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4-Vinylanisole promotes conspecific interaction and acquisition of gregarious behavior in the migratory locust

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Chemical signals from conspecifics are essential in insect group formation and maintenance. Migratory locusts use the aggregation pheromone 4-vinylanisole (4VA), specifically released by gregarious locusts, to attract and recruit conspecific individuals, leading to the formation of large-scale swarms. However, how 4VA contributes to the transition from solitary phase to gregarious phase remains unclear. We investigated the occurrence of locust behavioral phase changes in the presence and absence of 4VA perception. The findings indicated that solitary locusts require crowding for 48 and 72 h to adopt partial and analogous gregarious behavior. However, exposure to increased concentrations of 4VA enabled solitary locusts to display behavioral changes within 24 h of crowding. Crowded solitary locusts with RNAi knockdown of *Or35*, the specific olfactory receptor for 4VA, failed to exhibit gregarious behaviors. Conversely, the knockdown of *Or35* in gregarious locusts resulted in the appearance of solitary behavior. Additionally, a multi-individual behavioral assay system was developed to evaluate the interactions among locust individuals, and four behavioral parameters representing the inclination and conduct of social interactions were positively correlated with the process of crowding. Our data indicated that exposure to 4VA accelerated the behavioral transition from solitary phase to gregarious phase by enhancing the propensity toward proximity and body contact among conspecific individuals. These results highlight the crucial roles of 4VA in the behavioral phase transition of locusts. Furthermore, this study offers valuable insights into the mechanisms of behavioral plasticity that promote the formation of locust swarms and suggests the potential for 4VA application in locust control.

Locusta migratoria | olfactory perception | phase change | behavior | social interaction

Significance

Aggregation pheromones play crucial roles in locust swarm and plague formation. Although previous study demonstrated that 4-vinylanisole (4VA) attracts and recruits individuals, the mechanism by which 4VA triggers the behavioral change that occurs in the transition from the solitary phase to the gregarious phase remains unknown. Here, we demonstrate that 4VA exposure accelerates the acquisition of gregarious-like behaviors in solitary locusts during crowding by enhancing intraspecific social interactions. The absence of 4VA perception results in the loss of gregarious-like behaviors in crowded solitary locusts. Notably 4VA exposure enhanced the propensity toward proximity and body contact among locust conspecifics. Our findings reveal the 4VA-mediated behavioral plasticity in the establishment of gregarious behavior and offer important insights for behavioral intervention in pest management.

Living in groups is a widespread phenomenon among many insect species, such as cockroaches, crickets, locusts, termites, bees, ants, and certain flies. The interactions among conspecific individuals and their social environment are of great importance for their survival and reproduction (1). While a substantial number of studies have been conducted on social interactions between individuals in eusocial insects (2, 3), many other insects exhibit aggregative and collective behaviors without a real social hierarchy (4). Therefore, a good understanding of the factors driving the behavioral changes involved in interactions among conspecifics is crucial for comprehending group formation and maintenance in nonsocial insects.

Locusts have been considered one of the most destructive pests throughout human history because they can form large-scale swarms that cause immense damage to agricultural crops (5). As nonsocial insects, locusts display phase polyphenism or phase change, marked by the presence of gregarious and solitary phases in response to population density, with distinct morphological, physiological, and behavioral characteristics (5–9). While changes in morphological and physiological traits require a few days to several stadia to take place, behavioral changes can occur in just a few hours or a few days depending on the locust species (10–12). In the migratory locust (*Locusta migratoria*), behavioral change in nymphs from the gregarious phase to the solitary phase can rapidly occur; in contrast, the change from solitary to gregarious behavior is slow (12). Collective behaviors such as bands of nymphs marching and swarms of adults migrating are the most striking characteristics of gregarious locusts (13–15). The components of gregarious behavior, including the tendency to aggregate and high levels of activity, are essential in collective behaviors (9). Conversely, solitary locusts display low activity levels and avoidance of other individuals (10–12, 16). The switch from the mutual avoidance of solitary locusts to conspecific attraction is the initial step in the behavioral phase transition of locusts (9, 12). However, the mechanism by which solitary locusts acquire the propensity toward gregarious or collective behavior is unknown.

Aggregation pheromones are crucial intraspecific signals used in various contexts, including in the resting aggregations of cockroaches (17–19), the collective feeding of lepidopteran larvae (20, 21), and the mass attacks on host trees by bark beetles (22). The aggregation

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pheromone 4-vinylanisole (4VA) and its olfactory receptor have been identified in the migratory locust. The aggregation pheromone can attract and recruit both gregarious and solitary locusts, despite its emission from only gregarious locusts (23). The crowding of 30 solitary locusts for 24 h can initiate the release of 4VA (23), but during this period, the changes in behavior do not meet the typical standards of gregarious behaviors (12). Moreover, 4VA exposure plays key roles in inducing female sexual maturation synchrony in gregarious locusts (24). However, the mechanism by which 4VA affects the behavioral trait transition from the solitary phase to the gregarious phase remains to be elucidated.

In this study, we investigated the changes in behavioral traits of gregarious and solitary locusts in the presence and absence of 4VA. Behavioral assays were employed in a multi-individual system to assess the social interactions among individuals. Activation and suppression of 4VA perception significantly changed locust phase-related behavioral traits by altering individual interactions. These results demonstrate that the introduction of 4VA not only accelerates behavioral changes from the solitary phase to the gregarious phase but also plays a crucial role in the formation and maintenance of gregarious groups.

Results

Effectiveness of Crowding at Inducing Transition to Gregarious Behavior. We initially tested the behavioral status of fifth-instar solitary locusts in a well-established behavioral assay arena after subjecting them to crowding treatment for 24 h, 48 h, and 72 h. The degree of behavioral change was accurately evaluated using variables such as the probabilistic metric of gregariousness (P-*greg)* value, which assesses the transition toward typical gregarious behavior (Fig. 1 *A* and *B*). Solitary locusts displayed significant behavioral changes after 72 h of crowding treatment compared with the control solitary locusts, and 48 h of crowding treatment caused partial behavioral changes (median P-*greg* = 0.44 for 48 h; median P-*greg* = 0.61 for 72 h). However, compared to typical solitary behavior, applying the crowding treatment for 24 h did not lead to significant behavioral changes (median P-*greg* = 0.27) (Fig. 1 *C* and *D*). In addition, we analyzed three other behavioral variables contributing mostly to P-*greg*: total distance moved (TDM), total duration of movement (TDMV), and attraction index (AI). TDM and TDMV significantly increased following crowding treatment for 48 h and 72 h; however, they did not change after 24 h of crowding treatment (Fig. 1 *E* and *F*). Moreover, crowding treatment of the solitary locusts for 48 h and 72 h markedly changed the AI from repulsion to attraction (Fig. 1*G* and *SI Appendix*, Table S1). Therefore, solitary locusts must undergo a long crowding treatment period (48 h and 72 h) to exhibit gregarious behavior.

