Wolbachia-Driven Memory Loss in a Parasitic Wasp Increases Superparasitism to Enhance Horizontal Transmission

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ABSTRACT Horizontal transmission of the endosymbiont, Wolbachia, may occur during superparasitism when parasitoid females deposit a second clutch of eggs on a host. Wolbachia may increase the superparasitism tendency of Trichogramma wasps by depriving their memory. To test this hypothesis, we investigated the effects of conditioning experience and memory inhibitors (actinomycin D [ACD] and anisomycin [ANI]) on memory capacity, and expressions of memory-related genes (CREB1 and PKA), and superparasitism frequency of Wolbachia-infected (TDW) and uninfected (TD) lines of Trichogramma dendrolimi after conditioning with lemon or peppermint odor. We detected the presence of Wolbachia in eggs, larvae, pre-pupae, pupae, and adults of Trichogramma by using fluorescence in situ hybridization. The results showed that TDW females had a more reduced memory capacity than TD females after conditioning. Compared with TD females, TDW females showed a higher proportion of superparasitism and a downregulation of CREB1 and PKA genes after conditioning. TD females fed ACD or ANI showed a higher tendency for superparasitism and a downregulation of CREB1 and PKA, along with memory loss after conditioning than TD females fed honey solution only. The presence of Wolbachia was detected in the anterior region of the larva, pre-pupa, and pupa, but was not found in the head of the adult. The results provide evidence of host behavioral manipulation of Wolbachia by depriving memory of host Trichogramma wasps based on Poulin’s criteria. These host behavioral changes led by Wolbachia may be caused by the virulence of Wolbachia on the nervous system of the host.

IMPORTANCE The endosymbiotic bacteria, Wolbachia, live widely within cells of arthropods. Wolbachia are not only transmitted vertically from host mother to offspring, but are also transmitted horizontally among host individuals. Horizontal transmission is expected to occur during superparasitism when host parasitoid females deposit a clutch of eggs on a host previously parasitized by the same parasitoid species. Thus, a question is proposed regarding whether superparasitism behavior is a behavior modification induced by the symbiont to favor symbiont transmission. This study highlights behavioral mechanisms of Wolbachia-induced superparasitism in Trichogramma wasps and the manipulation of symbionts on host parasitoids.

KEYWORDS Trichogramma, Wolbachia, behavioral manipulation, memory loss, superparasitism

The endosymbiotic bacteria, Wolbachia, live widely within cells of insects and other arthropods. Wolbachia are predominantly transmitted vertically from host mother to offspring through eggs and often manipulate the reproduction of host arthropods in diverse ways, such as by inducing parthenogenesis, cytoplasmic incompatibility (CI), male killing (MK), and feminization (1). However, Wolbachia can also be horizontally...
transmitted among host individuals or even across species boundaries (1, 2). In nature, horizontal transfer (HT) of Wolbachia may occur when host individuals are in close contact, as Wolbachia can briefly persist outside host cells before traversing the cell membranes of the host (3, 4). Such close contact among parasitoid offspring individuals is expected to occur during superparasitism, i.e., when parasitoid females deposit a clutch of eggs on a host previously parasitized by the same parasitoid species (5, 6). In this situation, Wolbachia-infected parasitoid offspring and uninfected parasitoid offspring may share the same host (5, 7). The first natural case of high rates of HT in Trichogramma wasps was found in the egg parasite, Trichogramma kayki, when Wolbachia-infected and uninfected parasitoid eggs were deposited into the same host egg (8). Some of the uninfected T. kaykai larvae may acquire the infection of Wolbachia under such condition. Huigens et al. (9) further tested the intraspecific HT of Wolbachia in several Trichogramma species under superparasitism conditions, and detected intraspecific HT in Trichogramma kaykai and Trichogramma deion, but did not detect HT in Trichogramma atopovirilia. Our previous study also observed the intraspecific HT of Wolbachia in Trichogramma dendrolimi when the infected offspring egg was deposited into a host parasitized by an uninfected wasp for 1, 8, or 16 h; however, the HT was not detected when the infected offspring egg was deposited at 32 h after the host was parasitized by the uninfected wasp (10). This implies that Wolbachia may gain the opportunity for horizontal transmission among Trichogramma offspring by causing superparasitism.

