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A symbiotic gene stimulates aggressive behavior favoring the survival of parasitized caterpillars

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Animals often exhibit increased aggression in response to starvation, while parasites often manipulate host behavior. In contrast, underlying molecular mechanisms for these behavioral changes are mostly unknown. The diamondback moth, *Plutella xylostella*, is an agricultural pest that feeds on cruciferous plants as larvae, while *Cotesia vestalis* is a parasitoid wasp that parasitizes diamondback moth larvae. In this study, we determined that unparasitized diamondback moth larvae exhibit increased aggression and cannibalism when starved, while starved larvae parasitized by *C. vestalis* were more aggressive than unparasitized larvae. *C. vestalis* harbors a domesticated endogenized virus named Cotesia vestalis bracovirus (CvBV) that wasps inject into parasitized hosts. Starvation increased octopamine (OA) levels in the central nervous system (CNS) of diamondback moth larvae while a series of experiments identified a CvBV-encoded gene product named Assailant that further increased aggression in starved diamondback moth larvae. We determined that Assailant increases OA levels by activating *tyramine beta-hydroxylase* (*PxTβh*), which is a key enzyme in the OA biosynthesis pathway. Ectopic expression of *assailant* in *Drosophila melanogaster* likewise upregulated expression of *DmTβh* and OA, which increased aggressive behavior in male flies as measured by a well-established assay. While parasitized hosts are often thought to be at a competitive disadvantage to nonparasitized individuals, our results uncover how a parasitoid uses an endogenized virus to increase host aggression and enhance survival of offspring when competing against unparasitized hosts.

starvation | aggression | cannibalism | octopamine | parasite

Significance

Many animals exhibit increased aggression in response to starvation while some parasites manipulate host behavior. However, underlying molecular mechanisms for these changes in behavior are largely unknown. Here, we studied diamondback moth larvae, which are agricultural pests that often build to very high densities in crops. A wasp named *Cotesia vestalis* parasitizes diamondback moth larvae and harbors an endogenized virus named Cotesia vestalis bracovirus (CvBV). We show that parasitized hosts are more aggressive and cannibalistic under starvation than unparasitized hosts. We also show that a CvBV gene that is transferred to parasitized hosts elevates host aggression by increasing octopamine (OA) levels. Our results show how a parasite promotes its own survival under starvation conditions by manipulating the behavior of its host.

Starvation is common in nature, which can strongly modulate the physiological responses of animals and trigger multiple adaptive behaviors including aggressive behavior and cannibalism (1, 2). Aggressive behavior is also associated with other activities such as competition for mates by males where displays include wing threats, chasing, boxing, and other forms of attack (3, 4). A number of herbivorous insects cannibalize conspecifics when deprived of food, which benefit the cannibal by provisioning resources (5). Most studies of cannibalism also focus on the behavioral plasticity of starved animals (5, 6). However, insect herbivores and other animals are also commonly infected or parasitized by microbes, nematodes, and other insects such as parasitoid wasps (7–9). Some parasites have also evolved strategies for altering specific neural circuits of their hosts, which enables them to manipulate host behaviors that favor their own survival and transmission (5, 10). Whether parasites might also manipulate their hosts to defend against environmental stresses like starvation is unknown. Moreover, while several studies report strategies by which parasites alter the behavior of hosts to reduce cannibalism, predation, or parasitism by competitors (11–15), underlying molecular mechanisms are largely unclear.

Parasitoid wasps (order Hymenoptera) lay eggs in or on hosts that are other insects or related arthropods (16). After egg hatching, parasitoid larvae develop by consuming blood or host tissues. Endoparasitoids that lay eggs in hosts produce a range of virulence factors with functions that promote the development of wasp offspring. Some of these virulence factors are produced by wasp larvae while others derive from teratocytes or venom (17, 18). Venom produced in a venom gland contains many proteins (17), while teratocytes are cells deriving from the serosa surrounding the wasp embryo, and are released into the host's hemocoel when eggs hatch (18). Some endoparasitoids also harbor domesticated endogenous viruses (DEVs) which are permanently integrated in the germline of wasps but produce virions that adult females inject into hosts with eggs and venom (19–23). The DEVs associated with a monophyletic lineage of endoparasitoids in the family Braconidae evolved from a virus in the family *Nudiviridae* and are referred to as bracoviruses (BVs) (19). BVs and another group of endogenous viruses named ichnoviruses (IVs) have historically been referred

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to as polydnaviruses or more recently as polydnaviriformes (20–26). However, we do not use these terms because current evidence very strongly indicates BVs and IVs evolved from different ancestors (19).

The diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae), is an agricultural pest that feeds on cruciferous plants as larvae, while *Cotesia vestalis* (Hymenoptera: Braconidae) is a parasitoid wasp that parasitizes diamondback moth larvae and harbors Cotesia vestalis bracovirus (CvBV). Diamondback moth larvae commonly reach very high densities under outbreak conditions. We thus examined whether starvation conditions increase aggression and cannibalism in *P. xylostella* larvae, and how parasitism by *C. vestalis* affects these responses. We report that parasitized *P. xylostella* larvae are more aggressive than nonparasitized larvae. We also identified a gene encoded by CvBV that increases host aggression by elevating the biosynthesis of octopamine.

Results

Parasitization Elevates Host Aggression Under Starvation. Aggressive behavior is common in many herbivorous insects even when food resources are not limiting (5, 27, 28). However, initial studies indicated *P. xylostella* larvae only show aggressive behavior when food resources are limiting, which was further associated with reduced survival due to cannibalism under starvation conditions (*SI Appendix*, Fig. S1 *A*, *B*). At a starting density of 100 larvae per assay arena, aggressive behavior and cannibalism caused survival to decline beginning 24 h after *P. xylostella* larvae were deprived of food, whereas larvae with unlimited food exhibited almost no aggressive behavior or reductions in survival (Fig. 1*A* and Movie S1).

To test whether aggressive behavior differed between unparasitized hosts and hosts parasitized by *C. vestalis*, we used a one-on-one aggression assay under starvation conditions that resulted in one larva cannibalizing the other (Fig. 1*B*). Strikingly, parasitized larvae exhibited higher attack (biting) frequencies than unparasitized larvae (Fig. 1*C*). We also measured the survival rate of four groups of parasitized hosts (6 h, 12 h, 24 h, and 48 h postparasitization) in aggression assays with unparasitized hosts. Results showed that parasitized larvae in all four groups had higher survival rates than unparasitized larvae (Fig. 1*D* and *SI Appendix*, Fig. S1*C*). In contrast, no differences in survival were detected when two unparasitized or two parasitized larvae competed against one another (Fig. 1*D*). We also compared the foraging activity of parasitized and unparasitized larvae under starvation conditions.