4VA Accelerates the Transition to Gregarious Behavior in Solitary Locusts. To investigate the role of 4VA in behavioral changes, we assayed the behaviors of solitary locusts exposed to 4VA during crowding treatment (Fig. 2*A*). Sustained 4VA exposure without the crowding treatment did not induce statistically significant behavioral changes in solitary locusts compared to the control group (Fig. 2*B*). Sustained 4VA exposure with 24 h and 48 h of crowding significantly promoted behavioral changes toward the gregarious phase in the solitary locusts (Fig. 2 *C* and *D*). In noncrowding groups, 4VA exposure did not induce significant changes in TDM, TDMV, and AI (Fig. 2 *E*–*G*). 4VA exposure significantly changed AI but did not affect TDM and TDMV with crowding treatment for 24 h (Fig. 2 *H*–*J*). After crowding treatment for 48 h, TDM, TDMV, and AI all showed significant increases in the 4VA exposure groups compared with the control groups (Fig. 2 *K*–*M* and *SI Appendix*, Table S1). Thus, 4VA promotes the speed of solitary locust behavioral change toward the gregarious phase during crowding.

4VA Perception Is Necessary for the Initiation and Maintenance of Gregarious Behavior. To block the perception of 4VA, we applied RNAi knockdown of *Or35* in both solitary and gregarious locusts. After the ds*Or35* injection, the expression level of *Or35* significantly decreased compared with that in locusts injected with ds*GFP* (*SI Appendix*, Fig. S1 *A* and *B*). The electrophysiological responses of the basiconic sensilla, the specific response sensilla for 4VA (23), were significantly inhibited in both solitary and gregarious locusts after ds*Or35* injection (Fig. 3*A*). Compared to the control groups injected with ds*GFP*, the solitary locusts injected with ds*Or35* showed significantly reduced responses from the 4VA concentration of 0.5 ng/μL (Fig. 3*B*). Compared to the groups injected with ds*GFP*, the gregarious locusts injected with ds*Or35* showed significantly decreased responses to 4VA from 5 ng/μL (Fig. 3*C*). Furthermore, we found that solitary locusts injected with ds*Or35* did not display any attraction to 4VA (Fig. 3 *D* and *E*). Similarly, gregarious locusts showed the same behavioral results as solitary locusts after ds*Or35* injection (Fig. 3*F*). Thus, knockdown of *Or35* can effectively impede the perception of 4VA in solitary and gregarious locusts.

To further verify the impact of 4VA on the establishment of gregarious behavior, we performed RNAi knockdown of *Or35* in solitary locusts accompanied by crowding treatment (Fig. 3*G*). In the ds*GFP*-injected group, solitary locusts showed significant behavioral changes after crowding treatment for 72 h, and the median value of P-*greg* was 0.83, with 55% of locusts falling into the P-*greg* values ranging from 0.8 to 1.0, indicating typical gregarious behavior. However, the ds*Or35*-injected solitary locusts failed to exhibit gregarious behavior after 72 h of crowding treatment, without any locust falling into the P-*greg* values from 0.8 to 1.0, and the median value of P-*greg* decreased significantly to 0.18 (Fig. 3*H*). All three behavioral variables showed a significant decrease in the ds*Or35*-injected group compared with the ds*GFP*-injected group (Fig. 3*I*). Thus, the perception of 4VA mediates the development of gregarious behavior in solitary locusts by inducing attraction and increasing motility.

To verify the function of 4VA in the behavior of gregarious locusts, we examined the behavioral traits of gregarious locusts after knockdown of *Or35* by RNAi (Fig. 3*J*). In the ds*GFP*-injected gregarious locusts, the median value of P-*greg* was 0.83, with 46% of individuals falling into the values of P-*greg* within 0.8 to 1.0 (Fig. 3*K*). The gregarious locusts showed significant behavioral changes after injection of ds*Or35*, with the median P-*greg* of the ds*Or35*-injected group remarkably shifting to 0.46 (Fig. 3*K*). RNAi knockdown of *Or35* in gregarious locusts significantly reduced their TDM and TDMV and induced a significant decline in AI along with a shift from approaching to avoiding the stimulus locust group (Fig. 3*L* and *SI Appendix*, Table S1). Therefore, gregarious locusts cannot maintain gregarious behavior if they lose the ability to perceive 4VA, indicating the important roles of 4VA in the maintenance of gregarious behaviors.

4VA Accelerates Behavioral Transition by Increasing the Propensity for Body Contact. To investigate the mechanism underlying the 4VA acceleration of the locust behavioral transition from solitary to gregarious propensities, we developed a previously unreported behavioral paradigm for evaluating the process of crowding among multiple individuals from both spatial and temporal perspectives, as shown in Fig. 4 *A* and *B*. Five parameters representing social interactions, frequency of proximity (FP),

