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Early attainment of 20-hydroxyecdysone threshold shapes mosquito sexual dimorphism in developmental timing

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In holometabolous insects, critical weight (CW) attainment triggers pupation and metamorphosis, but its mechanism remains unclear in non-model organisms like mosquitoes. Here, we investigate the role of 20-hydroxyecdysone (20E) in CW assessment and pupation timing in *Aedes albopictus* and *Ae. aegypti*, vectors of arboviruses including dengue and Zika. Our results show that the attainment of CW is contingent upon surpassing a critical 20E threshold, which results in entrance into a constant 22 h interval and the subsequent 20E pulse responsible for larval-pupal ecdysis. Sexual dimorphism in pupation time arises from higher basal 20E levels in males, enabling earlier CW attainment. Administering 20E at 50% of L3/L4 molt, when most of males but not females pass the pulse, results in female-specific lethality. These findings highlight the pivotal role of 20E thresholds in CW, pupation timing, and sexual dimorphism, suggesting that manipulating 20E levels can skew populations male, offering a potential mosquito sex separation strategy.

Similar to human puberty, insect metamorphosis represents a period of sexual maturation sensitive to environmental cues[1]. In both cases, environmental stressors like malnutrition or disease can delay maturation until conditions improve[2–4]. Delayed or precocious maturation in human is often associated with serious health issues[5,6]. The mechanisms controlling the timing of sexual maturation, whether in humans or insects, remain complex and multifactorial, yet evolutionarily conserved[1–3,5–8]. In insects like *Drosophila* and mosquitoes, the adult body size is established at larval-pupal metamorphosis. To regulate this transition from growth to metamorphosis, insects need to determine when their body size is sufficient to initiate metamorphosis, a size called critical weight (CW)[9–12]. The CW is the size during the last instar larvae after which feeding is no longer required for a normal time course to metamorphosis[11–13]. Thus, it represents the commitment to metamorphosis, determines the duration of larval growth and the timing of metamorphosis, and is thus a key factor to understand the regulation of developmental timing in insects. Studying the regulation of CW in insects also provides valuable insights into the biology of puberty in various species[1].

In holometabolous insects, larvae undergo a series of ecdysis events triggered by the steroid hormone 20-hydroxyecdysone (20E), which is converted in peripheral tissues from ecdysone

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produced and released by the prothoracic glands (PGs). The conversion of cholesterol to ecdysone is facilitated by a series of enzymes, including Neverland and 'Halloween' genes, such as spook (spo), phantom (phm), disembodied (dib), and shadow (sad). In *Drosophila*, the growth of PGs is known to influence the developmental timing[14–18], while in imaginal discs ecdysone signaling is important in switching to a post-CW developmental program[17]. In insects, prothoracicotropic hormone (PTTH), a brain-produced neurosecretory peptide, regulates ecdysone synthesis and release[13,16,19–23]. Some species like *Manduca sexta* and *Bombyx mori* use juvenile hormone (JH) to inhibit PTTH release during larval development. Upon reaching CW, JH clearance allows PTTH release, triggering ecdysone production[13,24–26]. In *D. melanogaster*, JH appears to play a limited but essential role in metamorphosis control, as genetic ablation of the corpora allata or mutations in JH receptors result in developmental defects and lethality[13,27–30]. Consequently, factors triggered by CW that might be involved in the regulation of pulse ecdysone levels responsible for wandering and pupariation in this species remain unclear. It has been hypothesized that insect body size is monitored based on basal ecdysteroid concentration, with target tissues responding to ecdysteroids once their concentrations surpass a CW-specific threshold[25,31]. However, this threshold hypothesis is untested, leaving 20E's role in determining CW attainment in Diptera insects, including mosquitoes, speculative.

In mosquito, sexual dimorphism in pupation has been well recognized and widely used for sex separation to develop incompatible and sterile insect techniques (IIT-SIT) for mosquito population suppression in response to the recent surge in mosquito-borne diseases and the insufficiency of traditional control approaches in disease control[32–34]. This is based on the observation that the body size of female pupae is larger than males, and male develop faster than females during the immature stages of mosquito vectors, including *Aedes aegypti* and *Ae. albopictus*, the principal vectors of dengue, chikungunya, and Zika. Although extensive efforts have been made toward the understanding of the body size control during development in other insects and the critical functions of ecdysone signaling in the reproduction of adult mosquitoes[35–38], there is limited knowledge regarding the regulation of immature development in mosquitoes[39–41]. Evidence also suggests that models developed based on *Lepidoptera* and *Drosophila* may not be directly applicable to mosquitoes[41–43]. Addressing these gaps calls for an in-depth understanding of the mechanism determining mosquito development and its sexual dimorphism, particularly the assessment and regulation of CW—knowledge that can facilitate to develop innovative sex separation techniques in disease control.