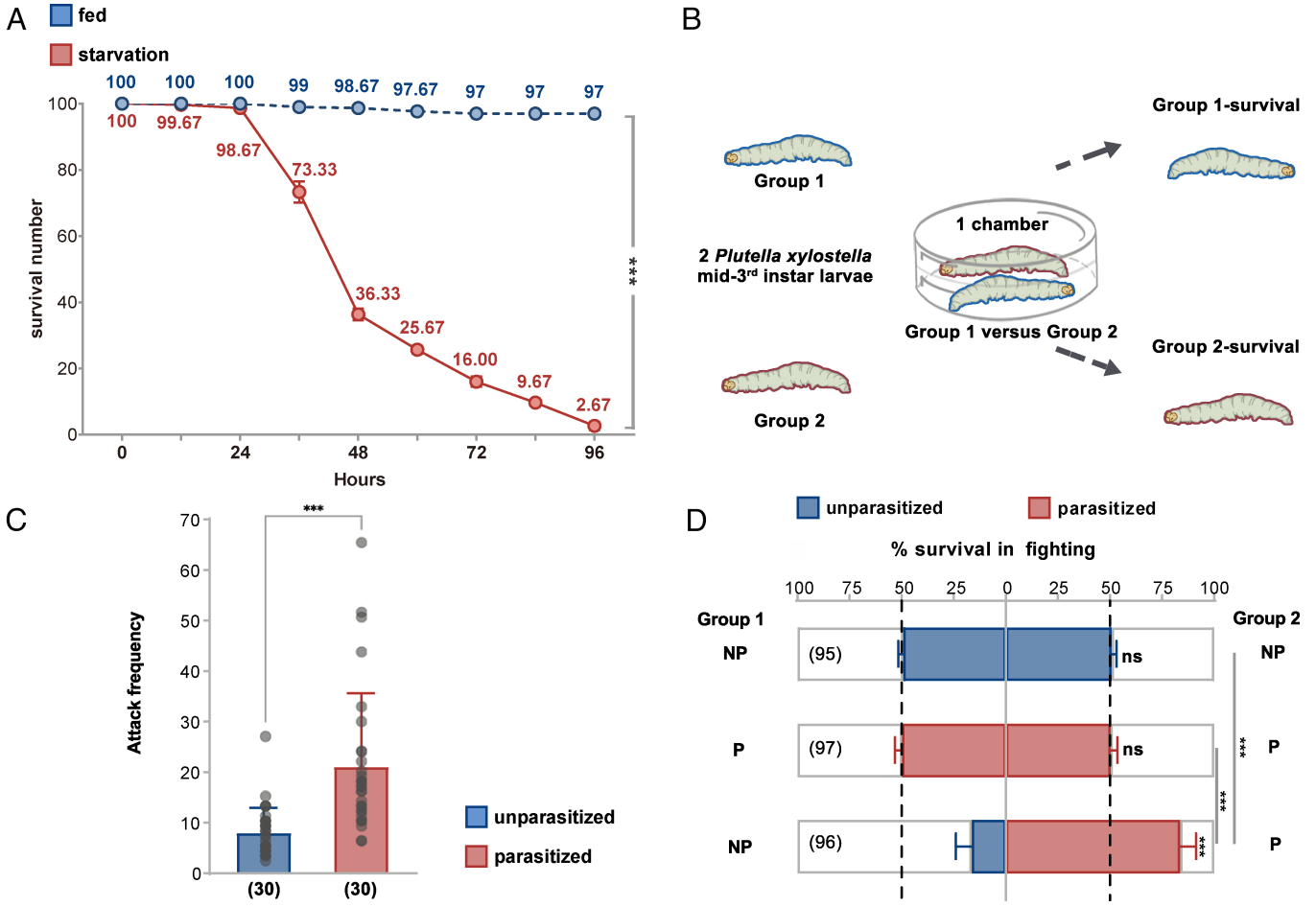

Fig. 1. *Cotesia vestalis* parasitization elevates host aggression and survival rate under starvation. (*A*) Survival rate of *P. xylostella* caterpillars in the presence of sufficient food or with food deprivation. Experiments were performed with three independent replicates. Data are presented as the mean values ± SD. Differences between groups were analyzed by the log-rank test (***$P < 0.001$). (*B*) Flow chart of the one-on-one aggressive behavior assay between *P. xylostella* caterpillars in two groups. (*C*) Attack frequency of *P. xylostella* caterpillars in a one-on-one aggressive behavior assay conducted between unparasitized and parasitized larvae in response to starvation. Differences between the two groups were analyzed by a two-sided unpaired Student's *t* test (***$P < 0.001$). (*D*) Survival of *P. xylostella* caterpillars in one-on-one aggression assays under starvation conditions between unparasitized and parasitized larvae. In the first two comparisons, individuals from the same treatment (either unparasitized or parasitized) were competed against each other as controls. In the third comparison, individual NP larvae were competed against individual P larvae. Each experiment contained 30 to 33 independent biological replicates, with three experimental replicates performed. Data are presented as the mean values ± SD. Differences between two groups in each comparison were analyzed by two-sided unpaired Student's *t* test, differences among three comparisons were analyzed by one-way ANOVA with Tukey's multiple comparisons test (***$P < 0.001$; ns: no significance).

This assay showed that parasitized *P. xylostella* larvae on average exhibited lower foraging activity levels than unparasitized larvae (*SI Appendix*, Fig. S1*D*). Thus, parasitism by *C. vestalis* was associated with *P. xylostella* larvae exhibiting higher attack frequencies and survival, but lower foraging activity under starvation conditions when compared to unparasitized larvae.

***P. xylostella* Larvae with Higher OA Levels Preferentially Survive in Aggressive Behavior Assays.** We next investigated why parasitism by *C. vestalis* increased the aggressive behavior of *P. xylostella* larvae. Since biogenic amines often stimulate aggressive behavior (29–37), we assessed whether biogenic amines mediate starvation-induced aggression in *P. xylostella* larvae. We analyzed the central nervous system (CNS) of parasitized and unparasitized host larvae by liquid chromatography and mass spectrometry (LC–MS) under fed and starved conditions to quantify three important biogenic amines: octopamine (OA), dopamine (DA), and serotonin (5–HT). Starvation increased the levels of two biogenic amines (e.g., DA and 5–HT) in the CNS (Fig. 2*A* and *SI Appendix*, Fig. S2 *A* and *B*) of unparasitized hosts. Contrastingly, depriving food from parasitized hosts induced a significant increase in OA (4.15-fold), no significant change in 5–HT, and a moderate increase in DA (1.92-fold) (Fig. 2*A* and *SI Appendix*, Fig. S2 *A* and *B*). To further investigate the effect of parasitism on OA content, we compared the CNS of parasitized and unparasitized hosts under fed and starved conditions by qRT–PCR. In fed hosts, parasitization modestly increased transcript abundance of tyramine beta-hydroxylase (*PxTβH*), which is a key enzyme in OA biosynthesis (36). However, transcript abundance for *PxTβH* was much higher in parasitized hosts when larvae were starved (Fig. 2*B*). These results suggested parasitization enhances synthesis of OA which was most increased when hosts were starved.

To test whether OA signaling affected starvation-induced host aggressive behavior postparasitization, we conducted two experiments. First, we examined whether injection of OA in PBS stimulated aggressive behavior under starvation in our one-on-one behavioral assay. Results showed that the survival rate of OA-injected larvae increased in comparison to control larvae injected with PBS alone (Fig. 2*C*). Next, we injected two antagonists of OA, phentolamine and mianserin (37), into larvae. Both decreased survival rates of parasitized hosts in aggression assays compared with PBS-injected controls (Fig. 2*D*). Taken together, these results suggested that upregulation of OA in the CNS enhances aggressive behavior and that OA levels and *PxTβH* expression are upregulated more in parasitized than unparasitized hosts when starved.