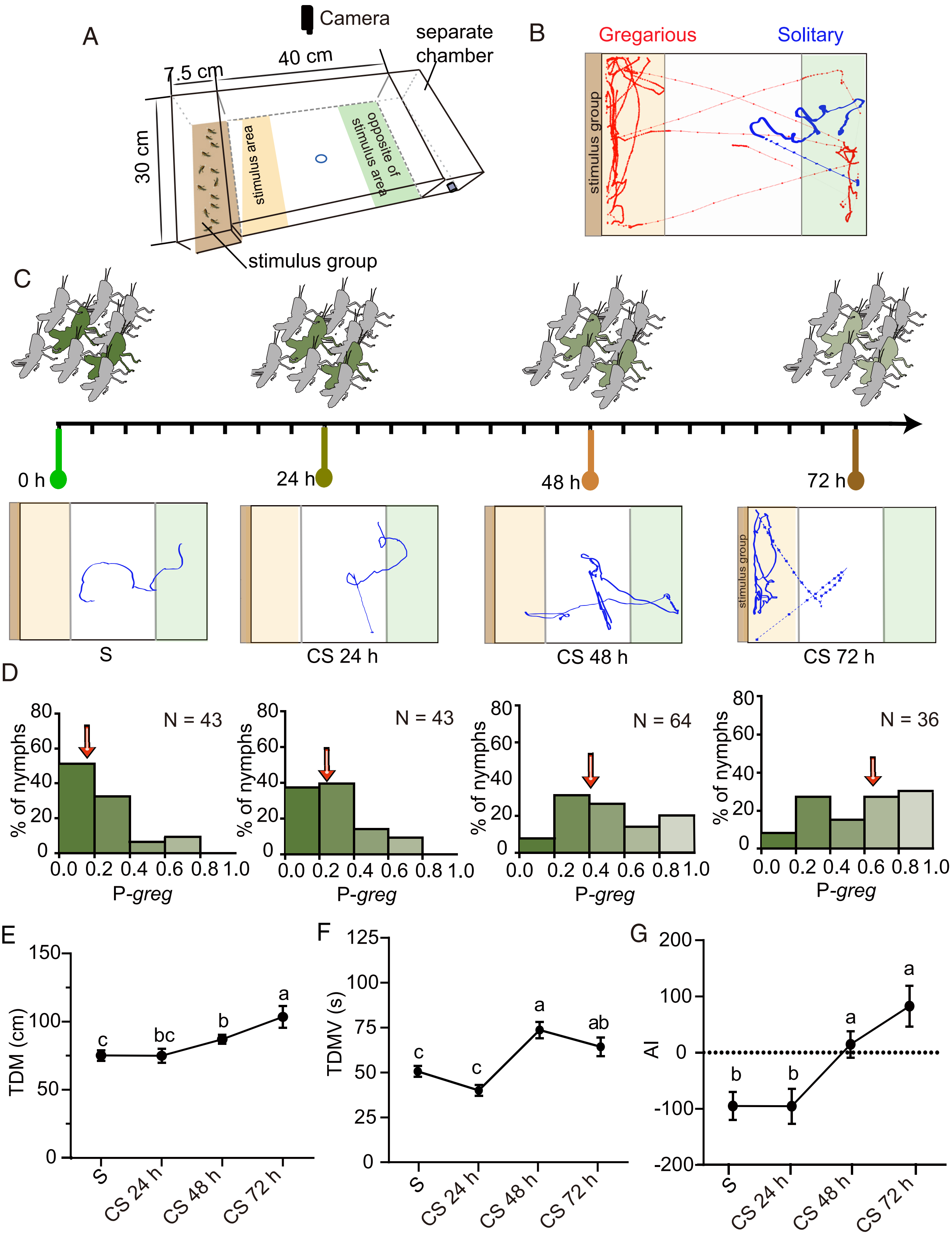

Fig. 1. Time course of behavioral changes in solitary locusts during crowding. (*A*) Schematic diagram of the behavioral assay. Rectangular arena with drawer for stimulus group under a camera that tracked the motion of the locusts. (*B*) Track visualization of gregarious locusts (red) and solitary locusts (blue). (*C*) Track visualization of solitary locusts during crowding treatments. (*D*) Behavioral phase states of solitary locusts after crowding for 24 h, 48 h, and 72 h. (*E*–*G*) Changes in total distance moved (TDM, *E*), total duration of movement (TDMV, *F*), and attraction index (AI, *G*) during the CS process. P-*greg*, probabilistic metric of gregariousness. Arrows indicate median P-*greg* values. Comparisons of median P-*greg* and AI were analyzed by the Mann–Whitney *U* test. Comparisons of TDM and TDMV were analyzed by one-way ANOVA (Tukey's multiple comparisons test), and different letters indicate statistically significant differences between groups. Data in *E*–*G* are presented as the mean ± SEM. CS, crowding of solitary locusts.

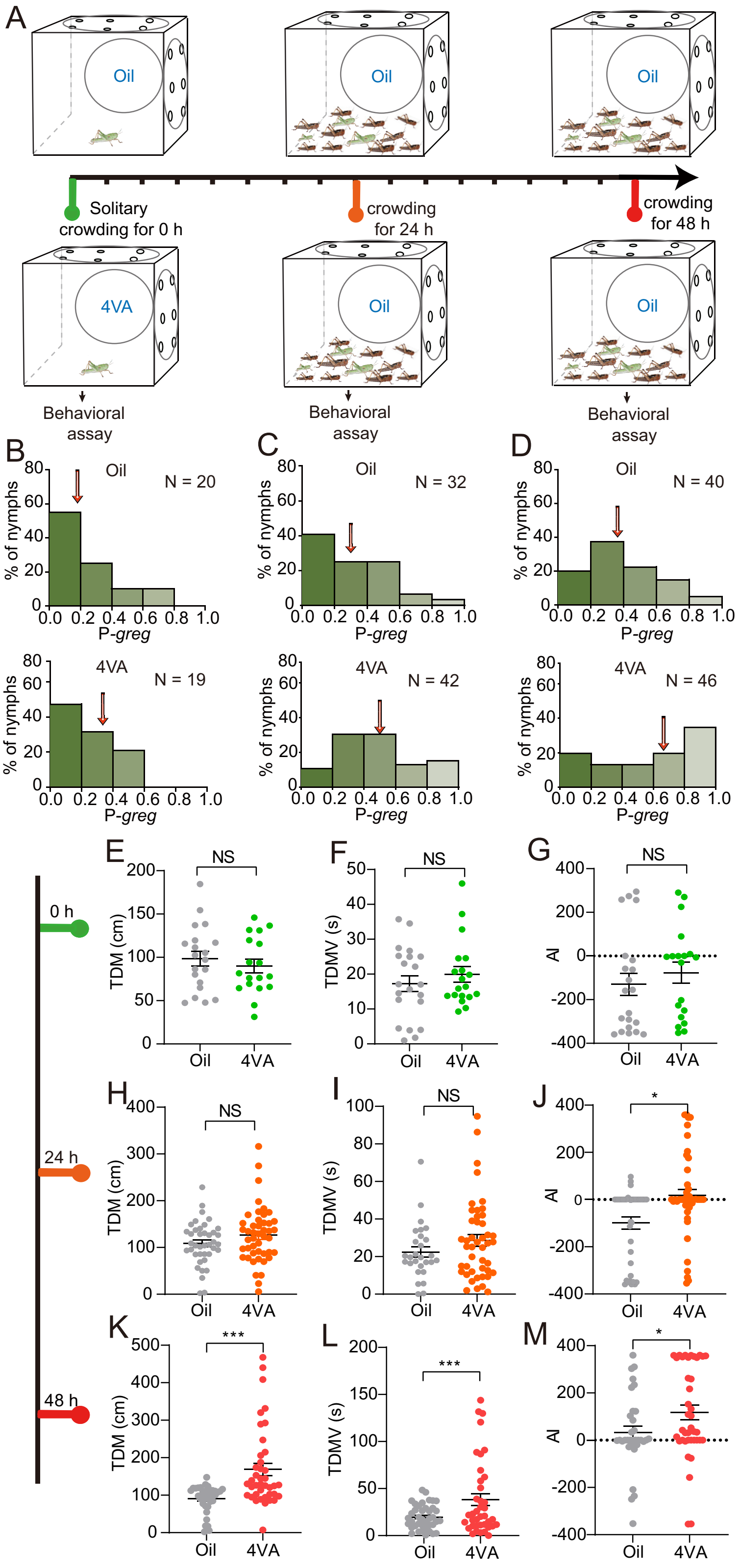

Fig. 2. 4VA accelerates behavioral changes in solitary locusts during crowding. (*A*) Schematic diagram of odor exposure treatment with crowding treatment for 0 h, 24 h, and 48 h of solitary nymphs. (*B*) Behavioral responses of solitary nymphs after sustained 4VA exposure or mineral oil. (*C*) Behavioral responses of solitary nymphs treated by exposure to 4VA or mineral oil with crowding for 24 h. (*D*) Behavioral responses of solitary nymphs treated by exposure to 4VA or mineral oil with crowding for 48 h. (*E*–*G*) Changes in TDM (*E*), TDMV (*F*), and AI (*G*) of solitary locusts sustained 4VA exposure or mineral oil. (*H*–*J*) Changes in the TDM (*H*), TDMV (*I*), and AI (*J*) of solitary locusts with sustained 4VA exposure and crowding for 24 h. (*K*–*M*) Changes in the TDM (*K*), TDMV (*L*), and AI (*M*) of solitary locusts with sustained 4VA exposure and crowding for 48 h. P-*greg*, probabilistic metric of gregariousness. Arrows indicate median P-*greg* values. Comparisons of AI were analyzed by the Mann–Whitney *U* test. Comparisons of TDM and TDMV were analyzed by Student's *t* test. TDM: total distance moved; TDMV: total duration of movement; AI: attraction index. NS, not significant; $^{*}P < 0.05$; $^{***}P < 0.001$.