Superparasitism is often viewed as a maladaptive mistake of wasps, as the parasitoid wasp is expected to allocate an optimal clutch size in a host to maximize the fitness according to Lack’s hypothesis (11–13). Interestingly, some symbionts can increase the superparasitism tendency of their host (parasitoid wasps), by allowing them to transmit to parasitoid offspring that share the same host insect (9, 14, 15). Thus, a question is immediately proposed that whether superparasitism behavior is a behavior modification induced by the symbiont to favor symbiont transmission, and viewed as “infectious behavior” (14–16). For example, a male-killing heritable symbiont, Arsenophonus naso-niae, benefits from superparasitism spreading in the offspring of the pupal parasitoid, Nasonia vitripennis (15). In Leptopilina boulardi, parasitoid females infected with the L. boulardi Filamentous Virus (LbFV) showed a higher tendency for superparasitism than their uninfected counterparts (17, 18). Farahani et al. (19) reported that Wolbachia-infected T. brassicae wasps superparasitized their hosts more often than their uninfected counterparts. Similarly, our previous studies also showed that T. dendrolimi females infected by Wolbachia exhibited a higher tendency of superparasitism than the uninfected females (10, 20). Although Wolbachia-infected females of T. brassicae (19) and T. dendrolimi (10, 20) exhibited a higher tendency for superparasitism than uninfected females, the mechanism behind this phenomenon is still unknown.

Poulin (21) proposed a series of criteria for testing whether the behavioral changes of a host are due to the adaptations of a parasite that is trying to enhance its transmission (“host manipulation hypothesis”): (i) the behavioral changes must be complex (because complex traits are unlikely to arise by chance); (ii) the behavioral changes must show purposeful design for a particular function; (iii) the behavioral changes are more likely to be an adaption if these changes arise in several lineages or related species (high superparasitism tendency of Wolbachia-infected Trichogramma females has been found in Trichogramma brassicae and T. dendrolimi); (iv) the manipulation should increase the transmission of the parasite. The higher superparasitism tendency in Wolbachia-infected Trichogramma females appears to fulfill the third criteria of the host manipulation hypothesis (10, 19, 20). Clearly, the most important criteria is that behavioral manipulation should be aimed at increasing the fitness of the parasite (criteria 4). The occurrence of superparasitism would result from benefit conflicts between Wolbachia and host wasps. For parasitoid wasps, the fitness of parasitoid larvae is entirely dependent on the quality of the host for their development (22). Superparasitism often results in the intraspecific competition of parasitoid larvae in most cases, leading to offspring fitness costs or even
The killing of offspring (5). This may come at the cost of a reduction in the vertical transmission of the parasite (23). Broadly speaking, the outcome of intrinsic intraspecific competition depends on the host usage strategy of parasitoid larvae. In "solitary" parasitoids (i.e., only one parasitoid offspring can successfully develop on or inside a host), the larva is seeking to monopolize the host resources; if more than one eggs have been laid in the same host, no more than one parasitoid offspring is expected to survive from the ensuing larval competition (5, 23). Trichogramma wasps can be viewed as a gregarious species, where more than one parasitoid offspring can successfully emerge from a host. The offspring of gregarious species can often survive under superparasitism conditions, although the offspring wasps may exhibit a small body size or short longevity (5, 22). Thus, Wolbachia may gain an opportunity for HT among these survival offspring.

Generally, the high tendency of superparasitism can be attributed to 2 broad explanations. One is that superparasitism is an adaptive strategy for short-lived wasps that rarely encounter healthy hosts in field conditions (22, 24, 25). This explanation predicts that wasps are less willing to accept a parasitized host when healthy hosts are common in the environment. Superparasitism is found more frequently when a wasp carries large supplies of unlaid eggs and is certain to die in a limited time (22, 25). However, the high tendency of superparasitism of Wolbachia-infected Trichogramma has been observed under laboratory conditions, where a certain number of hosts were supplied to newly emerged infected or uninfected wasps (10, 19, 20). This implies that the high tendency of superparasitism in Wolbachia-infected Trichogramma may be attributed to other factors. An alternative interpretation is that frequent superparasitism by Wolbachia-infected Trichogramma may be a result of the breakdown in the mechanism of host discrimination (25). Generally, parasitoid females prevent superparasitism by labeling their host with host marking pheromones (HMP) (13, 22, 25). Newly enclosed parasitoid females experience both healthy (unparasitized) and parasitized hosts, enabling them to discriminate between the 2 (24, 25). Previous studies reported that Wolbachia-infected Trichogramma exhibited a shorter memory than natural uninfected Trichogramma (26, 27). Thus, a hypothesis is proposed that, based on observations of the high tendency of superparasitism and memory loss in Wolbachia-infected Trichogramma, Wolbachia infection may select for reducing memory of females, causing a breakdown in the mechanism of host discrimination, and resulting in an increased tendency for superparasitism. This is called the "memory retention hypothesis" (27, 28).