Herein, we have demonstrated that, in the final instar larvae (4th instar, L4) of both *Ae. albopictus* and *Ae. aegypti*, the basal 20E level plays an essential role as a timing signal for pupation. We observed a gradual increase in 20E levels following ecdysis to L4, culminating in a small peak that consistently reached a fixed threshold level. This threshold level precisely coincided with the CW timing and pulse-like expression of spook, signifying the onset of pupation. Changes in the basal 20E levels were found to influence the pupation time by modulating the timing of this small 20E peak. Moreover, our results also showed that male larvae had a higher 20E levels than female during early development, underpinning the mosquito sexual dimorphism in development time. Finally, supplementation of 20E at 50% of ecdysis from the 3rd instar (L3) to L4 induced a high male bias in adults. Taken together, our data illustrated how the basal 20E levels modulated CW, pupation time, and their sexual dimorphism through a threshold 20E level and subsequent 20E pulse.

Results

The critical weight is attained around 30 h after ecdysis to the final instar larvae

To determine the timing of CW attainment in mosquito larvae, we conducted a starvation experiment at different time points after L3/L4 molt of *Ae. albopictus* and analyzed the resulting delay in pupation time. The results showed that starvation significantly delayed pupation, especially during early developmental stages. The delay in pupation decreased with prolonged feeding periods until 30 hrs after L3/L4 molt, beyond which starvation did not delay pupation (Fig. 1A). This indicated that the CW of *Ae. albopictus* larvae was reached approximately 30 hrs after L3/L4 molt under normal feeding conditions. To confirm the timing of CW attainment and identify the most crucial period for pupation time, we starved L4 larvae every 9 h after L3/L4 molt and compared the pupation times between the treatment and control groups. Our results demonstrated that the first two 9-hr periods after L3/L4 molt were the most critical for pupation time, and starvation only delayed pupation until 27–36 h after L3/L4 molt (Fig. 1B). Overall, these results suggest that the CW required for larval-pupal metamorphosis is achieved around 30 h after L3/L4 molt in *Ae. albopictus*.

The decrease in 20E levels leads to a delay in pupation induced by starvation

Previous studies have shown that a pulse of 20E production during the final larval instar's late developmental stage induces larval-pupal metamorphosis in holometabolic insects and nutrition is necessary to induce a small 20E peak at the early L3 stage of *Drosophila*[31]. To determine if the starvation-induced delay in pupation was related to 20E synthesis in *Ae. albopictus* L4, we examined the impact of starvation on 20E levels, firstly by measuring the expression of 20E receptor genes EcR and Usp, and the early induced gene E75, which are often used as proxies for 20E levels[39,44–46]. The results showed that starvation of larvae for 12 h post L3/L4 molt down-regulated E75 expression and up-regulated the expression of EcR and Usp (Fig. 1C). By analyzing the relationships between 20E levels and the expression of these genes, we found that only E75 expression was highly positively correlated with 20E levels (Supplementary Fig. 1A, B). Next, we measured ecdysteroid titer directly in hemolymph at 24 hafter starvation by a 20E EIA assay and the results showed that ecdysteroid levels were significantly decreased after starvation (Fig. 1D). Therefore, we used the E75 expression as a proxy for 20E levels in subsequent studies.

Since the 20E level was reduced by starvation in L4, we hypothesized that the decrease in 20E concentration might be responsible for the delay in pupation. To test this hypothesis, we supplemented newly emerged L4 with exogenous 20E at the first 12 h after L3/L4 molt under starvation conditions and compared pupation times between the treatment groups and control. The results demonstrated that 20E supplementation rescued the starvation-induced delay in pupation by boosting 20E levels, and both fed and starved larvae, after supplemented with 20E, pupated at similar time and earlier than control (Fig. 1E, F). Therefore, we conclude that starvation delayed pupation by reducing 20E production, indicating that basal 20E levels might play an essential role on pupation timing. Further experiments confirmed that starvation downregulated the expression of five steroidogenic enzyme genes, Nev, Spo, Phm, Dib and Sad (Fig. 1G). Taken together, these results suggest that starvation may decrease 20E levels by suppressing the expression of steroidogenic enzyme genes responsible for ecdysone synthesis.

