($F_{2,57} = 3.16$, $P = 0.05$). However, parasitism on secondary hosts showed a significant increase ($F_{2,57} = 15.41$, $P < 0.001$), while parasitism on primary hosts experienced a notable decrease ($F_{2,57} = 8.49$, $P < 0.001$). The daily average host feeding behavior also significantly increased with the rise in host density ($F_{2,57} = 5.49$, $P = 0.007$). Furthermore, at secondary host ratios of 0.5 and 0.8, compared to a secondary host ratio of 0.2 at the same host density, both total parasitism and overall parasitism significantly decreased (secondary host ratio 0.5, host density 30 hosts/9.6 cm²: $t = -3.21$, $df = 25.112$, $P = 0.004$; secondary host ratio 0.8, host density 50 hosts/9.6 cm²: $t = -4.23$, $df = 29.775$, $P < 0.001$) (Table 1).

3.2 Host processing behavior of *E. sophia* at different host resources

3.2.1 Time allocation for host processing behavior

Under the condition that the proportion of secondary host is 0.2 and the host density is 30, 50 and 70 hosts/9.6 cm² respectively, the proportion of each processing behavior of *E. sophia* female to the primary host and the secondary host in the total time within 1 hour is calculated. With the increase of host density, the proportion of host search time in the total time decreased, the proportion of secondary host processing time (examination and laying) in the total time increased, and the proportion of primary host feeding in the total time increased. Therefore, *E. sophia* females perceived the change of host density within 1 hour after entering the habitat. Among them, under the condition that the host density is 70 hosts/9.6 cm², the search time for hosts was significantly less than 30, 50 hosts/9.6 cm² ($F_{2,49} = 5.387$, $P = 0.008$). The feeding time accounts for the proportion of the total time was significantly higher than the host density of 30 hosts/9.6 cm² ($F_{2,58} = 3.518$, $P = 0.036$) (Fig. 3).

3.2.2 Encounter rate of secondary hosts

When females examine host in external, it was regarded as encountering the host. The proportion of secondary hosts provided in the experiment was 0.2, so the theoretical probability of encountering secondary hosts in the habitat (amount

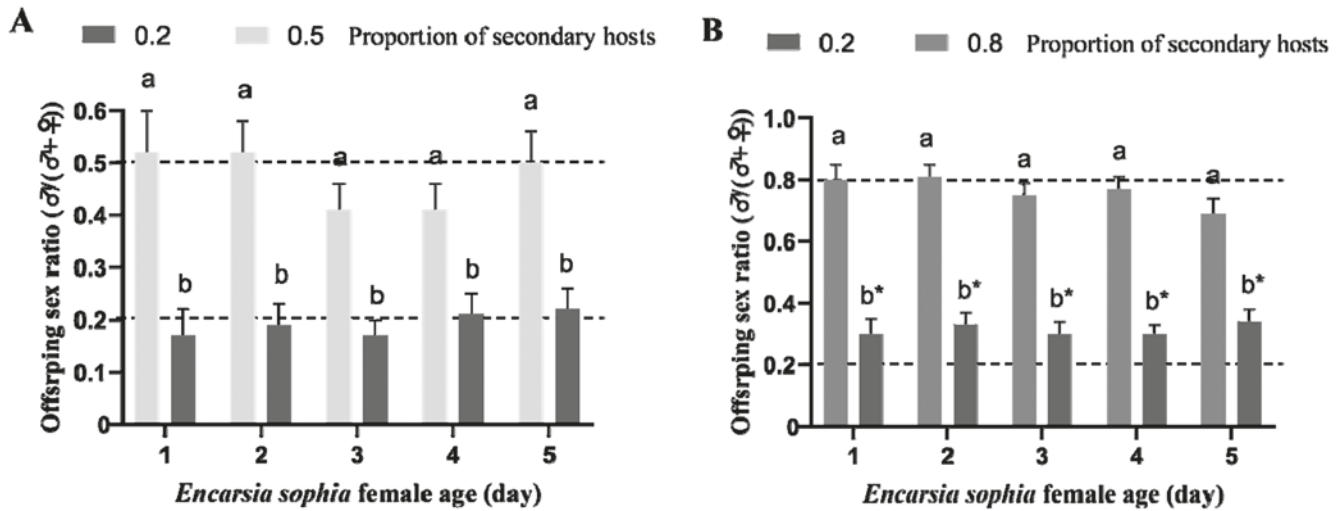


Fig. 2. Effect of different secondary host ratio and female age (1–5 days old) on offspring sex ratio of *Encarsia sophia* under the conditions of host densities at 30 (A) and 50 (B) hosts/9.6 cm², respectively. The secondary host ratio is calculated as the number of secondary hosts divided by the total number of hosts. Bar labels with distinct lowercase letters denote significant differences (t-test; $P < 0.05$) in the sex ratio of offspring produced by females of the same age across different host ratios. * denotes a significant difference between the actual offspring sex ratio and the expected ratio based on the secondary host proportion (t-test; $P < 0.05$). The lines at 0.2, 0.5, and 0.8 represent the expected values matching the ratio of sex allocation to the ratio of secondary hosts.