To test the hypothesis, Wolbachia-infected (TDW) and uninfected (TD) lines of T. dendrolimi were used as the model. This study followed the definition of superparasitism by van Dijken and Waage (29). Superparasitism is defined as the deposition of a clutch of eggs in or on a host that has already been parasitized by a female of the same species (29). Trichogramma wasps are often viewed as a facultative gregarious parasitoid species and have been widely used as an effective biological control agent against many lepidopteran pests in agriculture and forestry (30, 31). T. dendrolimi females often allocate tens of eggs on a larger lepidopteran egg (e.g., Antheraea pernyi and Samia cynthia) (31–33), and often allocate a single egg on a smaller lepidopteran egg (e.g., Corcyra cephalonica) (34). In this study, C. cephalonica eggs were used as the host eggs for a more exact determination of superparasitism (28).

Here, we examined the effects of Wolbachia infection on the memory of Wolbachia-infected (TDW) and uninfected (TD) females of T. dendrolimi after conditioning with lemon odor or peppermint odor and an egg card as a reward using a modified version of the methodology described by Farahani et al. (26, 27). The memory inhibitors, actinomycin D (ACD) (35) and anisomycin (ANI) (36), were applied to block the memory of TD and TDW females. The memory capacity of Trichogramma females was estimated by the proportion of choice (PCO) and the proportion of residence time (PRO) for the conditioning odor with a 24 h time interval after learning. The choice of the wasp was determined when the wasp crossed a line located 30 mm away from a tunnel pumping conditioning or control odor. The residence time of the wasp was defined as the
amount of time spent in the area with conditioning or the control odor before the wasp made the choice. Thereafter, we tested the memory capacity, frequency of superparasitism, and the expression of the indicator genes (CREB1 and PKA) related to memory formation (37, 38) in TD and TDW females with memory inhibition and Wolbachia infection. The results of this study are expected to bridge the gaps in understanding the behavioral mechanisms of Wolbachia-induced superparasitism in Trichogramma wasps and to highlight the manipulative effects of symbionts on host parasitoids.

RESULTS

To avoid side effects of ANI and ACD on the survival of Trichogramma females, a 15% honey solution mixed with 0.008 mg/mL ACD or 0.1 mg/mL ANI was used to block the memory of Trichogramma females according to the results of pre-experiments (Text S1 and Fig. S1). Compared with the experienced female wasps in TDW line, the experienced female wasps in TD line exhibited a higher memory capacity (a higher tendency to the conditioning odor) at 24 h after conditioning, but this was not true for those wasps at 12 and 36 h after conditioning (Text S1, and Fig. S2 and S3).