20E modulates pupation in a dose-dependent manner

To investigate the role of basal 20E levels in determining pupation time, we perturbed 20E signaling in L4 larvae by rearing newly molted larvae with different concentrations of 20E or its antagonist, Cucurbitacin (CucB), for 12 h post L3/L4 molt and examined the

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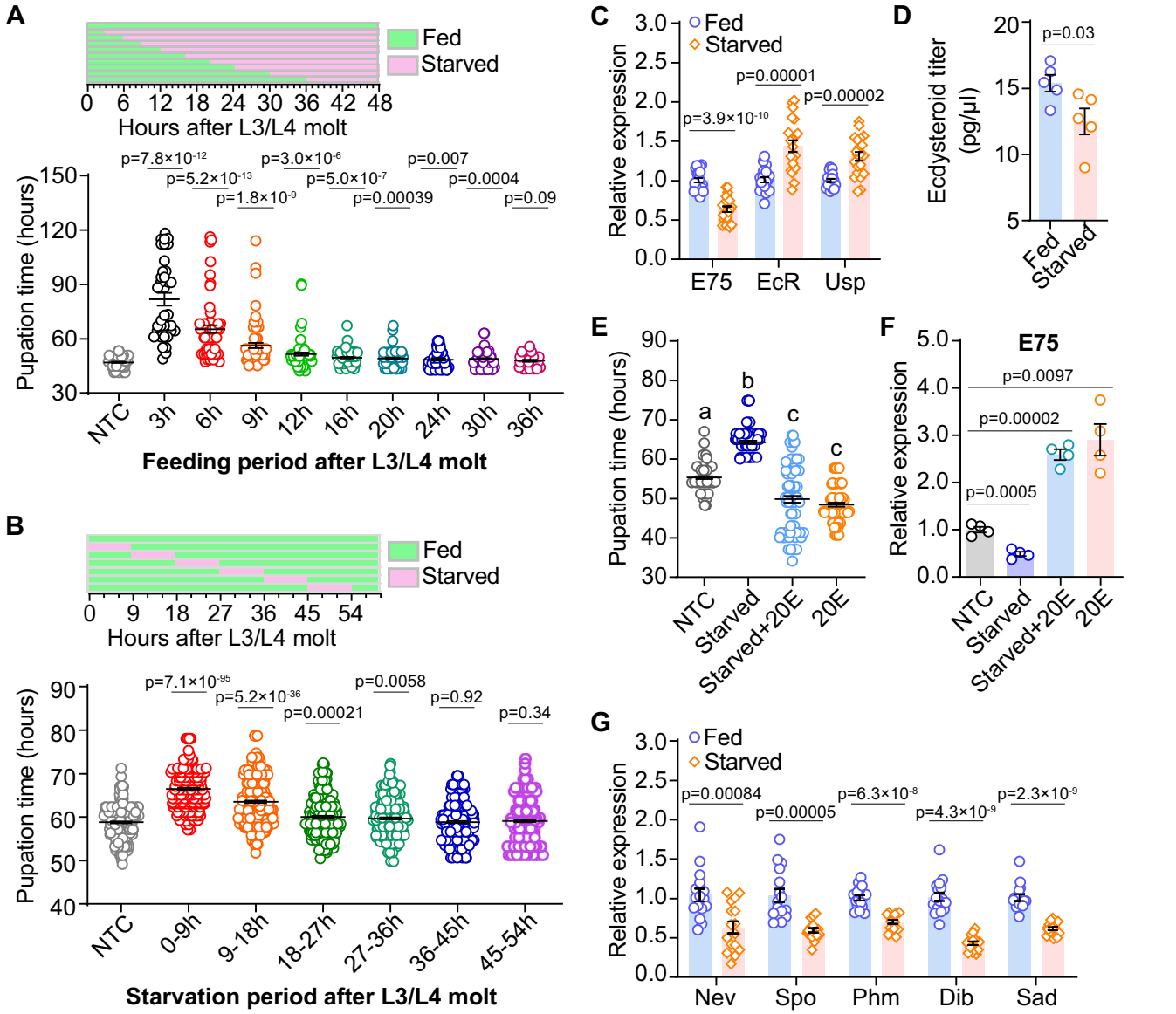