Table 1. Effect of different host density or host ratio on daily mean number (\pm SE) of hosts parasitized, parasitized rate and host feeding by *Encarsia sophia* female evaluated in first 5 days after emergence. Different lowercase letters following data in the same column indicate significant differences under different host proportions at the same host density (t-test; $P < 0.05$), while different uppercase letters indicate significant differences under the same host proportions at different host densities (HSD test; $P < 0.05$).

Host density	Secondary host ratio	No. of hosts parasitized			No. of hosts parasitized rate %			No. of hosts fed
		primary host	secondary host	primary host + secondary host	primary host	secondary host	primary + secondary hosts	primary host
30	0.2	6.4 \pm 0.5aA	1.5 \pm 0.2bB	7.9 \pm 0.6 aA	26.7	25.0	26.3	4.5 \pm 0.3B
	0.5	3.2 \pm 0.2 b	2.8 \pm 0.2a	6.0 \pm 0.2b	21.3	18.7	20.0	/
50	0.2	6.2 \pm 0.5aA	3.0 \pm 0.3bA	9.2 \pm 0.6aA	15.5	30.0	18.4	4.3 \pm 0.3B
	0.8	1.7 \pm 0.2b	49 \pm 0.3a	6.6 \pm 0.4b	17.0	12.3	13.2	/
70	0.2	5.1 \pm 0.3B	3.1 \pm 0.2A	8.2 \pm 0.5A	9.1	22.1	11.7	6.0 \pm 0.5A

of encountering secondary hosts/total amount of encountering hosts) is 0.2. While the actual observation shows that the actual probability of encountering secondary hosts under different host densities (30, 50 and 70 hosts/9.6 cm²) is 0.36 ± 0.05 , 0.55 ± 0.06 and 0.46 ± 0.06 , respectively, which are significantly higher than the theoretical value ($t = 3.34$, $df = 19$, $P = 0.003$; $t = 6.00$, $df = 22$, $P < 0.001$; $t = 4.68$, $df = 20$, $P < 0.001$).

3.2.3 The number of females treated and accepted to the host

Females of *E. sophia* enter the habitat and locate the host through searching. Once the host is externally examined, it is considered as the initiation of host-treatment behavior. After examining the host, females make the choice of either

accepting or rejecting it. In the case of an accepted primary host, females will either parasitize or feed, while for the accepted secondary host, females will hyperparasitize; however, feeding on secondary hosts is rarely observed. Based on statistical analysis, the proportion of females encountering the secondary host was 0.2, and the host density was set at 30, 50, and 70 hosts/9.6 cm², respectively. There was no significant difference in the treatment amounts for primary hosts, secondary hosts, and total hosts with the increase in host density (primary host: $\chi^2 = 0.74$, $df = 2$, $P = 0.929$; secondary host: $\chi^2 = 1.89$, $df = 2$, $P = 0.389$; total: $\chi^2 = 0.81$, $df = 2$, $P = 0.667$) (Supplementary Fig. 1).

There was no significant difference in the acceptance of the primary host, secondary host, and total host with the increase in host density (primary host: $\chi^2 = 1.28$, $df = 2$,