Effects of Wolbachia infection and memory blocking on memory of Trichogramma females. The PCOs of experienced TD females were significantly higher ($P = 0.0014$ [Lemon]; $P = 0.016$ [Peppermint]) than the theoretical value of 50%, but these were not true in case of experienced TDW females ($P = 0.86$ [Lemon]; $P = 0.58$ [Peppermint]). Regardless of Trichogramma line, the PCOs of naive females, experienced females fed ACD, and experienced females fed ANI were not different to the theoretical value of 50%. The PCOs ($z = 2.55$, $P = 0.011$ [Lemon]; $z = 2.27$, $P = 0.023$ [Peppermint]) and PROs of experienced TD females were significantly or marginally insignificantly higher ($z = 2.16$, $P = 0.031$ [Lemon]; $z = 1.64$, $P = 0.10$ [Peppermint]) than those of TDW females, but these differences were not true in case of naive females, experienced females fed ACD, and experienced females fed ANI (Fig. 1 and 2). The PCOs of experienced TD females conditioned to lemon odor were significantly or marginally insignificantly higher than those of naive females ($z = 2.99$, $P = 0.015$), experienced females fed ACD ($z = 2.99$, $P = 0.015$), and experienced females fed ANI ($z = 2.55$, $P = 0.052$). The PCOs of experienced TD females conditioned with peppermint odor were significantly higher than those of naive females ($z = 2.96$, $P = 0.016$), but were insignificantly higher than those of experienced females fed ACD ($z = 2.04$, $P = 0.17$), and experienced females fed ANI ($z = 2.27$, $P = 0.10$). In addition, regardless of the conditioning, the PCOs of experienced TDW females were not different from those of naive females ($z = 0.26$, $P = 0.99$ [Lemon]; $z = 0.51$, $P = 0.96$ [Peppermint]), experienced females fed ACD ($z = 0.40$, $P = 0.98$ [Lemon]; $z = 0.11$, $P = 0.9995$ [Peppermint]), and experienced females fed ANI ($z = 0$, $P = 1.00$ [Lemon]; $z = 0.26$, $P = 0.99$ [Peppermint]) (Fig. 1).
Regardless of *Trichogramma* line, the PROs of naive females, experienced females fed honey solution only, experienced females fed ACD, and experienced females fed ANI were not different to theoretical value of 50%. The PROs of experienced TD females conditioned with lemon odor were significantly or marginally insignificantly (*P* = 0.070) higher than those of naive females (*z* = 2.63, *P* = 0.043), experienced females fed ACD (*z* = 2.44, *P* = 0.070), and experienced females fed ANI (*z* = 2.83, *P* = 0.002). The PROs of experienced TD females with peppermint odor conditioning were significantly higher than those of naive females (*z* = 2.69, *P* = 0.036), but were insignificantly higher than those of experienced females fed ACD (*z* = 1.29, *P* = 0.57), and experienced females fed ANI (*z* = 1.27, *P* = 0.58). In addition, regardless of the conditioning, the PROs of experienced TDW females were not different from those of naive females (*z* = 0.34, *P* = 0.99 [Lemon]; *z* = 0.11, *P* = 0.9996 [Peppermint]), experienced females fed ACD (*z* = 0.24, *P* = 0.995 [Lemon]; *z* = 0.21, *P* = 0.997 [Peppermint]), and experienced females fed ANI (*z* = 0.030, *P* = 1.00 [Lemon]; *z* = 0.40, *P* = 0.98 [Peppermint]) (Fig. 2).

**Effects of *Wolbachia* infection and memory blocking on expression of CREB1 and PKA.** The expression levels of both memory-related genes, CREB1 and PKA, were significantly affected by the interaction of the conditioning treatment and the *Trichogramma* line (CREB1: *F*1,128 = 16.29, *P* < 0.001; PKA: *F*1,128 = 781.16, *P* < 0.001). The expression levels of CREB1 and PKA in TD or TDW females fed honey solution only (CK) were significantly higher than that in those fed ACD (CREB1: *z* = 11.55, *P* < 0.001 [TD], *z* = 3.84, *P* < 0.001 [TDW]; PKA: *z* = 54.68, *P* < 0.001 [TD], *z* = 6.64, *P* < 0.001 [TDW]) or ANI (CREB1: *z* = 5.22, *P* < 0.001 [TD], *z* = 3.43, *P* = 0.0017 [TDW]; PKA: *z* = 55.72, *P* < 0.001 [TD], *z* = 6.95, *P* < 0.001 [TDW]). The expression level of CREB1 in TD females fed ACD was significantly lower (*z* = 6.33, *P* < 0.001) than that in those fed ANI. Likewise, the expression level of CREB1 in TDW females fed ACD was lower than that in those fed ANI, but the difference was insignificant (*z* = 0.41, *P* = 0.91). The expression levels of CREB1 and PKA in TD females fed honey solution only (CREB1: *z* = 19.85, *P* < 0.001; PKA: *z* = 51.78, *P* < 0.001), ACD (CREB1: *z* = 12.14, *P* < 0.001; PKA: *z* = 3.74, *P* < 0.001), or ANI (CREB1: *z* = 18.06, *P* < 0.001; PKA: *z* = 3.01, *P* = 0.0026) were significantly higher than that in TDW females with the corresponding treatment (Fig. 3).