***Assailant* (*CvBV_19-7*) Triggers Starvation-Induced Host Aggression.** As earlier noted, parasitoids like *C. vestalis* inject an egg, which produces a larva and teratocytes, plus venom and CvBV virions into hosts at oviposition which all are potential sources of virulence factors. Venom and CvBV can be injected into third instar *P. xylostella* using a glass needle but too large a needle diameter is needed to inject *C. vestalis* eggs for larvae to survive due to their small size (4 to 7 mm in length, 1 to 2 mm width). We thus used an alternative approach called pseudoparasitism to assess whether wasp eggs versus venom or CvBV are sources of the factor that increases the aggression of parasitized hosts when

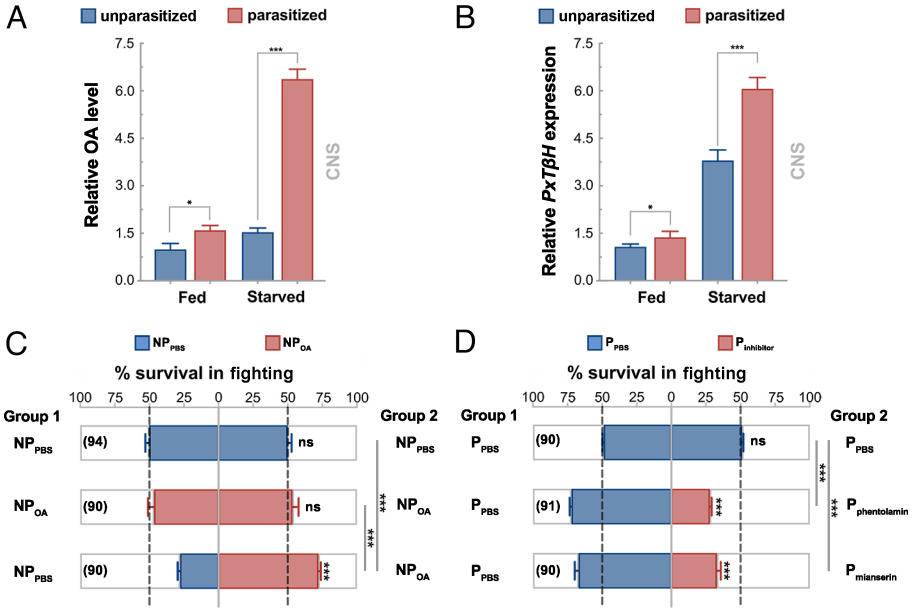

Fig. 2. Enhanced octopamine (OA) level contributes to starvation-induced host aggression postparasitization. (*A*) OA content in the CNS of unparasitized and parasitized caterpillars under fed and starvation conditions. The OA level in the CNS of fed, unparasitized caterpillars was used as the reference. Experiments were performed with three independent biological replicates. Data are presented as the mean values ± SD. Significance was determined by a two-sided unpaired Student's *t* test (***$P < 0.001$; *$P < 0.05$). (*B*) Transcript abundance of *PxTβH* under the same treatments as in (*A*). Experiments were performed with 3 independent biological replicates. Data are presented as the mean values ± SD. Significance was determined by a two-sided unpaired Student's *t* test (***$P < 0.001$; *$P < 0.05$). (*C*) Survival of unparasitized *P. xylostella* larvae in one-on-one aggression assays between OA treated (NP_{OA}) and controls (PBS-injected, NP_{PBS}) in response to starvation. In the first two comparisons, individuals from same treatment (either NP_{PBS} or NP_{OA}) were competed against each other as controls. In the third comparison, individual NP_{PBS} larvae were competed against individual NP_{OA} larvae.Each experiment contained 30 to 33 independent biological replicates, with three experimental replicates performed. Data are presented as mean values ± SD. Differences between two groups in each comparison were analyzed by a two-sided unpaired Student's *t* test, differences among three comparisons were analyzed by one-way ANOVA with Tukey's multiple comparisons test (***$P < 0.001$; ns: no significance). (*D*) Survival of parasitized *P. xylostella* larvae in one-on-one aggression assays conducted between phentolamine ($P_{phentolamine}$) or mianserin ($P_{mianserin}$)-treated larvae and controls injected with PBS (P_{PBS}) in response to starvation. In the first two comparisons, individuals from same treatment (either P_{PBS} or P_{OA}) were competed against each other as controls. In the third comparison, individual P_{PBS} larvae were competed against individual P_{OA} larvae. Each experiment contained 30 to 33 independent biological replicates, with three experimental replicates performed. Data are presented as the mean values ± SD. Differences between two groups in each comparison were analyzed by two-sided unpaired Student's *t* test, and differences among three comparisons were analyzed by one-way ANOVA with Tukey's multiple comparisons test (***$P < 0.001$; ns: no significance).

starved. In brief, earlier studies showed that braconid wasps harboring BVs can be gamma irradiated at a dose that prevents eggs from developing into larvae and producing teratocytes, but does not inhibit venom or BV activity which are commonly associated with altering host growth and/or immune defenses (38–43). The resulting females oviposit into hosts, which were referred to as pseudoparasitized, because they exhibited growth and immune alterations like parasitized hosts but do not contain a parasitoid larva or teratocytes because eggs do not hatch. We also earlier determined the same approach could be used with *C. vestalis* (44). We thus used gamma irradiated females in this study to produce pseudoparasitized *P. xylostella* larvae. We then paired pseudoparasitized hosts with unparasitized hosts in our aggressive behavior assay under starvation conditions. Results showed that pseudoparasitized larvae exhibited elevated aggression and higher survival rates when competing against unparasitized larvae under starvation conditions (*SI Appendix*, Fig. S3*A*). In contrast, no difference in survival was detected when two pseudoparasitized larvae competed against each other. This pattern was very similar to parasitized larvae consistently outcompeting and cannibalizing unparasitized larvae. In the absence of egg hatching in pseudoparasitized hosts, this finding also suggested increased aggression was due to venom and/or CvBV-produced factors rather than wasp larvae or teratocytes. To assess whether CvBV or venom alone increased host aggression, we injected a physiological dose of each into unparasitized host larvae separately. Aggression assays under starvation showed that aggression and survival of larvae injected with CvBV was significantly higher than for unparasitized larvae (Fig. 3*A*). In contrast, no differences in survival were detected when larvae injected with venom were competed against unparasitized larvae, or in control assays were two larvae injected with CvPV or venom were competed against one another (Fig. 3*A*).

We thus focused on CvBV as the source of the factor(s) that increases host aggressive behavior. To identify what this factor might be, we first conducted a transcriptome analysis to identify CvBV virulence genes that are potential candidates for promoting aggressive host behavior (Fig. 3*B*). Given the potential connection between increased host aggression and OA neuronal signaling, we also focused on CvBV genes that were expressed in the CNS of hosts, and that were significantly upregulated in starved larvae when compared to another host cell population, hemocytes, that is also infected by CvBV (Fig. 3*C* and *SI Appendix*, Fig. S3*B* and Tables S1 and S2). This approach led us to focus on three virlulence genes, *CvBV_19-3*, *CvBV_19-5,* and *CvBV_19-7*, that were among the top 10 most upregulated genes in the CNS of parasitized larvae when starved (Fig. 3*C*). Each of these genes belonged to a multigene family present in CvBV and BVs from other *Cotesia* wasp species, but shared no significant homology with other genes in public databases (*SI Appendix*, Fig. S4). We next conducted RNA interference (RNAi) experiments to determine whether each could be knocked down by injection of gene-specific siRNAs into *P. xylostella* larvae that were first injected with CvBV. Quantitative real-time PCR (qRT–PCR) assays showed that transcript abundance of each targeted gene was significantly reduced when compared to hosts injected with a nonspecific siRNA without off-target effects on other members of the same gene family (*SI Appendix*, Fig. S3*C*). We then conducted one-on-one aggression assays between hosts in which *CvBV_19-3, -5,* or *-7* was knocked down versus control hosts injected with a nonspecific siRNA. Despite the similarity between the three genes, only knockdown of *CvBV_19-7* lowered decreased aggression and survival of larvae when compared to the control (Fig. 3*D*). These findings thus suggested expression of *CvBV_19-7* increases the aggressive behavior of parasitized larvae. We thus renamed *CvBV_19-7 "assailant"* as an inducer of aggressive behavior in diamondback moth larvae parasitized by *C. vestalis*. Comparison of *CvBV_19-7* to other genes in public databases indicated their presence in other closely related wasp species but not in other microgastrine braconids that also harbor BVs (*SI Appendix*, Fig. S4). This finding indicated genes closely related to *assailant* are present in BVs other *Cotesia* species inject into hosts, which suggests they potentially also have roles in modifying aggression or other behaviors.