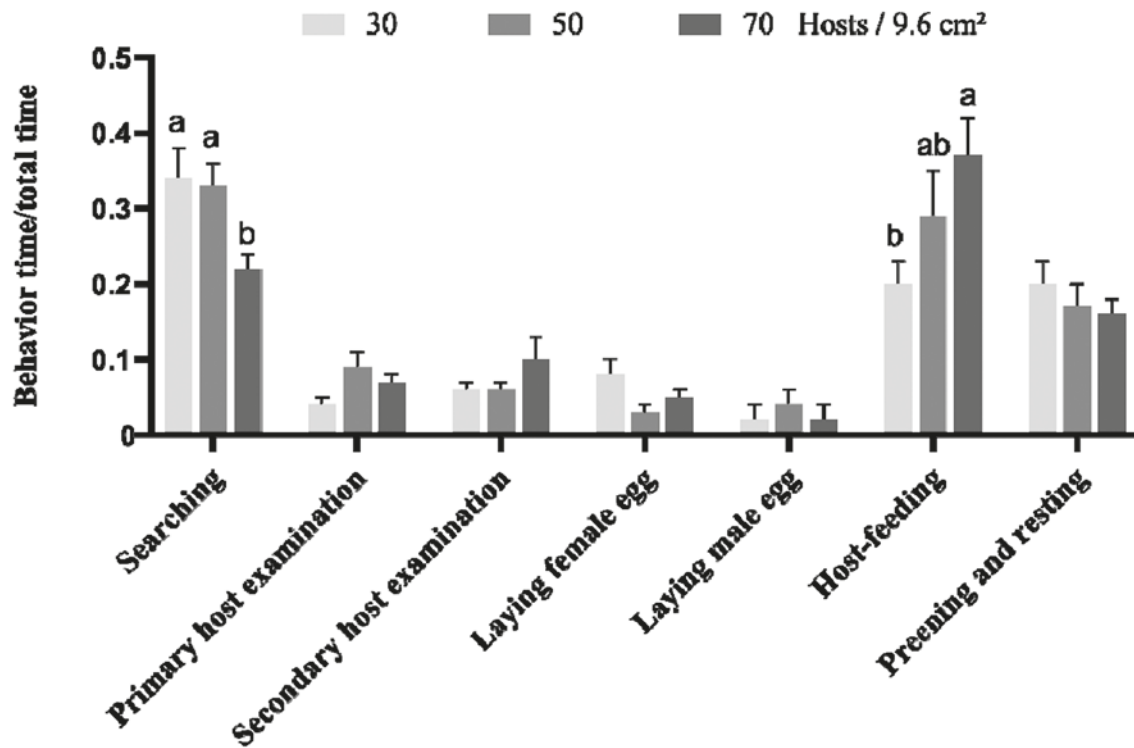


Fig. 3. Relative time (in %) spent by *Encarsia sophia* female on host treating under the condition of different host density (the proportion of secondary host was 0.2). Bar heads with different lowercase letters indicate significant differences (HSD test; $P < 0.05$) in the rate of behavior time among different host density.

$P = 0.526$; secondary host: $\chi^2 = 1.63$, $df = 2$, $P = 0.443$; total: $\chi^2 = 0.73$, $df = 2$, $P = 0.696$). However, there was an adjustment in oviposition and feeding behavior after receiving the primary host. With the increase in host density, females shifted more of their egg-laying behaviors towards host-feeding behaviors ($F_{2,61} = 3.80$, $P = 0.028$) (Fig. 4).

4 Discussion

As an important biological control agent, the sex allocation of heteronomous hyperparasitoids remains a subject of debate. Here, we focus on *E. sophia*, the dominant parasitoid of *B. tabaci*. Our results, diverging from both Godfray et al.'s findings and those of Walter and Donaldson, indicated that under conditions where the ratio of secondary hosts exceeds that of primary hosts or in situations of low host density (host limitation), the offspring sex ratio of *E. sophia* adjusts based on the relative abundance of primary and secondary hosts. However, when the ratio of secondary hosts is low (< 0.5), with an increase in host density, the offspring sex ratio tends toward 1:1. Godfray and colleagues proposed that the sex ratio of heteronomous hyperparasitoid tends toward 1:1 with increasing host density, independent of the relative abundance of primary and secondary hosts. However, their findings did not encompass situations with different host ratios at the same host density, limiting the applicabil-

ity to diverse host scenarios for heteronomous hyperparasitoid (Godfray & Waage 1990; Godfray & Hunter 1992, 1994). Also, Donaldson and Walter's results suggested that the sex ratio of heteronomous hyperparasitoids is solely influenced by the relative abundance of primary and secondary hosts (Walter & Donaldson 1994). Yet, this can be attributed to an experimental design focusing exclusively on a host density of 50, lacking consideration for other host densities. Therefore, drawing conclusions that the sex ratio of heteronomous hyperparasitoid offspring is entirely or unrelated to host ratio based on existing results may be premature.