**Effects of *Wolbachia* infection and memory blocking on superparasitism of *Trichogramma* females.** After conditioning and a 24 h time interval, the differences in parasitism rate between TD and TDW experienced females fed honey solution only (*z* = 1.56, *P* = 0.12) or ANI (*z* = 1.07, *P* = 0.28) were insignificant. However, the parasitism rate by TD experienced females fed ACD was significantly higher than that by TDW experienced females (*z* = 2.08, *P* = 0.038) (Fig. 4A). The proportion of superparasitism by TDW
experienced females fed honey solution only ($z = 5.97, P < 0.001$) was significantly higher than that by TD experienced females, but this difference was not true for the experienced females fed ANI ($z = 1.35, P = 0.18$) or ACD ($z = 1.32, P = 0.19$). Additionally, the proportion of superparasitism by TD experienced females fed ANI ($z = 3.47, P = 0.0015$) or ACD ($z = 3.06, P = 0.0063$) was significantly higher than that by those fed honey solution only.

FIG 3 Expression level of CREB1 (A) and PKA (B) of TD and TDW females fed ACD, ANI, or honey solution only (CK). Error bars indicate 95% confidence interval. The different uppercase letters indicate significant differences among TD females fed ACD, ANI, or honey solution only. The different lowercase letters indicate significant differences among TDW females fed ACD, ANI, or honey solution only. "ns," "**," and "****" indicate nonsignificant differences, significant differences at $P < 0.01$, and $P < 0.001$, respectively, between TD and TDW females. This experiment was performed thrice.

FIG 4 Parasitism rate (A) and proportion of superparasitism (B) of TD and TDW females fed honey solution only, ACD, or ANI at 24 h after conditioning. Error bars indicate 95% confidence interval. The different uppercase letters indicate significant differences among TD females subjected to different treatments. The different lowercase letters indicate significant differences among TDW females subjected to different treatments. "ns," "**," and "****" indicate nonsignificant differences, significant differences at $P < 0.01$, and $P < 0.001$, respectively, between TD and TDW females. This experiment was performed once.
The proportion of superparasitism by TDW experienced females fed honey solution only was significantly lower than that by those fed ACD ($z = 3.09$, $P = 0.0057$), but was not lower than that by those fed ANI ($z = 1.61$, $P = 0.24$) (Fig. 4B).

**FISH detection of *Wolbachia* in the egg, larva, pre-pupa, pupa, and adult of *Trichogramma* offspring.** *Wolbachia* signals were highly abundant in the posterior part of the eggs, larvae, pre-pupae, and pupae, and the abdomen of the adult. The presence of *Wolbachia* was detected in the anterior region of the larvae, pre-pupae, and pupae, but was not found in the head of the adult (Fig. 5).

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DISCUSSION

Our observations regarding the effect of Wolbachia infection on the memory capacity of Trichogramma were similar to the observations of Farahani et al. (27). We confirmed that Wolbachia-infected Trichogramma wasps showed a reduced memory capacity compared to their uninfected counterparts. Both of the indicator genes for memory formation, CREB1 and PKA, were downregulated in infected females when compared to those in the uninfected counterparts. Similar to our previous studies (10, 20) and to the observation by Farahani et al. (19), we re-confirmed that infected females showed a higher superparasitism tendency than their uninfected counterparts. By blocking the memory of infected and uninfected females using ACD and ANI, we revealed the association between the higher tendency for superparasitism and the memory retention in infected females. We found that uninfected females fed ACD or ANI showed a higher tendency for superparasitism, memory loss, and downregulation of memory-related genes, which is similar to the observation in Wolbachia-infected females. The results support the “memory retention hypothesis”.