Assailant Is Necessary for OA Induction in Parasitized Hosts Under Starvation. To determine whether *assailant* triggers OA biosynthesis under starvation, we analyzed CNS extracts by qRT–PCR and LC–MS analysis after RNAi knockdown of *CvBV_19-3*, *CvBV_19-5,* or *CvBV_19-7* in parasitized larvae. Expression of *PxTβH* was significantly reduced by knockdown of *assailant* but not *CvBV_19-3* and *CvBV_19-5* (Fig. 4*A*). Accordingly, only knockdown of *assailant* reduced OA content in parasitized host caterpillars (Fig. 4*B*). We next used Assailant as a bait protein to screen its interactors with *P. xylostella* proteins using a yeast cDNA library and a yeast-two hybrid (Y2H) assay. After sequencing the positive clones, we identified 46 candidate host proteins that interacted with Assailant (*SI Appendix*, Table. S3). However, one-on-one yeast experiments found only a TATA-binding protein associated factor 4 (TAF4) homolog of *P. xylostella* (PxTAF4) that interacted with Assailant (Fig. 4*C*). TAF4 is a component of the TFIID complex that recognizes promoters and regulates gene-specific expression. It also plays a key role in initiating gene transcription, maintaining the stability of the complex, and modulating responses to specific cellular signals (45). The interaction between PxTAF4 and Assailant (Fig. 4*C*) suggested the latter could play a role as a transcription factor. Antibodies generated against Assailant and PxTβH detected both proteins in a subset of OA neurons in the CNS of *P. xylostella* larvae (Fig. 4*D*), which was also consistent with Assailant increasing PxTβH transcription (Fig. 4*E*).

We further assessed the effects of Assailant on OA biosynthesis by using the GAL4/UAS binary expression system to ectopically express this protein in *D. melanogaster* (*SI Appendix*, Fig. S5*A*). *Assailant* expression was driven in specific OA neurons (*Tdc2-GAL4*) as verified by PCR, immunoblotting, and immunostaining (*SI Appendix*, Figs. S5 *B–D*). We then determined by qRT–PCR and LC–MS analysis that *DmTβH* gene expression and relative OA levels were significantly increased in fly brains expressing Assailant compared to controls (*SI Appendix*, Fig. S5 *E* and *F*). These findings further supported the conclusion that Assailant stimulates *TβH* gene expression and is functional in this heterologous model. We noted that ectopically expressed Assailant did not influence the body size of adult flies compared with control individuals (Fig. 4*G*) as measured by body weight, wing size, hind leg length, thorax length, and abdomen length, which otherwise could impact outcomes of aggressive behavior. *Drosophila* male flies are well known to fight and occupy food resources (29). We thus used a previously developed aggression assay and fight chamber (46) that measured occupancy rates by males overexpressing *assailant* versus controls (*SI Appendix*, Fig. S5*H*). Results showed that flies expressing *assailant* exhibited a significantly higher occupancy rate and won the majority of fights for food resources with control individuals (*SI Appendix*, Fig. S5*I* and Movie S3). Lunging is another important feature of aggressive behavior in fighting between male flies (46). Ectopically expressed *assailant* in OA neurons significantly increased the duration of lunges compared with control individuals (*SI Appendix*, Fig. S5*J*). Taken together,