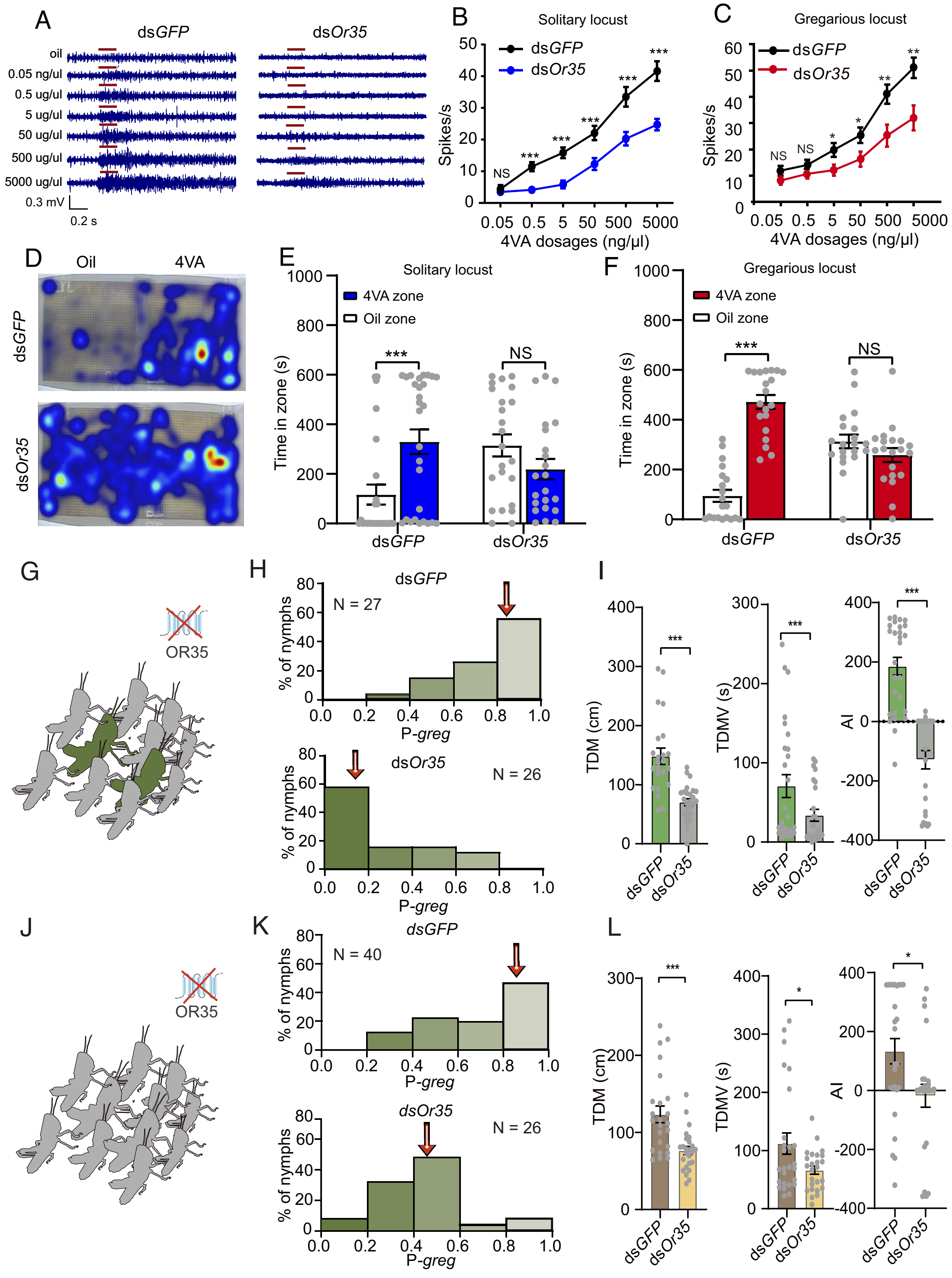

Fig. 3. Locusts cannot obtain and maintain gregarious behavior without the ability to perceive 4VA. (*A*) Representative spike traces of basiconic sensilla of locusts after injection of ds*GFP* and ds*Or35*. (*B*) The responses of basiconic sensilla to 4VA at different concentrations in solitary locusts after RNAi of *Or35* (n = 15 for the ds*GFP* group, n = 17 for the ds*Or35* group). (*C*) The responses of basiconic sensilla to 4VA at different concentrations in gregarious locusts after *Or35* RNAi knockdown (n = 50 for the ds*GFP* group, n = 40 for the ds*Or35* group). (*D*) Behavioral tracing of locusts injected with ds*GFP* and ds*Or35*. (*E*) The olfactory responses of solitary locusts to 4VA with *Or35* knockdown (n = 29 for the ds*GFP* group, n = 27 for the ds*Or35* group). (*F*) The olfactory response of gregarious locusts to 4VA injected with ds*Or35* (n = 20 for the ds*GFP* group, n = 20 for the ds*Or35* group). (*G*) Experimental processing schematic of solitary locusts. (*H*) Behavioral phase state of crowded solitary locusts after RNAi knockdown of *GFP* and *Or35*. (*I*) Changes in TDM, TDMV, and AI in crowded solitary locusts after *Or35* RNAi knockdown. (*J*) Experimental processing schematic of gregarious locust. (*K*) Behavioral phase state of gregarious locusts after RNAi knockdown of *GFP* and *Or35*. (*L*) Changes in TDM, TDMV, and AI in gregarious locusts after injection of ds*Or35*. *P* values in the SSR assay were determined by two-tailed unpaired *t* test. Behavioral data are the mean ± SEM. *P* values in behavioral assays (*E* and *F*) were determined by the Wilcoxon signed-rank test. P-*greg*, probabilistic metric of gregariousness. Arrows indicate median P-*greg* values. Comparisons of AI were analyzed by the Mann–Whitney *U* test. Comparisons of TDM and TDMV were analyzed by Student's *t* test. NS, not significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001. TDM, total distance moved, TDMV: total duration of movement; AI, attraction index.