The sex ratio of insects denotes the proportion of female and male phenotypes within the insect population during a specific period (Abe et al. 2021). Consequently, the sex ratio of parasitoids is most directly reflected in the ratio of female to male offspring, representing the proportion of females producing female and male eggs. Heteronomous hyperparasitoids, owing to their robust host feeding ability, primarily feed on primary hosts and infrequently on secondary hosts (Yang et al. 2012). This feeding behavior has an impact on the relative abundance of primary and secondary hosts. To elucidate the sex ratio regulation mechanism of heteronomous hyperparasitoids, we investigated the parasitism and feeding behavior of females under varying primary and secondary host resources. The results contribute to the sex ratio theory outlined above. Under conditions of low host density (insufficient number of hosts), the parasit-

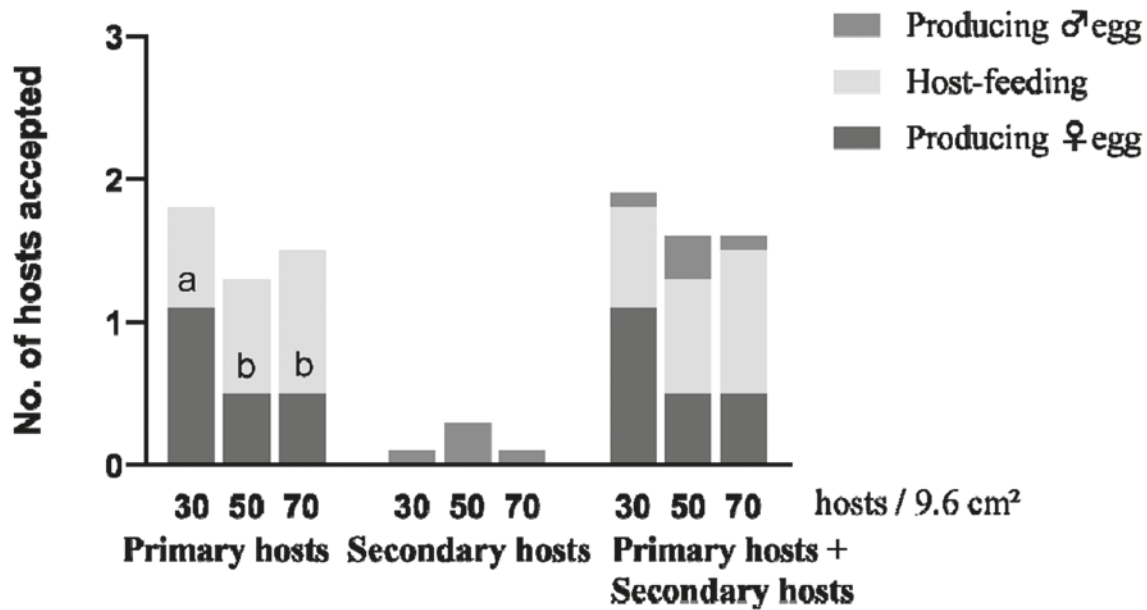


Fig. 4. Abundance of hosts accepted by *Encarsia sophia* female in 1 h under the condition of different host density (with a secondary host proportion of 0.2).

Note: Bar heads with different lowercase letters indicate significant differences (HSD test; $P < 0.05$) in the number of female eggs among different host density.

ism rate on primary and secondary hosts is equivalent, and the sex ratio of female offspring is determined by the relative abundance of the two hosts. This finding aligns with previous studies by Kuenzel (1975), Williams (1977), and Hunter (1989), confirming a significant positive correlation between the number of male offspring of *Encarsia pergandiella* and the proportion of suitable-age secondary hosts based on field and laboratory data (Kuenzel 1975; Williams 1977; Hunter 1989). However, through statistical analysis of the parasitism and feeding levels of *E. sophia* under different host conditions, we observed that with an increase in host density (sufficient number of hosts) and a low proportion of secondary hosts, the parasitism rate on secondary hosts significantly surpassed that on primary hosts. Consequently, there was a decrease in female production and an increase in male production. Simultaneously, there was an escalation in feeding on primary hosts, resulting in a reduction in the number of eggs laid by female offspring and an increase in the number of eggs laid by male offspring. Therefore, the sex ratio of offspring tended to be 1:1. Furthermore, under the same host density, an increase in the proportion of secondary hosts results in a significant decrease in both the total parasitism quantity and parasitism rate of *E. sophia* females. This suggests that an environment with excessively high proportions of secondary hosts negatively influences the parasitism rate of the parasitoid, potentially linked to the parasitoid's preference for different host types. Subsequent experiments could explore the dwell time of the parasitoid in various environments, confirming whether females are inclined to reduce their stay when exposed to environments with exces-

sively high proportions of secondary hosts, thereby leading to a potential early departure. In essence, this behavior may mitigate the occurrence of intense interspecific competition among heteronomous hyperparasitoids.