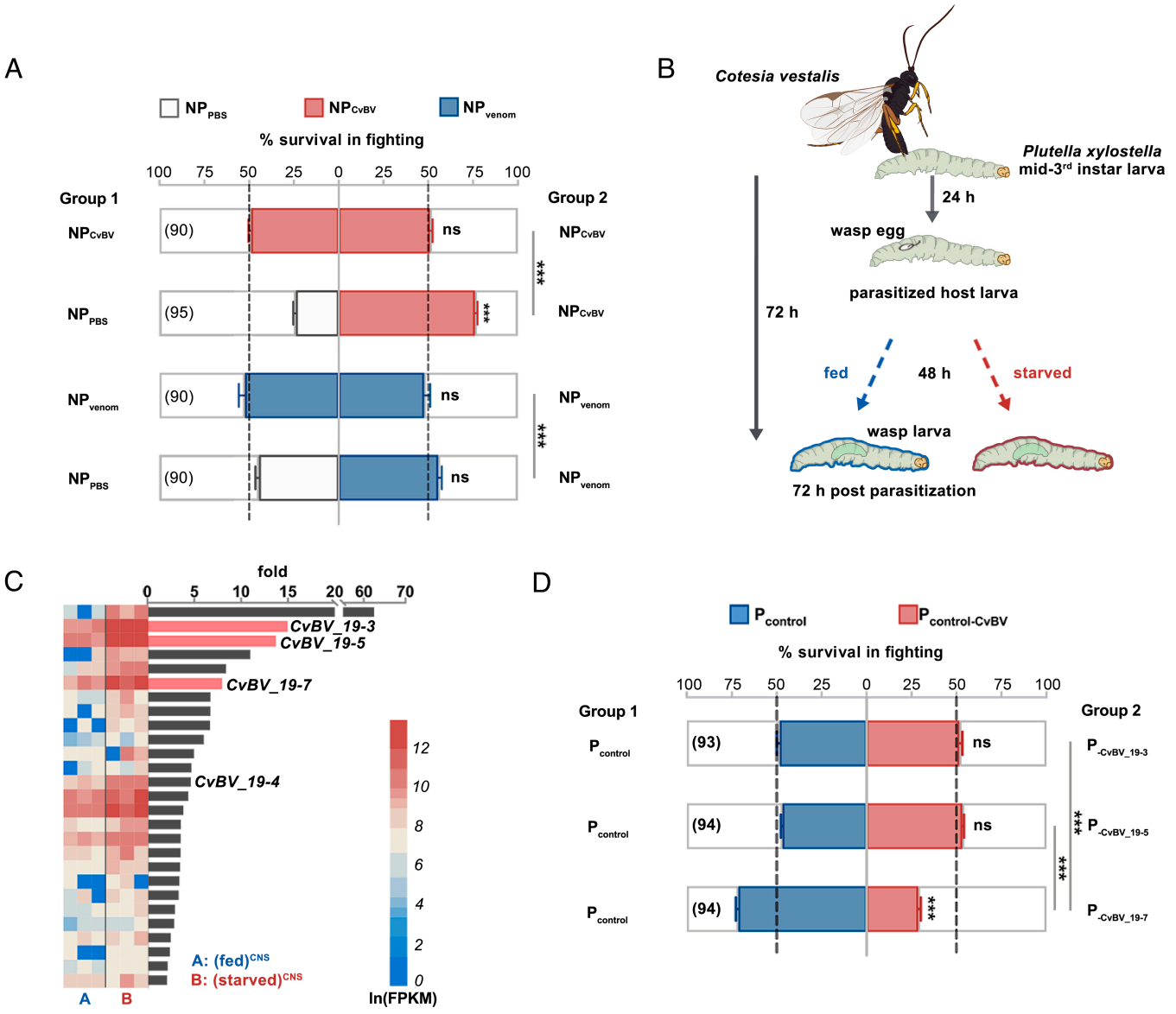

Fig. 3. *CvBV_19-7* promotes starvation-induced host aggression. (*A*) Survival of *P. xylostella* larvae in one-on-one aggression behavior assays between unparasitized larvae injected with CvBV (NP_{BV}), venom (NP_{venom}), or PBS (NP_{PBS}) in response to starvation. In the first and third comparisons, individuals from same treatment (either NP_{BV} or NP_{venom}) were competed against each other as controls. In the second comparison, individual NP_{venom} larvae were competed against individual NP_{PBS} larvae while in the fourth comparison individual NP_{venom} larvae were competed against individual NP_{PBS} larvae. Each experiment contained 30 to32 independent biological replicates, with three experimental replicates performed. Data are presented as the mean values ± SD. Differences between two groups in each comparison and differences between two comparisons were analyzed by two-sided unpaired Student's *t* test (***$P < 0.001$; ns: no significance). (*B*) Flow chart for how *C. vestalis*-parasitized caterpillars were prepared for CNS transcriptome analysis. (*C*) Transcription levels and fold changes for CvBV genes in the CNS under fed and starvation conditions in three biological replicates. Red bars highlight the transcriptional fold change between starved and fed conditions for three CvBV genes that were further studied. Black bars indicate other CvBV genes that were upregulated in the CNS under starved conditions. (*D*) Survival of parasitized caterpillars in one-on-one aggression assays under starvation conditions where each of the red-highlighted genes in C were knocked down by injection of specific siRNAs ($P_{control}$-CvBV) and compared to control larvae injected with a nonspecific siRNA ($P_{control}$). Experiments were performed with 30 to 32 independent biological replicates. Data are presented as the mean values ± SD. Differences between two groups in each comparison were analyzed by two-sided unpaired Student's *t* test, and differences among three comparisons were analyzed by one-way ANOVA with Tukey's multiple comparisons test (***$P < 0.001$; ns: no significance).

expression of *assailant* in OA neurons enhanced OA levels in the brain and increased aggression in adult male *Drosophila*.

Discussion

Cannibalism can be elicited by density-dependent factors such as overcrowding and limitations in food quantity or quality as well as by density-independent factors such as injury or infection (5, 27, 28). Examples of density-dependent factors affecting cannibalism in insects include studies of migratory locusts like *Locusta migratoria* where crowded conditions increase cannibalism (11). Scarce food resources and starvation also trigger cannibalism in the beetle *Leptinotarsa decemlineata* (47). Examples of density-independent factors affecting insects include studies from *Drosophila* where parasitoid-induced injury can promote cannibalism (48). Infection by the bacterium *Pseudomonas aeruginosa* has also been shown to increase cannibilism in the mealworm *Zophobas morio* (49).

Many parasitoid wasps parasitize and develop in the bodies of larval-stage insects. Hosts parasitized by larval endoparasitoids usually must also continue to feed in order for wasp offspring to pupate and emerge as adults (16). Successful development of wasp offspring thereafter is almost always followed by the host dying, while adult female parasitoids usually mate and disperse to