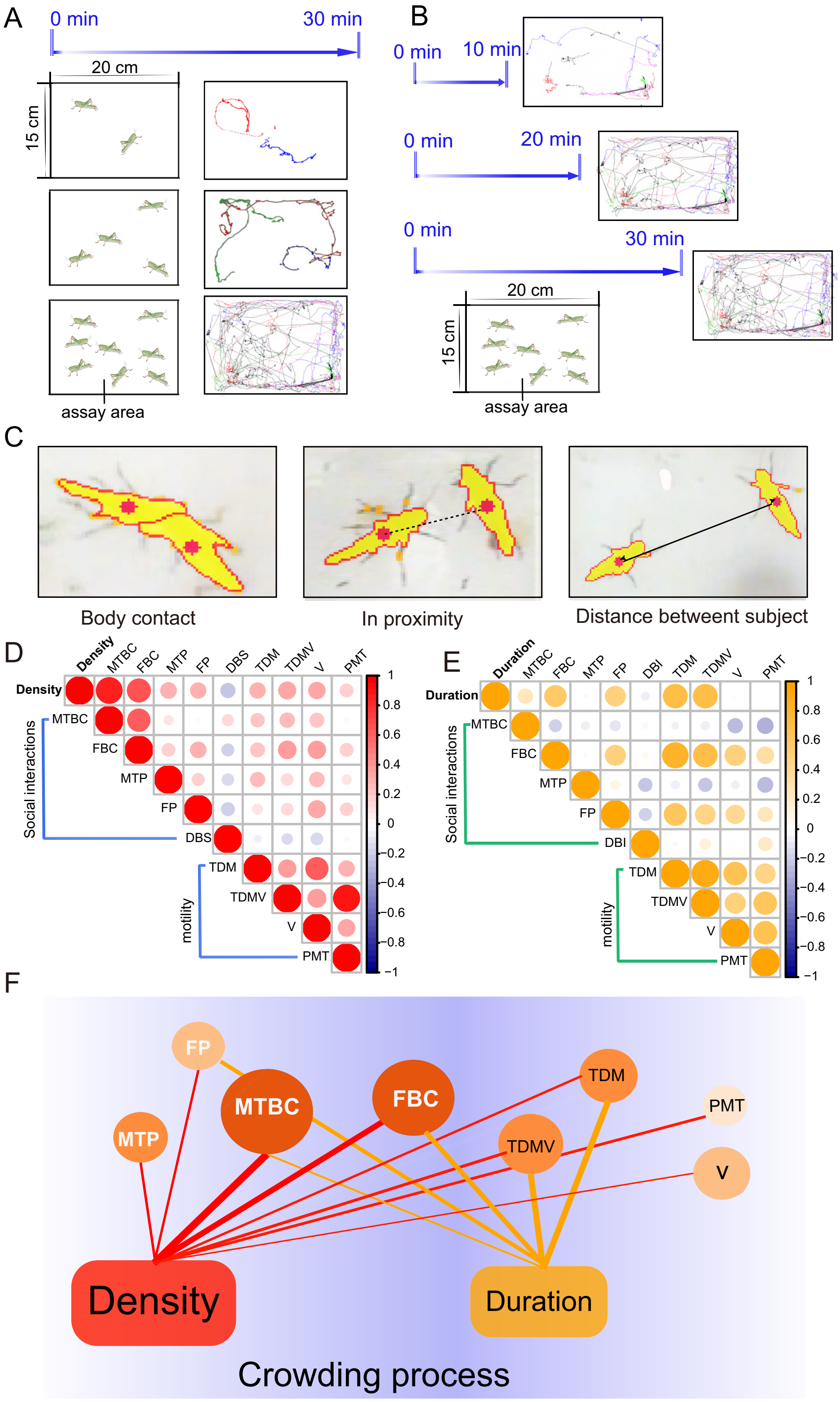

Fig. 4. Social interactions mainly contribute to the process of crowding. (*A*) Schematic diagram of behavioral assays of two, four, and eight solitary locusts for 30 min. (*B*) Schematic diagram of behavioral assays of eight solitary locusts for different durations. (*C*) Diagram of social interaction parameters, body contact, proximity, and distance between subjects. The proximity threshold is less than a body length (4 cm). (*D*) Correlation matrix of density and behavioral parameters. (*E*) Correlation matrix of duration and behavioral parameters. (*F*) Network diagram of behavioral parameter interactions in the aggregation process. TDM, total distance moved, TDMV: total duration of movement; FP: frequency of proximity, MTP: mean time of proximity, FBC: frequency of body contact, MTBC: mean time of body contact, DBS: distance between subjects, PMT: proportion of moving time, V: velocity.

mean time of proximity (MTP), frequency of body contact (FBC), mean time of body contact (MTBC), and the distance between the subjects (DBS) (Fig. 4*C* and *SI Appendix*, Fig. S2), and four parameters related to motility, TDM, TDMV, velocity (V), proportion of moving time (PMT) were collected. We first evaluated the correlation between density and the nine parameters and found that eight parameters were significantly correlated with density. The results indicated that FBC and MTBC exhibited the highest correlation with density, and MTP, FP, TDMV, TDM, V, and PMT also showed different degrees of correlations with density (Fig. 4*D*). On the other hand, five parameters, including TDMV, TDM, FBC, FP, and MTBC, showed a gradual increase over time and a positive correlation with duration (Fig. 4*E* and *SI Appendix*, Fig. S2 *C* and *D*). However, DBS was independent of changes in density and duration (Fig. 4 *D* and *E*). The parameter network diagram illustrates the relevance between eight behavioral parameters and the crowding process (Fig. 4*F*). The diagram of the parameter network highlights the contribution of behavioral parameters related to social interaction to the process of crowding.

We next evaluated the interactions among multiple individuals after 4VA exposure treatment using this paradigm. After two individuals were introduced into the arena exposed to 4VA (Fig. 5*A*), the FBC and the MTBC increased significantly compared with those of the control group that was not exposed to 4VA. The frequency and MTP between individuals also significantly increased compared with those of the control group (Fig. 5*B*). When four individuals were introduced into the arena (Fig. 5*C*), the four variables of social interactions, i.e., the proximity and body contact parameters, were also markedly increased in the 4VA exposure group (Fig. 5*D*). Moreover, when eight individuals were introduced into the same arena, the FBC of the 4VA-exposed locusts was observably higher than that of control locusts, while the MTBC was slightly reduced after 4VA exposure. The proximity parameters showed the same change trend as the body contact parameter (Fig. 5 *G* and *H*). In addition, there were significant increases in the four locomotion indexes. The TDM, V, TDMV, and PMT were all observed to increase in the 4VA exposure groups (*SI Appendix*, Fig. S3). Thus, 4VA induces a higher frequency of locusts to approach and make contact with each other and facilitates their behavioral changes to those typical of gregarious traits.

To further validate the necessity of 4VA in promoting social interaction, we conducted behavioral assays after knocking down *Or35* in solitary locusts and exposing them to 4VA. After introducing two ds*Or35*-injected individuals into the arena sprayed with 4VA, statistically significant decreases in the FBC and MTBC between individuals were observed compared to the ds*GFP* group (Fig. 5 *G* and *H*). The frequency and MTP also decreased significantly after injection of ds*Or35* (Fig. 5*I*). When four ds*Or35*-injected individuals were introduced into the arena, the FBC and MTBC were significantly higher than those of ds*GFP*-injected locusts, and the FP and the MTP also markedly decreased compared with those of the ds*GFP* group (Fig. 5 *I* and *J*). Moreover, the FBC, MTBC, FP, and MTP when there were eight locusts in the arena also significantly declined in comparison to those of the ds*GFP*-injected group (Fig. 5 *K* and *L* and *SI Appendix*, Table S1). In the ds*Or35*-injected group, all four behavioral variables, TDM, TDMV, V, and PMT, showed significant decreases compared with those of the ds*GFP*-injected group (*SI Appendix*, Fig. S4). Therefore, 4VA is crucial in maintaining the traits of gregarious behavior by promoting social interactions among locust individuals.