Fig. 1 | Starvation delays pupation by decreasing 20E levels. Determination of the attainment of critical weight by starving newly emerged L4 larvae at different times after L3/L4 molt (**A**) or every 9 hrs during the development of L4 larvae (**B**). Pupae were collected every 3 hrs after the first pupa emerged and pupation time was the duration of L4 larval development. For **A**, $n = 88, 37, 70, 80, 86, 86, 87, 85, 84$ and 46 for NTC, 3 h, 6 h, 9 h, 12 h, 16, 20 h, 24 h, 30 h and 36 h, respectively. For **B**, $n = 365, 358, 367, 361, 357, 368$ and 360 for NTC, 0–9 h, 9–18 h, 18-27 h, 27–36 h, 36–45 h and 45–54 h, respectively. **C** The effects of starvation on expression of E75, EcR and Usp were assessed in L4 larvae starved for 12 h after L3/L4 molt. $n = 21$ biological replicates with 3 larvae/replicate for each group. **D** The ecdysteroid titer in hemolymph was assayed at 24 hrs after starvation. Each sample contained hemolymph from 10 larvae and 5 biological independent samples were collected for each group. **E**, **F** The newly emerged L4 larvae were starved and treated with 80 μg/mL of 20E for 12 h, and then fed under normal condition. For **E**, $n = 98, 98, 96,$ and 90 for group of NTC, Starved, Starved+20E and 20E, respectively. **F** The concentration of 20E after starvation or 20E treatment for 12 h after L3/L4 molt was assayed by expression of E75; $n = 4$ biological replicates with 8 larvae/replicate. **G** The effect of starvation on expression of steroidogenic genes responsible for ecdysone synthesis. The newly emerged L4 larvae were starved for 12 h after L3/L4 molt. The relative expression levels were detected by using qRT-PCR ($n = 16$ biological replicates with 3 larvae/replicate). Statistic: (**A–D, F, G**) two-tailed Student's *t*-test; (**E**) one-way ANOVA with Bonferroni's multiple comparison, the exact *p*-value was provided in Source Data. NTC: non-treatment control (without starvation or 20E treatment). Data shown are means ± SEM. Source data are provided as a Source Data file.

corresponding pupation times. The results showed that pupation was both significantly accelerated with the increase of 20E and delayed with the increase of CucB to weaken 20E reactions (Fig. 2A, B). Further analysis showed a strong negative correlation between 20E levels and pupation time (Spearman's test, $R = -0.736$, $P < 0.0001$), and a strong positive correlation between CucB concentrations and pupation time (Spearman's test, $R = 0.554$, $P < 0.0001$). To determine whether the onset of pupation in *Ae. albopictus* is modulated by 20E levels in a dose-dependent manner, we starved L4 immediately after L3/L4 molt and supplemented them with different concentrations of 20E for 12 h. Starved larvae without 20E or supplementation with a low concentration (20 μg/mL) of 20E could not pupate and eventually died (Fig. 2C). However, with increasing concentrations of 20E from 40 μg/mL to 120 μg/mL, the percentage of L4 successfully initiating pupation increased from 13.3% to more than 70% (Fig. 2C). As expected, the pupae and adults that emerged from these larvae were miniature compared to the control groups without starvation and 20E supplementation (Fig. 2D). These results suggest that both pupation time and rate of *Ae. albopictus* are determined by 20E levels in a dose-dependent manner.