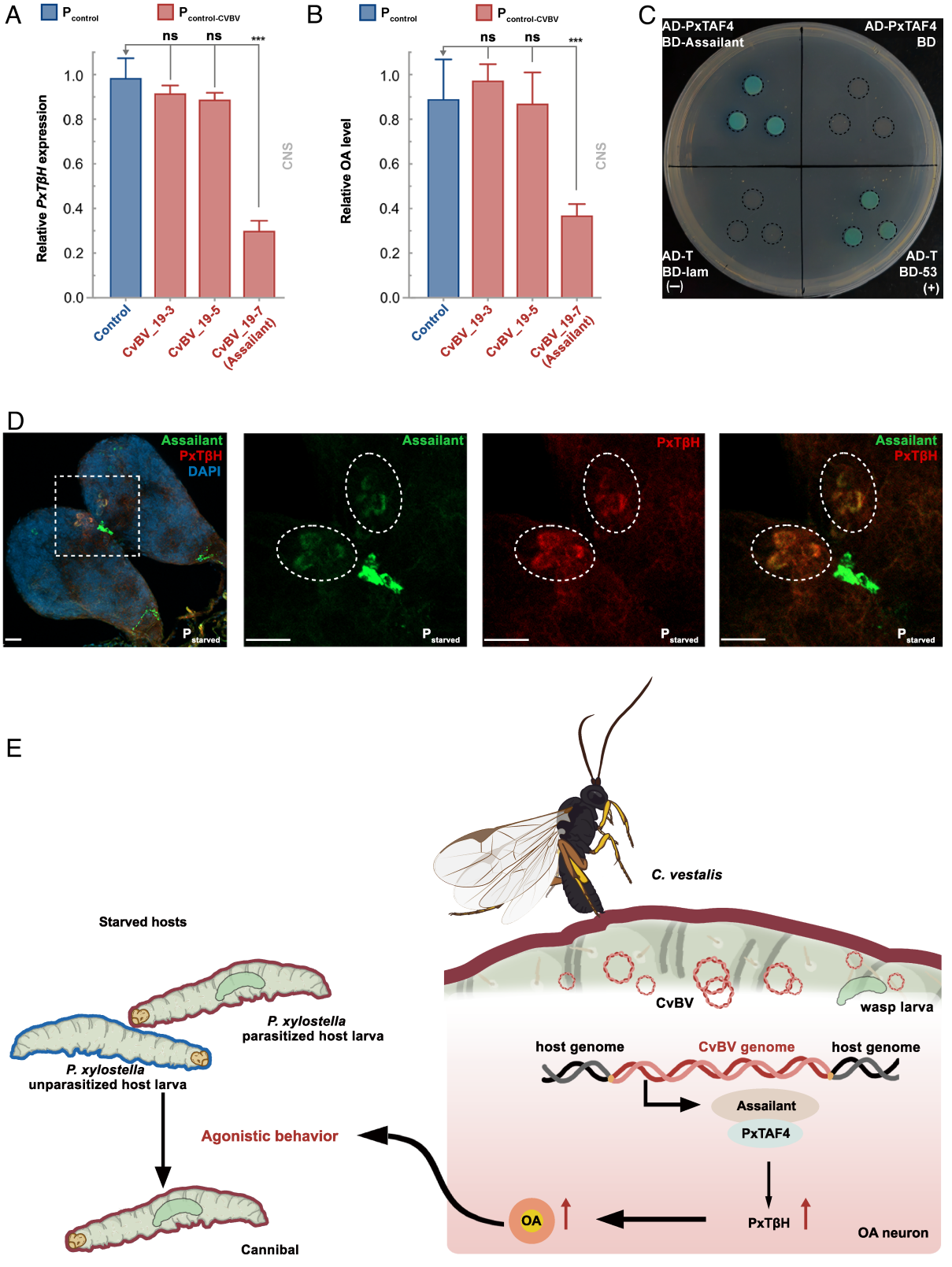

Fig. 4. CvBV_19-7 (*assailant*) expression in octopaminergic neurons increases OA in parasitized *P. xylostella* larvae under starvation. (*A*) PxTβH transcript abundance in the CNS of *C. vestalis*-parasitized larvae (control) versus parasitized larvae in which *CvBV_19-3*, *CvBV_19-5*, and *CvBV_19-7* were knocked down by RNAi. Experiments were performed with three independent biological replicates per treatment. Data are presented as mean values ± SD. Differences between groups were analyzed by one-way ANOVA with Tukey's multiple comparisons test (***$P < 0.001$; ns: no significance). (*B*) OA content in the CNS of parasitized larvae for the same treatments as in A. Experiments were performed with three independent biological replicates. Data are presented as the mean values ± SD. Differences between groups were analyzed by one-way ANOVA with Tukey's multiple comparisons test (***$P < 0.001$; ns: no significance). (*C*) Interaction between Assailant and PxTβH by Y2H analysis. The bait vector pGBKT7-Assailant (BD-Assailant) and the prey vector pGADT7-PxTFIID (AD-PxTAF4) were cotransformed into Y2H Gold yeast cultured on SD/-Trp-Leu-his-Ade yeast medium with X-α-Gal and Aba toxin (−QDO/X/A). Cotransformation of the blank vector pGBKT7 and the prey vector pGADT7-PxTAF4 was used as the experimental control, pGBKT7-53 and pGADT7-T as the positive control (+), and pGBKT7-lam and pGADT7-T as the negative control (−), respectively. (*D*) Colocalization of Assailant (green) and PxTβH (red) in the CNS of parasitized larvae under starvation. Nuclei stained with DAPI (blue). (Scale bar, 20 μm.) (*E*) Proposed model illustrating how parasitism by *C. vestalis* increases aggression in starved *P. xylostella* larvae.

parasitize new hosts (16). Aggression between hosts and cannibalism can thus adversely affect the survival of wasp offspring and the abundance of adult parasitoids. As agricultural pests that feed on a range of cruciferous crops, *P. xylostella* commonly experiences crowded feeding conditions during outbreak conditions including densities exceeding more than 1,000 larvae per plant, which result in complete defoliation and starvation conditions (50–52). Results reported in this study likewise indicate density-dependent starvation increases aggression and cannibalism in *P. xylostella* larvae. A few studies report examples of parasitized hosts being eaten by unparasitized conspecifics, which has also been suggested to reduce the impacts of parasitism in the next generation (53–55). However, results reported in this study present a different perspective in that *P. xylostella* larvae parasitized by *C. vestalis* become more aggressive than unparasitized hosts, which increases the survival of *C. vestalis* offspring under starvation conditions. We thus suggest *C. vestalis* increases the aggression of parasitized hosts as an adaptation to promoting offspring survival under stress-associated conditions that occur with resource limitation.

Prior studies identify several BV genes with functions in suppressing host immune defenses, which otherwise kill wasp eggs and larvae (56–58). Some studies also identify BV genes with roles in altering host growth and molting (summarized in 21). BVs, other endogenous viruses, and selected other viruses that infect parasitoids are also known to affect other processes including plant responses to herbivorous hosts, host recognition by hyperparasitoids, and changes in host behavior (54, 59–64). In contrast, the viral genes and molecular mechanisms underlying these latter alterations are unknown. Results from this study thus advance understanding of parasitism-associated shifts in host behavior by identifying a CvBV gene (*assailant*) that is highly expressed in the CNS of parasitized *P. xylostella* larvae and presenting experimental evidence this gene product elevates aggression in parasitized hosts by further increasing OA. Our results additionally indicate Assailant and PxTAF4 proteins interact, which likely underlies increased transcription of *PxTβh* and higher amounts of OA in the CNS. While some host alterations after parasitism by BV-producing wasps involve interactions between BVs and other factors like venom and teratocytes (16, 21, 59, 60), other alterations have clearly been shown to be due to particular BV genes that are transcribed in hosts (21, 56, 58). Results reported in this study likewise identify *assailant* as the primary factor that increases aggression of parasitized larvae under starvation conditions when compared to unparasitized larvae.

Finally, we extend our results beyond *P. xylostella* by showing that heterologous expression of Assailant in *D. melanogaster* also increases OA in the CNS, which increases aggression in adult males in the context of competing for mating sites. This finding lends evidence that Assailant increases aggression in another insect without any other factor *C. vestalis* females naturally introduce when parasitizing *P. xylostella.* That Assailant increases aggression in adult male flies is also interesting in the context of an earlier study showing that a bacterium, *Wolbachia,* reduces aggression in male flies by negatively influencing the production of OA (65). Our finding that other parasitoid species in the genus *Cotesia* harbor BVs that also encode *assailant*-like genes suggest this family of virulence factors also potentially affects aggression or other behaviors in additional host species. As earlier noted, prior studies well-document that *P. xylostella* populations commonly increase to outbreak levels in agroecosystems, which is also associated with larvae feeding at high densities that defoliate host plants. That *C. vestalis* increases the aggression of parasitized hosts clearly suggests increased survival of parasitized hosts under conditions of starvation benefits wasp offspring, which in turn could also potentially promote increased conservation of these natural enemies as biological control agents. However, we also recognize reported results were conducted under controlled laboratory conditions that were essential to identifying *assailant*. Thus, a key need going forward is complementary field studies to assess whether parasitized hosts likewise outcompete unparasitized hosts under starvation or outbreak conditions and whether *C. vestalis* populations benefit from Assailant activity.

Materials and Methods

Insects. *C. vestalis* and *P. xylostella* were reared at 25 °C with a relative humidity of 65% under a 14:10 light: dark cycle. *P. xylostella* larvae were fed an artificial diet from Keyun Bio Company. Adult *P. xylostella* and *C. vestalis* were fed a 20% honey/water (V/V) solution. *Drosophila melanogaster* was reared at 25 °C with a relative humidity of 50% under a 16:8 light: dark cycle. All strains used in this study were maintained on standard cornmeal/molasses/agar medium at 25 °C in 6-ounce square-bottom plastic fly bottles. To obtain *CvBV_19-7* transgenic flies, the full length of *CvBV_19-7* was first cloned into the pUAST-attb vector. The transgenic *Drosophila* line carrying the *UAS-19-7* gene was obtained by phiC31 integrase-mediated insertion into the attP2 landing-site locus on the 2nd chromosome (66).

Aggression and Foraging Activity Assays. Blue and red dyes were applied to the body surface of unparasitized and parasitized mid-third instar *P. xylostella* of uniform body size. Two individuals were placed into each recording chamber [10 mm (Diameter) × 4 mm (Height)] and separated by a plastic membrane in the first 6 h. After the membrane was removed, two starved individuals met and recording was started using a Sony FDR-AX100 camera (Sony). Aggressive encounters between two first-encounter individuals were recorded by manually quantifying the frequency of aggressive attacks under standard light conditions over a 1 h period. Attacks were defined as previously described (67). Survival rate was calculated for each assay. Experiments were performed with at least 30 independent biological replicates. For foraging activity assays, starved unparasitized and parasitized caterpillars were transferred equidistantly to opposite ends of a 35 mm diameter arena with fresh artificial diets placed in the center. The time it took for each caterpillar to find the food was recorded using a Sony FDR-AX100 camera (Sony).