Discussion

In the present study, we revealed that 4VA plays a key role in the establishment of gregarious behavior in solitary locusts by enhancing the social interactions among individuals. Upon the loss of the ability to perceive 4VA, crowded solitary locusts remain socially isolated and fail to display gregarious behavior, and gregarious locusts cannot maintain gregarious characteristics (Fig. 6). These results demonstrate the behavioral mechanism by which 4VA exposure leads to the formation of gregarious behavior and swarms in locusts.

4VA is necessary in the acquisition and maintenance of gregarious behavior in locusts. The behavioral transition between the solitary and gregarious phases in the migratory locust is not symmetrical in that the behavioral change from the solitary to gregarious phase is a slow process, differing largely from the rapid solitarization process that can occur within 1 h (12, 25, 26). Our study has shown that an increase in 4VA exposure concentration significantly shortened the duration of the behavioral transition in solitary locusts during crowding. Exposure to 4VA only significantly increased AI in solitary locusts after 24 h of crowding, but it increased AI, TDM, and TDMV after 48 h of crowding. These results indicate that 4VA initially alters sensory responses to other individuals, followed by enhancing the activities among individuals. Although 4VA is specifically released by gregarious locusts, crowding of four to five solitary locusts can initiate the release of 4VA (23), indicating that 4VA signals the existence of other conspecific individuals. Therefore, locusts actively engage in searching for and making contact with each other, exhibiting heightened activity levels as a result, ultimately leading to behavioral phase changes and aggregations. On the other hand, a deficiency in 4VA perception not only impedes the gregarious-like behavior of solitary locusts under crowding treatment but also decreases gregariousness in gregarious locusts at a high population density. These results further emphasize the significance of olfactory stimuli in regulating phase-related behavioral changes in locusts. The locusts will attenuate their search for and contact with other individuals when they lose the ability to perceive 4VA, even if there are locusts remaining in the vicinity.

The multi-individual behavioral assay system developed in the present study determined the behavior traits of individual–individual interactions during the crowding process. Despite the effective induction of behavioral change toward gregarious-like behavior through crowding treatment (10, 12), the specific behavioral details of the crowding process have not been thoroughly investigated. This study has demonstrated that four parameters representing individual interactions are positively correlated with crowding in this system, whereas this may not be feasible in other behavioral assay systems (10–12, 16). Additionally, locomotion exhibited a significantly positive correlation with the FP and body contact, indicating that activity levels changed with social interactions. The multiindividual behavioral assay system together with the phase evaluation system (10–12, 16) enabled the elucidation of the complete process and outcomes of locust phase change.

Enhanced individual interactions facilitated by 4VA have a profound impact on locust behavior and colony formation. Unlike eusocial insects, locust swarms have no social hierarchy, no division of labor, and no leader. The occurrence of locust aggregation and collective behavior may arise from solitary locusts becoming active through multiple contacts with other locusts. Locust individuals integrate information gathered from conspecifics to quickly and accurately assess their surroundings and obtain information that may not be available to them as individuals. Thus, we propose a positive feedback model for locust aggregation. The model suggests that locusts encounter each other during concentrated egg-laying or synchronic hatching, and the initially random body contact triggers the release of 4VA. 4VA preliminarily attracts locusts to gather in one place, but these individuals still display disorderly movements and remain socially isolated. As the number of individuals grows and the perception of 4VA increases, the locusts begin to interact socially, learn from each other, imitate each other's movements and change their behavioral traits to those of the