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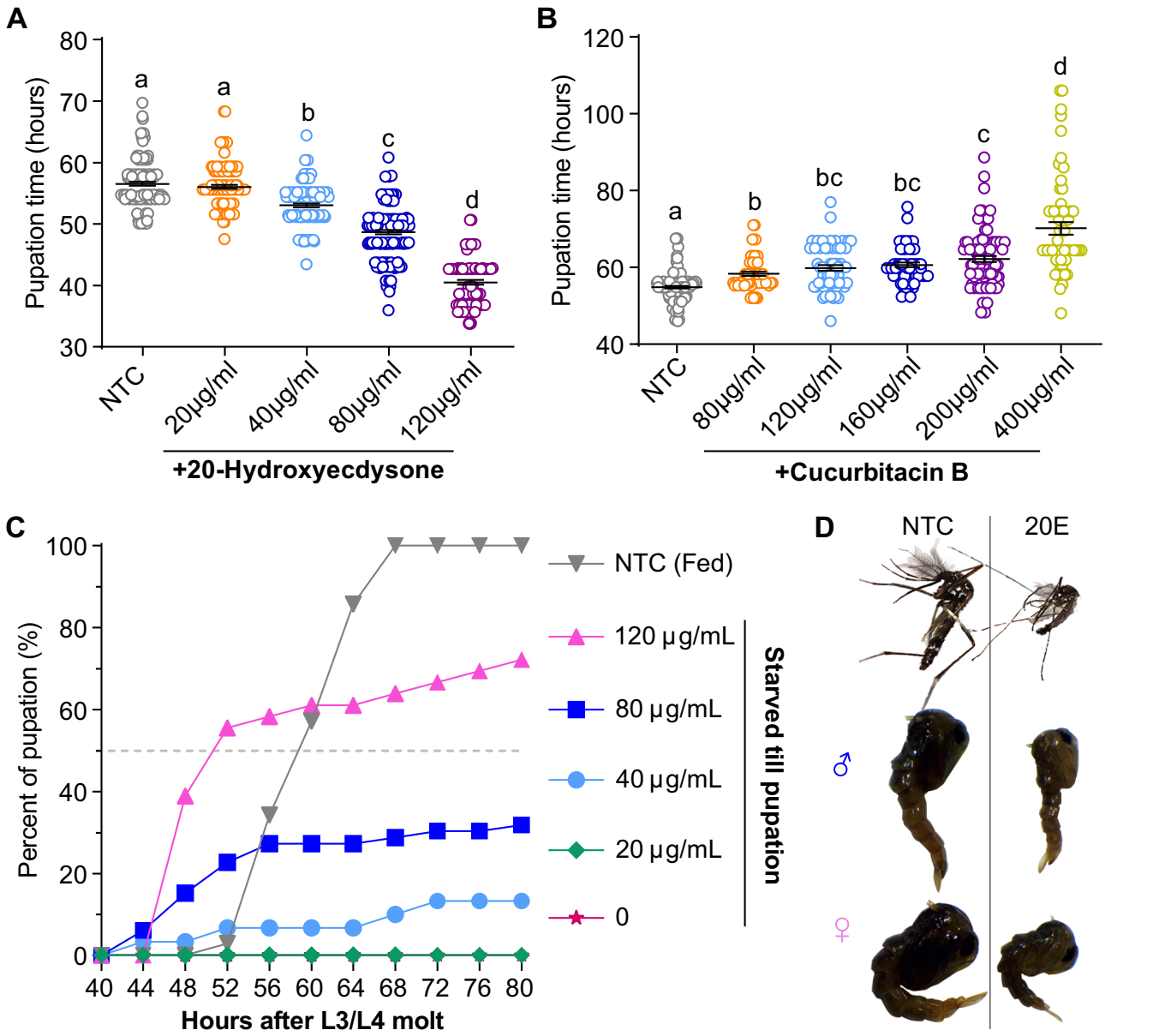

Fig. 2 | Basal 20E levels determine the pupation time and rate in a dose-dependent manner. Newly emerged L4 larvae were treated with different concentrations of 20E (**A**) and CucB (**B**) for 12 h after L3/L4 molt and then reared under normal condition. For **A**, $n = 171, 117, 119, 175$ and 89 for group of NTC, 20, 40, 80 and 120 μg/ml of 20E. For **B**, $n = 133, 60, 61, 62, 90$ and 61 for group of NTC, 80, 120, 160, 200 and 400 μg/ml of CucB. **C** Newly emerged L4 larvae were starved and treated with different concentrations of 20E for 12 h after L3/L4 molt. Then they were reared individually in 24-well plates and starved till pupation ($n = 35, 44, 35, 30, 66$ and 36 for group of NTC, 0, 20, 40, 80 and 120 μg/ml of 20E). **D** Adult male (upper), male (middle) and female (lower) pupae emerged from larvae of (**C**). Statistics: (**A** and **B**) one-way ANOVA with Bonferroni's multiple comparison, bars with different letters are significantly different from each other, the exact *p*-value was provided in Source Data. NTC: non-treatment control (without drug treatment). Data shown are means ± SEM. Source data are provided as a Source Data file.