Though many studies have reported that some symbionts can increase the superparasitism tendency of their host (parasitoid wasps) to enhance the spread of the symbiont in the population of host wasps, the mechanisms behind this symbiont-induced behavioral change are poorly understood. According to the results of Fish detection, Wolbachia infection in the nervous system at the immature stages may be one underlying reason for the behavioral changes in this study. It is worth noting that Wolbachia signals in adult heads were weak. Similarly, our previous study also found that Wolbachia density was low in the heads of Trichogramma pretiosum and T. dendrolimi based on qPCR and Fish detection (39). Though Wolbachia signals were weak in adult heads, we still detected a significant downregulation of memory-related gene expression in infected adults compared with their uninfected counterparts. This raises an additional question: how would there be a sustained reduction in memory-related gene expression in the adult stage if Wolbachia is no longer present in the head? Previous studies showed that Wolbachia could colonize and replicate in the central nervous system (CNS) of the host insects, such as D. melanogaster (40, 41), Folsomia candida (42), Spalangia endius (43), and Eurema hecabe (44), and this may account for the effects of Wolbachia on host behaviors, including locomotive behaviors, sleep, feeding behaviors, mating preference and frequency, learning, and memory capacity (45, 46). The decrease in memory induced by Wolbachia infection is found not only in Trichogramma species, but also in Armadillidium vulgare (47). Min and Benzer (48) found that Wolbachia can cause a widespread degeneration of tissues, including the brain and retina. Malkeyeva et al. (49) reported that Wolbachia-induced a macroautophagy in the neurons of D. melanogaster. However, without another probe for the CNS demonstrating co-localization of nervous tissue and Wolbachia, it is still unclear if there is a direct interaction. According to the observation on the development of a Trichogramma species, the formation of the CNS of Trichogramma telengai occurs at the late larval stage (50). The co-localization of nervous tissue and Wolbachia at immature stages may negatively affect the formation of the CNS. We assume that memory loss may be a side effect of the virulence of Wolbachia on the central nervous system. Contradicting our results, Bi et al. (51) reported that Wolbachia may improve the learning and memory capacity of Drosophila melanogaster and Drosophila simulans by upregulating the expression of CREB through microRNA. The different results may be due to the different host species and Wolbachia strains used in the studies. Different from Trichogramma, species such as Drosophila do not parasitize as wasps do, so memory reduction may not be beneficial. Future studies should focus on determining whether host behavioral changes are directly caused by the virulence of Wolbachia on the host nervous system.

Although this study reveals that Wolbachia may benefit from HT by inducing superparasitism through memory loss in Trichogramma wasps, the experiments were conducted under laboratory conditions. Many questions need to be answered regarding
In conclusion, our results enrich the evidence indicating the behavioral mechanisms for host manipulation in Wolbachia and support the “host manipulation hypothesis” based on Poulin’s criterion 1–4 and on the observations of previous studies (10, 19, 20, 27). However, as the tests were conducted under laboratory conditions, further studies should be encouraged to test the effect of the behavioral manipulation of Wolbachia on the epidemic of Wolbachia and the dynamic coexistence of both infected and uninfected populations of Trichogramma in fields.

MATERIALS AND METHODS

Insects. All insects, including a Wolbachia-uninfected bisexual isofemale line (TD) and a Wolbachia-infected thelytokous isofemale line (TDW) of T. dendrolimi, and their host C. cephalonica were reared under the following conditions: 26 ± 1°C temperature, 75% ± 5% RH, and a 16 h/8 h light/dark cycle. The TD line, which has the same genetic background as the TD line, was established by artificially transferring Wolbachia into females of the TD line (7). Wolbachia infection in the TDW line was detected by amplifying the Wolbachia surface protein gene (wsp) according to the protocol described in our previous studies (10, 20). The Wolbachia-uninfected population of C. cephalonica was reared on a semi-artificial diet. The C. cephalonica eggs were glued onto a card using gum arabic. These egg cards were used for parasitization by T. dendrolimi.

Conditioning procedure. The conditioning procedures of TD and TDW females were conducted according to the methods described by Farahani et al. (27) and Smid et al. (35). The TD or TDW naive females (emerged within 24 h), in a group of 100 individuals, were supplied with an egg card with ca. 200 host eggs for 30 min to obtain oviposition experience. These wasps were then individually transferred into a training pipe (Diameter = 25 mm, Length = 95 mm) with a conditioning odor at an airflow of 1 m/s and an egg card with 200 host eggs as a reward, and lasted for 120 min. Lemon odor and peppermint odor were used as the conditioning odor. The tested odor was presented on a circular filter paper (Diameter = 20 mm) with 1 μL peppermint or lemon solution (99% pure) in a pipe (Diameter = 20 mm, Length = 70 mm) connected with the training pipe. Half of the wasps in each group underwent the training procedure using lemon odor, while the other half was trained using peppermint odor. The procedure described above was defined as a single conditioning event. To obtain experienced females with a long-term memory, TD or TDW females were subjected to 3 conditioning events with an interval of 30 min between events.