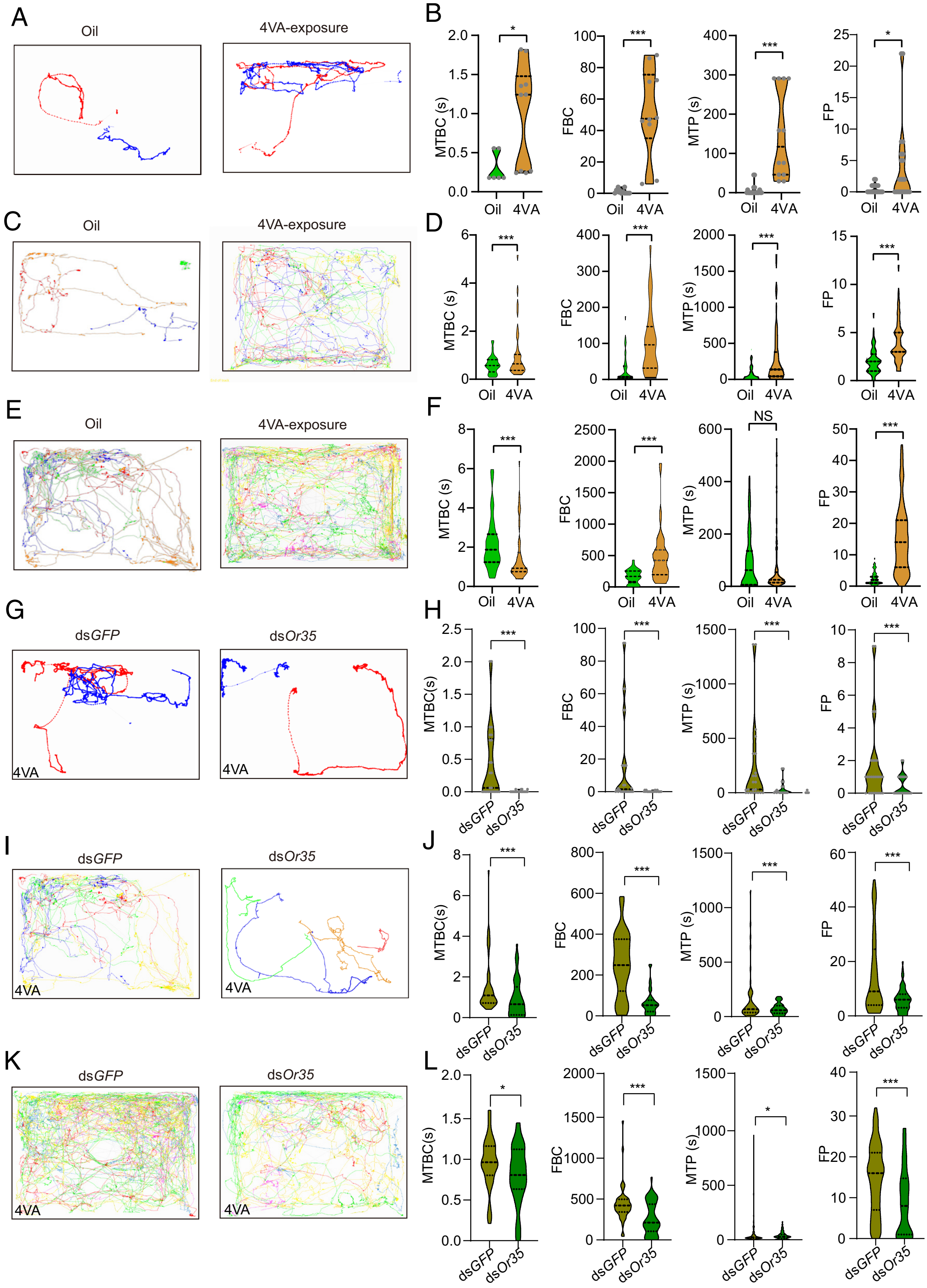

Fig. 5. 4VA accelerates locust behavioral change by increasing social interactions. (*A*) Track visualization of two solitary locusts exposed to oil and 4VA. (*B*) Changes in MTBC, FBC, MTP, and FP of two solitary locusts with 4VA exposure. (*C*) Track visualization of four solitary locusts exposed to oil and 4VA. (*D*) Changes in MTBC, FBC, MTP, and FP of four solitary locusts with 4VA exposure. (*E*) Track visualization of eight solitary locusts exposed to oil and 4VA. (*F*) Changes in MTBC, FBC, MTP, and FP of eight solitary locusts with 4VA exposure. (*G*) Track visualization of solitary locusts injected with ds*GFP* and ds*Or35* under 4VA exposure. (*H*) Changes in MTBC, FBC, MTP, and FP of two solitary locusts with *Or35* knockdown. (*I*) Track visualization of four solitary locusts injected with ds*GFP* and ds*Or35* under 4VA exposure. (*J*) Changes in MTBC, FBC, MTP, and FP of four solitary locusts with *Or35* knockdown. (*K*) Track visualization of eight solitary locusts injected with ds*GFP* and ds*Or35* under 4VA exposure. (*L*) Changes in MTBC, FBC, MTP, and FP of eight solitary locusts with *Or35* knockdown. Comparisons of FBC and FP were analyzed by the Mann–Whitney *U* test. Comparisons of MTBC and MTP were analyzed by Student's *t* test. FBC: frequency of body contact, MTBC: mean time of body contact, FP: frequency of proximity, MTP: mean time of proximity; $*P < 0.05$; $***P < 0.001$.

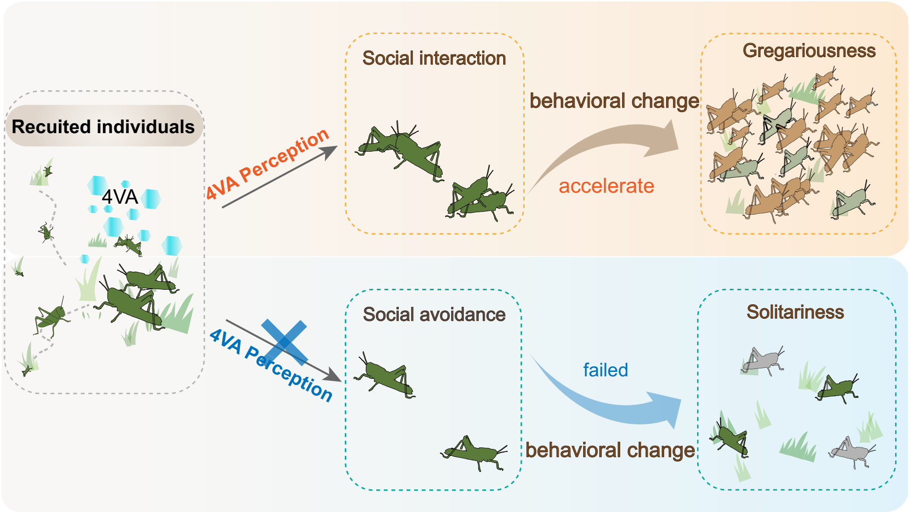

Fig. 6. 4VA accelerates locust gregariousness by increasing social interactions among individuals. After the locust individuals are attracted and recruited together, perception of 4VA increases the frequency of solitary locusts to approach and contact each other, leading to a behavioral shift toward gregarious behavior and ultimately accelerating population formation. In the absence of 4VA perception, solitary locusts maintain social isolation and exhibit no changes in their behavioral traits, ultimately resulting in solitariness.

gregarious phase. This process is further amplified as more locusts aggregate, resulting in the formation of locust swarms.

A multitude of crucial genes in the peripheral and central systems have been implicated in the behavioral phase change mediated by 4VA, operating at both sensory and motility levels. Previous studies have established the role of various olfactory genes, such as odorant-binding proteins (OBPs), chemosensory proteins, and *takeout* genes, in regulating locust attraction and repulsion behaviors during phase change (12, 27). Biogenic amine neurotransmitters, including dopamine (25, 28), octopamine, and tyramine (26), are also reported to regulate behavioral phase transition by altering the attraction and repulsion responses. Two dopamine receptors (Dop1 and Dop2) (25), miR-8-5p—regulated glutamate/GABA balance (29), and the neuropeptide F/nitric oxide signaling pathway (30) have also been confirmed to influence locust motility and trigger/hamper locust aggregation behavior. However, the signal connection and transduction of 4VA with neurotransmitters, neuropeptides, and other related modulators in locusts will need to be the subject of further investigation.

Our results also have significant implications for developing alternative strategies for locust control. As important ecological components, locusts and grasshoppers play important roles in the material cycle and energy flow in ecosystems. The widespread use of chemical pesticides against migratory locusts not only poses risks to nontarget insects but also has negative ecological consequences. Consequently, relying solely on the application of chemical pesticides is insufficient for effective locust control. By developing new behavioral-modifying agents targeting 4VA perception during the early stage of locust aggregation, we can effectively control locust aggregation and swarm formation in an eco-friendly manner. These potential applications hold promising prospects for developing sustainable locust control measures.

Materials and Methods

Insects. The gregarious and solitary locusts used in these experiments were obtained from colonies of the same strain at the Institute of Zoology, Chinese Academy of Sciences, China. Gregarious locusts were reared in large, well-ventilated cages (30 × 30 × 30 cm^3) at densities of 800 to 1,000 insects per cage. The solitary locusts were reared in a separate well-ventilated room, with each being in a single cage (10 × 10 × 25 cm^3) with charcoal-filtered compressed air. All colonies were reared under a 14:10 light/dark cycle at 30 ± 2 °C with a humidity of 60 ± 5% and were provided with a diet of fresh greenhouse-grown wheat seedlings and wheat bran.

Crowding Treatment of Solitary Locusts. Ten fifth-stadium solitary locusts were introduced to an optic perplex-made box (10 × 10 × 10 cm^3) with 20 gregarious locusts as the stimulating group in the same cage. After being crowded for 24 h, 48 h, and 72 h, the behaviors of treated solitary locusts were tested. Odor exposure with crowding treatment was conducted in an optic perplex-made box (10 × 10 × 10 cm^3) surrounded by odor-absorbing filter paper.

4VA Exposure Treatment. A 2.0-mL uncovered glass jar with 4VA (50 ng/μL) diluted in mineral oil was placed with a solitary locust in each cage. Mineral oil alone was used for the control group. These two groups were placed in separate locations to prevent cross-contamination. To investigate the function of 4VA alone in the phase change of solitary locusts, the behaviors of treated solitary locusts were directly tested after treatment with volatilized 4VA for 72 h. To evaluate the role of 4VA in the gregarization of solitary locusts under crowding conditions, 4VA-exposed solitary locusts were subjected to crowding for 24 h and 48 h prior to performing the behavioral assay. Simultaneous 4VA fumigation was conducted during crowding treatment to ensure that the total time of 4VA fumigation included the time of crowding treatment, totaling 72 h.