Accumulating 20E to a threshold is required for transition to a constant period for pupation

As basal 20E levels can regulate the pupation time and rate in a dose-dependent manner, we thus hypothesize that, in mosquito larvae, the concentration of 20E is utilized as an intrinsic indicator of critical weight, and once a threshold is exceeded, it initiates a process leading to pupation. To test it, we monitored the temporal profile of 20E concentrations during the L4 development by measuring the expression of E75. We observed that 20E levels gradually increased with growth until reached a small peak at 30 h after L3/L4 molt, which coincided with the timing of attainment of CW (Fig. 1A, B), and there was a pulse production of 20E around 51 h after L3/L4 molt that responsible for larval-pupal ecdysis (Fig. 3A). We then monitored how the timing of this small peak would change by modulation of basal 20E levels using either supplementation or starvation. As a result, supplementation with 20E (80 μg/mL) or treatment with starvation for 12 h either speed up or delay the peak 8 h, respectively, as compared to the non-treated control group (The concentration of 20E supplementation in the subsequent experiment was always 80 μg/mL, unless otherwise stated). Except for that, the dynamic of 20E levels in both treatment groups followed the same pattern as the control group. Specifically, according to the E75 expression relative to the beginning of L4 stage, 20E levels increased over time, with E75 expression reaching ~ 6-fold of its level at the small peak, followed by a constant 22 h intervals and the subsequent 20E pulse responsible for larval-pupal ecdysis (Fig. 3A, B). These results indicate that 20E levels at the small peak, corresponding

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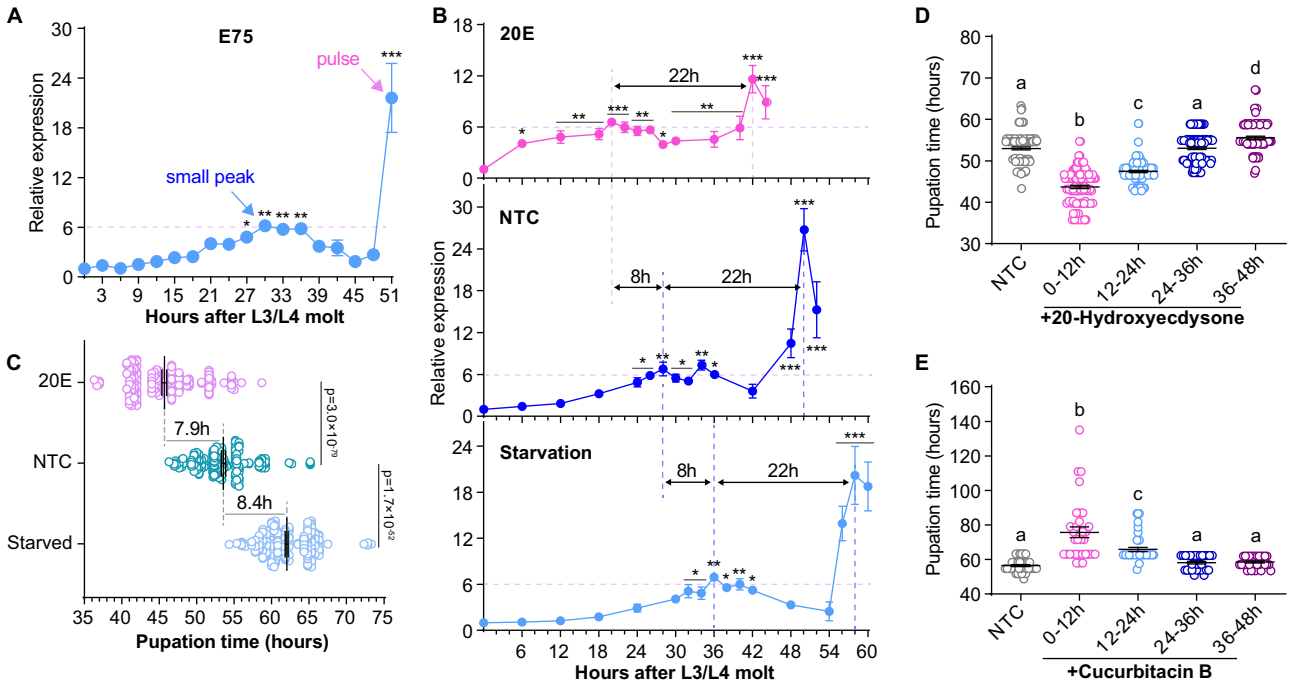