Through the aforementioned results, we have established that heteronomous hyperparasitoids can maintain the stability of offspring sex ratios by regulating female egg production, male egg production, and feeding behaviors. This leads us to the next question: how does the hyperparasitoid adjust female production, male production, and host feeding through behavioral changes upon perceiving host density, ultimately influencing the sex ratio of offspring? By observing the host-handling behavior of *E. sophia* females after entering the habitat, we found that females can perceive host density within one hour of entering the habitat. Host processing behaviors under the three host densities exhibited significant temporal variations. With the escalation of host density, the proportion of time allocated to host searching decreased, while the proportion of processing time (examination and oviposition) on secondary hosts and feeding time on primary hosts significantly increased. Additionally, Hunter (1993) suggested that the sex ratio of *E. pergandiella* might be linked to the proportion of females encountering secondary hosts, and Avilla (1987) proposed that differences in parasitism between primary and secondary hosts may be attributed to variations in the encounter rate and treatment time of hyperparasitoids toward the two hosts. Analyzing the encounter rate of *E. sophia* females with secondary hosts under different host densities, we observed an increase in the encounter rate with host density, surpassing the theoretical

probability. Hence, the likelihood of females encountering secondary hosts was higher, aligning with Avilla's observations on *Encarsia tricolor* and Hunter's findings with *E. pergandiella*. Females exhibit a preference for secondary hosts in both primary and secondary hosts (Avilla & Copland 1987; Hunter 1993).

Contrastingly, when comparing the encounter rate of secondary hosts under the three host densities with the corresponding offspring sex ratio, it was noted that the encounter rate of secondary hosts was higher than the corresponding offspring sex ratio. This implies that the offspring sex ratio of hyperparasitoids is not solely determined by the encounter rate of secondary hosts. Hunter proposed that if females are more prone to accepting or rejecting a host frequently, the offspring sex ratio of parasitoids may not directly reflect the proportion of primary hosts to secondary hosts in the habitat (Hunter 1989). We further analyzed the number of *E. sophia* females treating and accepting two hosts under three host densities. The results showed no significant difference in the number of primary hosts, secondary hosts, and the total number of hosts, which contradicts the observed sex ratio of *E. sophia*'s offspring. Consequently, we delved into understanding how *E. sophia* makes behavioral choices between primary and secondary hosts, ultimately influencing the number of male and female offspring. Upon further analysis, it was discovered that although the number of females treating and accepting primary hosts did not significantly differ with changing host density, the treatment behavior of primary hosts changed with increasing host density. Partial oviposition behaviors on primary hosts transformed into feeding behaviors. Consequently, when the number of secondary hosts is low, the number of female offspring is reduced, leading to a trend toward an equal ratio of male and female offspring. Hunter's sex allocation study of *E. pergandiella* revealed that the female oviposition sex ratio was affected by the proportion of secondary hosts, but to a lesser extent than predicted solely from the proportion of secondary hosts. This discrepancy may be attributed to the oversight of female feeding behavior on primary hosts (Hunter 1993).

Furthermore, research on the sex ratio adjustment capability of heteronomous hyperparasitoids not only enriches the theoretical understanding of the sex ratio in this special type of parasitoid wasps but also provides a reference for evaluating their application and large-scale rearing. Our study demonstrates that heteronomous hyperparasitoids can adjust the sex ratio of their offspring under different host resource conditions, which is crucial for population stability. Huang and Warsi have suggested that adjusting the parasitoid-to-host ratio can reduce the population fluctuation range of parasitoids and increase their survival probability (Huang et al. 2016; Warsi et al. 2023). This could be one reason why such parasitoids become dominant populations in the wild, making them excellent candidates for biological control (Yang et al. 2022; Tize et al. 2023). However, in large-scale rearing, the high male-to-female ratio due to parthenogen-

esis and hyperparasitism characteristics of these parasitoids poses challenges (Katono et al. 2023). Maximizing female production and ensuring the establishment of released parasitoids are fundamental to optimizing biological control programs (Riccardo et al. 2018; Hougardy et al. 2022). Our results indicate that for heteronomous hyperparasitoids, higher host density does not necessarily lead to more female offspring. At a secondary host proportion of 0.2 and a host density of 30 hosts/9.6 cm², i.e., under host limitation with ample parasitoid eggs, the maximum number of female offspring was obtained with minimal consumption of secondary hosts (primary parasitoid larvae). This condition minimizes costs and maximizes yield, representing the optimal rearing strategy. We can use this information to calculate the ratio of maternal parasitoids to primary and secondary hosts, achieving large-scale production of female offspring.

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Table S1, S2; Figure S1