Bioassay method. To test the memory capacity of Trichogramma females after condition, a four-quadrant olfactometer was used to test the preference of Trichogramma females to the conditioning odor. The experiments were conducted in a climate room (26 ± 1°C) with scattered light (2000 lx). The device contained an empty square room space (100 mm × 100 mm) divided into 4 equally shaped areas. Each area was connected to a Teflon capillary holder containing activated carbon and the capillaries to supply conditioning or control odor. The conditioning odor (1 m/s) was pumped into the area from two tunnels placed opposite to each other along a diagonal line. The control odor (clean air) was pumped from the other 2 tunnels. To reduce the effects of previous trials, the device was cleaned with distilled water after each trial. After each trial, the conditioning or control odor in a tunnel was altered by counterpart odor, and the device was rotated clockwise at 90°.

In a trial, a single female was introduced from the hole at the center of the area. The female wasp then allowed to walk freely in the area. The trial was stopped when the wasps made a choice, or the
time reached 900 s. The choice of the wasp was determined when the wasp crossed a line located 30 mm away from a tunnel pumping conditioning or control odor. The residence time of the wasp was defined as the amount of time spent in the area with conditioning or control odor before the wasp crossed the line. The wasps that held still for 180 s were abandoned, and excluded from analysis. The wasps used for different treatment levels were obtained from the same generation of the Trichogramma populations.

**Effects of Wolbachia infection and memory blocking on memory of Trichogramma females.** To reduce side effects of ANI and ACD on the survival of Trichogramma females, a 15% honey solution mixed with 0.008 mg/mL ACD or 0.1 mg/mL ANI was selected to block the memory of Trichogramma females according to the results of the toxicology test (Text S1 and Fig. S1). The effective memory durations of experienced female wasp in TD or TDW line was determined by the preference to the odor at 12 h, 24 h, and 36 h after conditioning according to the procedure of bioassay in a four-quadrant olfactometer described above. Compared with experienced females in the TDW line, experienced females in the TD line exhibited a higher tendency to the conditioning odor at 24 h after conditioning. Naive females did not display a significant difference in the preference between the conditioning odor and control air (Text S1, and Fig. S2 and S3).

In this experiment, Trichogramma females subjected to 4 different treatments were used: (i) Naive females without training; (ii) Experienced females that were conditioned with lemon odor or peppermint odor; (iii) Experienced females fed a honey solution mixed with ACD; (iv) Experienced females fed honey solution mixed with ANI. After a 24 h time interval, 60 naive females, experienced females, and experienced females fed ACD or ANI of each Trichogramma line (i.e., 30 females for lemon odor and 30 females for peppermint) were tested according to the procedure of bioassay in a four-quadrant olfactometer described above. The experiments were conducted from 7 am to 11 am on a day, and were accomplished within 5 days. The entire experiments were replicated using different individuals.

**Effects of Wolbachia infection and memory blocking on expression of CREB1 and PKA.** The orthologs of CREB1 and PKA were identified by aligning the sequences of T. dendrolimi genomic assembly (Text S2, Table S1 and Fig. S4 and S5). The available CREB1 protein sequence from D. melanogaster (Q9VWW0), N. vitripennis (XP_032455913), A. mellifera (CAD23075) and Bombyx mori (ADM32514), along with the available PKA protein sequence from D. melanogaster (CAAA3841), N. vitripennis (NP_001164381), A. mellifera (XP_02695426) and B. mori (NP_001104823) were used as queries. The primers for CREB1 and PKA were designed using the Primer Premier 5.0 software (Premier Biosoft) (Table S2).

The TD or TDW 1-day-old females fed honey solution only, ACD, or ANI, in groups of 100 offspring individuals, were collected and stored at ~80°C. Each treatment involved 24 replicates. The entire experiments were performed using different individuals. The total RNA content of these females was extracted using Trizol reagent (Ambion, Life Technologies) according to the protocol described in our previous study (52). The RT-qPCRs were carried out in the Bio-Rad CFX96 Real-time PCR Detection System (Bio-Rad, Hercules,) according to the procedure described in our previous study (52). The expressions of CREB1 and PKA were normalized using a reference gene, RPL18, which is considered an appropriate reference gene for the adult stage of T. dendrolimi (52).