RNA Interference Knockdown of *Olfactory Receptor 35* (*Or35*). Double-stranded RNA (dsRNA) molecules targeting green fluorescent protein (GFP) and *Or35* were synthesized by using the T7 RiboMAX Express RNAi system (Promega, WI, P1700) according to the manufacturer's instructions. The fragments used in the experiment were blasted against the genome database to detect gene homologies and avoid nonspecificity in RNAi knockdown. The primers for dsRNA synthesis are listed in *SI Appendix*, Table S2. Ten micrograms of dsRNA was directly injected into the thoracic hemocoels of fifth-instar gregarious locusts and solitary locusts. The gregarious locusts were returned to cages for rearing, and the solitary locusts were reared under the crowding treatment. The behavior assay was performed 72 h after injection. The effects of RNAi on relative mRNA levels were detected through qPCR after the behavioral assay.

RNA Preparation and qPCR. Total RNA was extracted from the locust antennae by using TRIzol reagent (Invitrogen) in keeping with the manufacturer's protocol. DNase was applied to eliminate DNA contamination in the RNA samples. Two micrograms of total RNA was obtained from every sample and reverse transcribed using a FastKing RT Kit (with gDNase) (Tiangen) according to the manufacturer's instructions. qPCR was performed by using a RealMaster-Mix (SYBR Green) kit (Tiangen) with a Roche Light Cycler 480. Amplification was started with incubation at 95 °C for 5 min followed by 40 cycles at 95 °C for 20 s, 58 °C for 20 s, and 68 °C for 20 s. The amplification specificity of the target gene was confirmed by detecting melting curves. The primers used in the qPCR assay are listed in *SI Appendix*, Table S3. The reference gene employed to normalize and calculate the expression levels of the target genes was the *RP-49* gene. All qPCR analyses were performed in six to eight biological replicates.

Phase Change Behavioral Assay. The behavioral assay experiment was performed in a rectangular arena (40 × 30 × 10 cm^3) with a clear top, an opaque plastic wall, and two separate chambers (7.5 × 30 × 10 cm^3) on both sides (Fig. 1*A*). One of the chambers contained 30 fifth-instar gregarious locusts as the stimulus group, and the other chamber was left empty. The floor of the arena was covered with filter paper to avoid contamination during the assay. Next, the locusts were released into the arena through a tunnel. An EthoVision system (Noldus Inc.) was used to record their behavior and the data analysis extraction. Each locust was monitored for 6 min and tested only once (12, 31). Five different behavioral parameters were extracted from the video: TDM, TDMV, total duration in stimulus area, total duration in opposite area, and AI (AI = total duration in stimulus area-total duration in opposite area). The binary logistic regression model described in a previous study was used to evaluate the phase state of the locusts. The regression model was as follows:

$$P_{greg} = \frac{e^{\eta}}{(1+e^{\eta})},$$

$$\eta = -2.11 + 0.005 \times \text{AI} + 0.012 \times \text{TDM} + 0.015 \times \text{TDMV},$$

where P-*greg* shows the probability of a locust in the gregarious phase (P-*greg* = 1 means the locust is fully gregarious, whereas P-*greg* = 0 means individuals display solitary behavior).

Behavioral Assays in a Multi-individual System. Unlike the popular behavioral phase change assay (10–12), the social interaction of multiple locusts was tested in an arena (20 × 15 × 10 cm^3) without a stimuli group chamber. Filter paper was placed on the floor of the arena to reduce interference from environmental contaminants, and it was replaced once per group. During each experiment, two, four, or eight solitary locusts were introduced into the arena, and their social interaction and walking behavior were recorded by video camera for 30 min. Five parameters representing social interactions, FP, MTP, FBC, MTBC, and the DBS and four parameters related to motility, TDM, TDMV, V, and PMT were automatically exacted and calculated by the Movement and Social modules of the EthoVision system (Noldus Inc.). The details are provided in Table 1. The correlation analysis between the parameters and the density/duration was performed using the corrplot package in the R platform (v.3.2.3).

Dual-Choice Behavioral Assays. A dual-choice arena system described in detail in previous studies was used to perform the behavioral assay (23, 32). A clean oil vertical airflow served as the control zone, while the adjacent 4VA vertical steam was the treatment provided by the behavioral apparatus. We conducted a dual-choice behavioral assay with fifth-instar gregarious and solitary locusts injected with dsRNA to verify the blocking effect of *Or35* on the perception of 4VA by locusts. A drop of 10 μL diluted 4VA (50 ng/μL) was dropped onto the filter paper, and an equal volume of mineral oil drops was added to the other side of the funnel as a control treatment. Each locust stayed in the olfactometer for 10 min. After testing 15 individuals, the positions of the filter papers on the two sides of the funnel were interchanged to cancel out the positional bias. At least 30 locusts were tested for each group. The locusts' behaviors were recorded for 10 min at 25 frames per second using VCR software (v.2, Noldus Information Technology) with a Panasonic video camera. Then, EthoVision XT (v.11.5, Noldus Information Technology) software was used to analyze the video and objectively measure the total distance of movement (unit: cm) and the total time spent on each side (unit: s).

Single-Sensillum Recordings (SSRs). Fifth-instar gregarious and solitary nymphs were used for SSRs. A narrow plastic tube with a diameter of 1 cm was used to stabilize the locust, while dental wax was employed to fix the antennae. To record the sensillum responses, a sharpened tungsten wire electrode was inserted into the sensillum using a micromanipulator (Narishige). A reference electrode was placed in the compound eye. Signals were amplified using IDAC4 amplifiers (Syntech) and analyzed with Autospike32 (v.3.9, Syntech) to characterize the frequency variation in 4VA concentrations ranging from 10^{-5} to 1 (v/v) dilutions. Ten microliters of 4VA solution was added to a Pasteur glass tube containing a piece of filter paper.

Table 1. Definition and calculation of parameters in the multi-individual behavioral assay system

Variable	Definition and calculation
FP	Frequency of proximity; Proximity refers to the situation where the distance between two objects is less than or equal to 4, but greater than 0. FP represents the frequency of occurrence of this condition.
MTP	Mean time of proximity; MTP represents the mean time of occurrence of this condition within a total of 30 min.
FBC	Frequency of body contact; Body contact refers to the situation where the distance between two objects is less than or equal to 0. FBC represents the frequency of occurrence of this condition.
MTBC	Mean time of body contact; MTBC represents the mean time of occurrence of this condition within a total of 30 min.
DBS	Distance between subjects; The mean distance between any two subjects within a total of 30 min.
TDM	Total distance moved.
TDMV	Total duration of movement.
V	Velocity.
PMT	Proportion of moving time in total duration.

Statistical Analysis. The P-*greg* during crowding of solitary locusts was analyzed by the Mann–Whitney *U* test. AI was analyzed by the Mann–Whitney *U* test. TDM and TDMV were analyzed by one-way ANOVA (Tukey's multiple comparisons test). In the 4VA exposure and RNAi experiments, TDM and TDMV were analyzed by Student's *t* test. Student's *t* test was also used to analyze the effects of RNAi. The data are expressed as the mean ± SEM. $P < 0.05$ was considered significant. The probabilistic metric of solitariness (P-*greg*) was presented as median values. Correlation analysis was performed using the R programming language and the corrplot package. Data were analyzed using SPSS 18.0 (SPSS Inc.).

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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