Pseudoparasitism Assays. *C. vestalis* adult females (2 d old) were gamma irradiated at a dose of 100 grays (Gy) using previously established methods (44). Hosts were pseudoparasitized by placing mid-third instar *P. xylostella* larvae in a 10 mm (diameter) × 80 mm (height) glass vial with individual irradiated *C. vestalis* female wasps that were allowed to oviposit. We dissected a subset of hosts 36 to 48 h postoviposition by 30 gamma irradiated females, which confirmed that no eggs laid by these individuals hatched. We also maintained another cohort of pseudoparasitized hosts, which showed reduced growth and an inability to pupate as normally occurs when hosts are parasitized by nonirradiated females or when hosts are injected with CvBV (68), which supported that virions from irradiated females retained functionality through the ability to infect and express virulence gene products.

CvBV and Venom Microinjection. To collect CvBV virions and venom from female wasps, ovaries and venom glands from mated two-day-old *C. vestalis* female wasps were dissected in cold PBS. Ovary calyces were punctured, which released CvBV virions into the saline, which was passed through a 0.22 μm filter to remove cellular debris and prevent contamination. After centrifuging at 20,000×*g* for 1 h, the supernatant was discarded and the CvBV particles were resuspended in PBS. Venom gland reservoirs were punctured individually in PBS. Cellular debris from venom glands was removed by centrifuging at 10,000×*g* for 5 min. Virions and venom collected from one single female wasp are defined as one female equivalent (FE). We injected 0.1 FEs of CvBV virions or venom into mid-third instar *P. xylostella* larvae, while larvae microinjected with PBS of the same volume served as control (69, 70).

Transcriptome Analysis. The CNS (brain and ventral nerve cord) of parasitized early-fourth instar *P. xylostella* were collected in TRIzol reagent at 72 h postparasitization under normal fed condition and following a 48 h-starvation condition with three biological replicates, respectively. RNA extraction, cDNA library

construction, and paired-end RNA-seq (Illumina) were carried out by Annoroad Gene Technology Co., Ltd. Transcriptome sequencing data statistics are listed in *SI Appendix*, Table S2. CvBV-related reads were taken from each Illumina library by mapping reads to the CvBV genome (71). The CvBV genome index was built using Bowtie (v2.1.0) (72), and paired-end clean reads were aligned to the CvBV genome using TopHat (v2.1.1) (73). Cuffdiff (v2.2.1) (74) was used to calculate fragments per kilobase of exon per million fragments mapped (FPKMs) for the coding genes in each sample, and the FPKM was calculated based on the length of the fragments and the read count mapped to each fragment. In comparison of the total average FPKM of all CvBV genes in one tissue with three biological replicates, the average FPKMs of each CvBV gene were used to determine whether it was highly expressed in this tissue when hosts were fed (*SI Appendix*, Table S1). The transcriptional change multiple of each CvBV gene in CNS 48 h poststarvation was from the calculated average FPKMs. CvBV gene with more than a twofold increase in FPKM was defined as a starvation-induced CvBV gene in the *P. xylostella* CNS. The heatmap plot of starvation-induced CvBV genes in CNS was generated using the pheatmap R package (v1.0.12) with FPKMs standardized by natural logarithm (75).

CvBV Gene Cloning. Total RNA was isolated from homogenized parasitized and unparasitized mid-third instar *P. xylostella* using TRIzol and reverse transcribed into cDNA using the PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara). The entire coding region of CvBV genes of interest was cloned and sequenced using cDNA from parasitized *P. xylostella* as template and gene-specific primers, while host genes were amplified using cDNA of unparasitized *P. xylostella* and gene-specific primers. Primer sequences are listed in *SI Appendix*, Table S4.

CvBV Gene Knockdown. To knock down CvBV genes by RNAi, a specific 25-bp RNA oligos were designed based on the sequence of CvBV genes and synthesized by Sangon Biotech. These sequences are listed in *SI Appendix*, Table S4. The miRCURY LNA miRNA mimic (siNC, EXIQON 479903-001) was used as a negative control. A total of 5 pmol of siRNA was injected into each mid-third instar *P. xylostella* larva using an Eppendorf FemtoJet 4i Microinjector with the following parameters: injection pressure = 900 hPa and injection time = 0.15 s. *P. xylostella* larvae were then parasitized 6 h postinjection and subsequently reared for 24 h under fed conditions followed by rearing for 48 h under starvation condition. Knockdown of candidate CvBV genes in CNS of *P. xylostella* was assessed by measuring relative transcript abundance in treatment and control larvae by qRT–PCR. We then used treatment and control larvae in one-on-one aggression assays.

Quantitative Real-Time PCR (qRT–PCR). Total RNA was extracted and reverse transcribed into cDNA using the ReverTra Ace qPCR RT kit (Toyobo) according to the manufacturer's protocol. qRT–PCRs were performed in a CFX Connect real-time system (Bio-Rad) with THUNDERBIRD qPCR Mix (Toyobo). Reactions were carried out for 60 s at 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. The *Px-β-Actin* gene (GenBank accession number: NM_001309101) and *Px-β-Tubulin* gene (GenBank accession number: EU127912) were used as internal controls, and the relative concentrations were determined using the $2^{-\Delta\Delta Ct}$ method. All the primers used in qRT–PCR assays are listed in *SI Appendix*, Table S4.

Phylogenetic Analysis. BLASTP was performed against the NCBI NR database to search for potential homologs of CvBV_19-7. A phylogenetic tree was constructed using default parameters by IQTree v2.2.0 (76) with 1,000 bootstrap replicates and embellished in Figuretree v1.4.4.

Biogenic Amine Measurements. Amine levels in the CNS (brain and ventral nerve cord) of early-fourth instar *P. xylostella* after different treatments were estimated by mass spectrometry. In brief, the CNS from 30 larvae for each treatment and replicate were homogenized in 1 × PBS and then mixed with an equal volume of acetonitrile. The mixture was centrifuged at 10,000×*g* for 10 min at 4 °C the supernatant was collected and immediately frozen at −80 °C. Samples were thawed immediately before analysis. An Agilen 6460 triple quadrupole mass spectrometer (Agilent Technologies) equipped with an electrospray ionization (ESI) source was used to quantify OA, DA, and 5-HT concertation under the positive ion multiple-reaction monitoring (MRM) mode. Agilent Mass Hunter Workstation was used for data acquisition and processing. Nitrogen was used as the sheath gas and drying gas. The nebulizer pressure was set to 45 psi, and the flow rate of drying gas was 5 L/min. The flow rate and temperature of the sheath gas were 11 L/min and 350 °C, respectively. Chromatographic separation was carried out on a Zorbax SB C18 column (150 × 2.1 mm, 3.5 μm). Mass spectrometric detection was completed by use of an ESI source in positive ion MRM mode.

Microinjection of OA and Antagonists. We prepared a 100 nM octopamine hydrochloride (Sigma-Aldrich) solution in 1 × PBS which was used to inject 0.1 μL into early-fourth instar unparasitized *P. xylostella* larvae. Unparasitized host larvae injected with the same volume of 1x PBS served as the control. This dose corresponded to earlier estimates of OA in lepidopteran hemolymph after starvation or parasitism by a parasitoid in the genus *Cotesia* (77, 78). We prepared stock solutions (1 mM) of two OA antagonists, phentolamine and mianserin (Sigma-Aldrich), in DMSO that were stored in the dark at −20 °C. We then diluted the phentolamine stock to 1 μM and the mianserin stock to 10 nM in 1 × PBS. A 0.1 μL volume of each antagonist was then injected into unparasitized *P. xylostella* larvae followed by parasitization 2 h postinjection. These amounts were used because prior results determined a 10 times higher dose of phentolamine and a10 times lower dose of mianserin inhibited the effects of OA when injected into lepidopteran larvae (78, 79). An Eppendorf FemtoJet 4i microinjector was used to inject larvae with the following parameters: injection pressure = 900 hPa and injection time = 0.15 s. To test the effect of octopaminergic signaling inhibition on starvation-induced aggression and cannibilism, unparasitized host larvae microinjected with OA or PBS only (control) were bioassayed 3 h postinjection. To test the effects of each antagonist on starvation-induced aggression and cannibalism in parasitized hosts, mid-third instars were parasitized followed 24 h later by injection of each antagonist or DMSO diluted in PBS (control) followed by bioassay 3 h postinjection.