**Effects of Wolbachia infection and memory blocking on superparasitism of Trichogramma females.** To obtain the oviposition experience, TD or TDW naive females (emerged within 24 h) were individually supplied with a card with 10 host eggs for 1 h. Thirty of each of naive females, experienced females fed honey solution only, experienced females fed ACD, and experienced females fed ANI, of TD and TDW lines, were supplied with an egg card with 10 host eggs for 1 h after 24 h. The oviposition behaviors were recorded under the stereo zoom microscope (Zeiss SV6) through a T. dendrolimi female often deposit one offspring egg into a C. cephalonica egg by a time of oviposition, a few host eggs may not have contained an offspring egg or may have contained 2 offspring eggs after a single oviposition. A single egg from a female wasp was deposited into a host egg only when a single oviposition behavior occurred with fluctuating abdominal movements (29, 53, 54). The location of the parasitized host egg in the egg card was marked by a surgical skin marking pen (0.5 mm tips, T3023; Tondaus) soon after a trial. The host eggs were maintained until the eggs blackened, indicating the parasitization of the host egg (55). The blackened eggs were cut off from the egg card and individually transferred into a new Durham glass tube for emergence. After the emergence of the wasp offspring, the host eggs were dissected to determine the presence of dead Trichogramma offspring. The superparasitized host eggs were defined as eggs with 2 Trichogramma offspring deposited by two times of oviposition (28). The entire experiments were replicated using different individuals.

**FISH detection of Wolbachia in eggs, larvae, prepupae, pupae, and adults of Trichogramma.** To investigate the presence of Wolbachia in the neurotrophic part of Trichogramma, the fluorescence in situ hybridization (FISH) was applied to detect Wolbachia signals in the eggs, larvae, pre-pupae, pupae, and adult wasps of Trichogramma. The procedure of FISH detection was conducted according to the description of Zhao et al. (56) and Wang et al. (57). A group of ca. 500 TD or TDW females were transferred into a glass tube (75 mm length × 12 mm diameter) containing the egg card with ca. 200 host eggs for 10 min. The eggs were individually cut out from the cards and then dissected at 0.5 h (egg stage), 48 h (larval stage), 96 h (prepupal stage), 144 h (pupal stage), 240 h (newly emerged adult) after parasitization under a stereomicroscope (SV6; Zeiss). To target the 16S rRNA (rRNA) of Wolbachia, two 5′-rhodamine-labeled Wolbachia probes were used (W1, 5′-AATCCGGCCGAACCGACC-3′ and W2, 5′-CTCTGAGTAGCGCACTATC-3′) (58). The location of Wolbachia in the Trichogramma offspring or adults was photographed under a laser scanning confocal microscope (Olympus FV3000, Monolith). The images in Fig. 5 are representative images of 16 eggs, 10 larvae, 10 pre-pupae, 10 pupae, and 10 adults. The entire experiments were replicated using different female individuals.
Data analysis. To examine memory inhibition in TD or TDW females fed ACD or ANI, the GLM with quasi-binomial distribution was used to analyze the PCO and PRO, with 24 h time interval after conditioning in naive females and experienced females fed honey solution only, ACD, or ANI. Exact binomial tests were applied to test PCO and PRO against the theoretical value of 50%. To test the effects of memory inhibition and Wolbachia infection on the superparasitism tendency of Trichogramma females, the frequency of superparasitism by TD or TDW females fed honey solution only, ACD, or ANI was also analyzed using GLM with quasi-binomial distribution as well. The GLM with Gaussian distribution was applied to analyze the expression of memory-related genes, CREB1 and PKA, in TD or TDW females fed honey solution only, ACD, or ANI. All data calculations and analyses were conducted using R software version 4.0.2 (57).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, DOCX file, 0.02 MB.
TEXT S2, DOCX file, 0.01 MB.
FIG S1, TIF file, 2.2 MB.
FIG S2, TIF file, 2.1 MB.
FIG S3, TIF file, 2.4 MB.
FIG S4, TIF file, 1 MB.
FIG S5, TIF file, 1.2 MB.
TABLE S1, DOCX file, 0.01 MB.
TABLE S2, DOCX file, 0.01 MB.

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