Y2H Assays. The Yeastmaker™ transformation system (Clontech) was used to screen the *P. xylostella* proteins interacting with CvBV_19-7 according to the manufacturer's instructions. To examine the interaction among CvBV _19-7 and PxTAF4, the full-length ORF of *CvBV_19-7* was inserted into bait vector pGBKT7 to generate pGBKT7-*CvBV_19-7*, and the full-length ORF of PxTAF4 was inserted into prey vector pGADT7 to generate pGADT7- PxTAF4. The bait and prey plasmids were cotransformed to the Y2H Gold yeast strain. The pGBKT7-53 and pGADT7-T interaction served as a positive control, and the pGBKT7-lam and pGADT7-T served as a negative control. Transformants were subsequently cultured on SD/-Trp-Leu-his-Ade with X-α-Gal and Aba toxin (QDO/X/A) yeast medium. Primers used in Y2H assays are listed in *SI Appendix*, Table S4.

Immunohistochemistry. The CNS was dissected from *P. xylostella* larvae in 1 × PBS as described above and then fixed in 4% paraformaldehyde at 4 °C overnight. Fixed CNSs were washed in 1 × PBST (PBS containing 0.1% Triton X-100 and 0.05% Tween 20) 3 times for 5 min at room temperature and then blocked in 1% BSA in 1 × PBST for 2 h at room temperature. Primary antibodies diluted in 1 × PBST were applied at 4 °C for 20 to 24 h. The samples were washed in 1 × PBST 3 times for 5 min at room temperature and subsequently incubated in secondary antibodies diluted in 1 × PBST for 2 h at room temperature. The samples were washed again in 1 × PBST 3 times for 10 min at room temperature and DAPI was added for 20 min at room temperature. After washing in 1 × PBST, the samples were mounted in ProLong Gold Antifade Mountant with DAPI (Invitrogen) for confocal imaging (Zeiss LSM800). An antibody against CvBV_19-7 was generated in rabbits while an antibody against PxTβH was generated in mice by AtaGenix Laboratories. Antibodies and dyes were used at the following dilutions: rabbit anti-CvBV_19-7 (1:200, AtaGenix, generated against the YSGQYGTDNQQATL+Cys peptide), mouse anti-PxTβH (1:200, AtaGenix, generated against PxTβH protein without the signal peptide), Alexa Fluor 488 goat anti-rabbit (1:1,000, Life Technologies), Alexa Fluor 594 goat anti-mouse (1:1,000, Life Technologies) and DAPI (2 μg/mL, Roche).

Immunoblotting. Total protein from approximately 30 brains of transgenic male *D. melanogaster* was extracted by Minute™ Total Protein Extraction Kit for Insects (Invent) for western blot according to the manufacturer's protocol. Samples were diluted in 5 × Protein Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis Loading Buffer (Sangon) and then boiled for 10 min. Proteins were separated in a denaturing polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After blocking and washing, membranes were then incubated with primary antibodies against CvBV_19-7 (1:500, AtaGenix) or primary antibodies

against actin (1:2,500, CWBIO) for 2 h at room temperature. Secondary antibody horseradish peroxidase–conjugated goat anti-rabbit IgG (1:5,000, ABclonal) and secondary antibody horseradish peroxidase–conjugated goat anti-mouse IgG (1:5,000, ABclonal) were used to incubate the membrane with primary antibodies against CvBV_19-7 and actin, respectively, in Tris-buffered saline with 0.05% Tween-20 for 2 h at room temperature. After five washes, membranes were then incubated with the enhanced chemiluminescence western blotting substrate for imaging (Promega).

Insect Size Measurement. Newly emerged male flies were collected on the first day posteclosion and size measurement was carried out on the fifth day. Body weight, area of right wing, lengths of the abdomen, thorax, and right leg were performed as the five indicators of adult fly size (80). The total body weight of 10 individuals was measured and the average was recorded. For measurement of adult appendage sizes, fixed adult flies in 95% ethanol were rinsed and dissected in 1 × PBS and then mounted in a drop of 5% glycerol in PBS on a glass slide. Images were captured with an Olympus SZX16 microscope and processed in FIJI2.

Adult Male Fly Aggression Assays. Newly emerged male flies were collected on the first day of eclosion and kept in single vials. One-on-one fighting assays were carried out on 5-d-old male flies at the standard rearing condition. For the food occupation measurement between male transgenic flies, the fighting chambers were carefully settled as follows: standard food or 1.2 % agarose was melted and placed into the lid of a 1.5-mL Eppendorf tube (radius: 4 mm), then the lid was put into a chamber of a well plate with a radius of 6 mm and surrounded by 1.2% agarose. The height from the surface of the food patch to the upper edge of the chamber was measured at 2 mm. On the day of the assay, 10 μl of 75% apple juice (v/v) with 20% sucrose (w/v) and 20% yeast (w/v) was added onto the surface of the food patch, and two male flies were placed into the prepared fighting chamber with light CO_2 anesthetization and allowed to stay for 30 min before the assay was conducted. A cover glass slip was used to close the fighting chamber for a 10-min recording (Canon). Male flies overexpressing *CvBV_19-7* were identified with the dark red color of eyes from the controls with orange color. The winner tended to push the other one away from the food patch and succeeded in food monopolization. The winning rate of food occupancy was defined as the proportion of winners with different treatments in total chambers (29). Duration rate of lunges in one-on-one fighting assays between male flies were measured as follows: 1.2% agarose with 50% apple juice (v/v), 2.5% sucrose (w/v) and 2.5% yeast (w/v) was added into a chamber of a well plate as described above. Two male flies were placed into the prepared fighting chamber with light CO_2 anesthetization and allowed to stay for 30 min before the assay was conducted. A cover glass slip was used to close the fighting chamber for a 10-min recording (Canon). Male flies overexpressing *CvBV_19-7* were identified from controls by eye color. A lunge bout was defined as raising the front legs and upper body of the behaving fly, subsequently slamming down onto another fly (46). Recorded videos were tracked with Caltech FlyTracker and corrected manually to determine the moving trails of two individuals in each fighting chamber (81). Resulting tracking data were analyzed using behavior classifiers trained from the Janelia Automatic Animal Behavior Annotator (JAABA) to determine the lunge bout (82). The classifier performance is listed in *SI Appendix*, Table S5.

Statistical Analysis. Statistical analyses were performed with GraphPad Prism (v9.0). Data are expressed as the means ± SD. Log-rank tests were used to analyze the survival rate change of *P. xylostella* larvae in different rearing densities or under starvation conditions. For comparison of the aggressive competitiveness of *P. xylostella* larvae or aggression of male flies in different treatments, we performed one-way ANOVAs with Tukey's multiple comparisons test. The two-tailed nonpaired Student's *t* test was conducted for other experiments. Significant values are indicated as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Data, Materials, and Software Availability. RNA-Seq data have been deposited in NCBI Sequence Read Archive under PRJNA799668 (58) and PRJNA818058 (83). All study data are included in the article and/or supporting information.

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