Fig. 3 | The basal 20E levels determine the timing of a 20E threshold for critical weight. The concentrations of 20E were assayed by E75 expression levels for (**A**, **B**). **A** Temporal expression of E75 was detected in L4 larvae using qRT-PCR. L4 larvae were collected individually every 3 h from L3/L4 molt till pupation ($n = 10$ for each time point). **B** L4 larvae were treated with 80 μg/mL of 20E or starved for 12 hrs after L3/L4 molt, and then reared under normal condition. Larvae were collected every 2–6 h from L3/L4 molt till pupation. The first and the second vertical dashed lines indicate the small peak and the pulse of 20E, respectively, for each group. ($n = 6$ pools for 0 hrs, $n = 4$ pools for others). **C** Pupation time were compared between groups ($n = 197$ for NTC, $n = 220$ for starved, $n = 185$ for 20E). **D**, **E** Newly emerged L4 were reared in 6-well cell culture plates and treated with 80 μg/mL of 20E or 200 μg/mL of CucB for 12 h at different time periods during development of L4 ($n = 128$, 116, 124, 123 and 89 for NTC, 0–12 h, 12-24 h, 24–36 h and 36–48 h of 20E treatment, respectively; $n = 39$, 33, 40, 30 and 30 for NTC, 0–12 h, 12–24 h, 24–36 h and 36–48 h of CucB treatment, respectively.). Statistics: (**A**, **B**) One-way ANOVA with LSD multiple comparison, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ compared to 0 hrs; (**C**) two-tailed Student's *t*-test; (**D**, **E**) one-way ANOVA with Bonferroni's multiple comparison, bars with different letters are significantly different from each other. For (**A**, **B**, **D**, **E**) the exact *p*-value was provided in Source Data. Data shown are means ± SEM. Source data are provided as a Source Data file.

to the CW, is the threshold to exceed in order to enter into a constant 22 h transition period for pupation. Furthermore, 20E supplementation or starvation treatment either accelerated or delayed the pupation approximately 8 h, respectively (Fig. 3C), similar to their impacts on the timing of the small peak (Fig. 3B). We then modulated 20E levels by rearing L4 with exogenous 20E or CucB every 12 hrs after L3/L4 molt and analyzed their impacts on pupation time. Consistent with the role of 20E levels as intrinsic assessment of CW, changes in 20E levels could alter the pupation time only before but not after the threshold was attained (Fig. 3D, E), with the exception that supplement with 20E at 36–48 h − very close to the natural pulse at ~51 h − resulted in significant delay in pupation compared to the control, with 67.8% died as intermediate or pupae (Fig. 3D, Supplementary Fig. 4A). The latter is likely caused by an extended duration of 20E pulse resulted from detrimental effect of combining supplementation and the intrinsic pulse of 20E[44]. Similar results were also observed in *Ae. aegypti* (Supplementary Fig. 2, 4A). Overall, these results indicate that the CW of L4 is determined by a threshold 20E level and accumulating 20E to this threshold triggers the transition to a constant period for pupation.

A pulse-like expression of spook is synchronized with the attainment of the threshold 20E

The observation of a threshold 20E level timing the onset of pupation implies that reaching this threshold initiates a cascade of gene expression, which subsequently leads to the synthesis and release of 20E pulses that are responsible for the transition from larva to pupa during ecdysis. To explore the genes regulated by this threshold level of 20E, we compared the transcriptome profiles between larvae at 30 h after ecdysis to L4, which corresponds to the timing of CW, and newly emerged L4, and between L4 treated with exogenous 20E for 12 h and those reared under normal conditions using RNA-seq. As a result, we observed the two comparison groups shared 1090 differentially expressed genes (DEGs), including 931 genes regulated in the same direction (Fig. 4A). In the subsequent KEGG pathway analysis of these DEGs, eight pathways were significantly enriched, with RNA polymerase regulated to the great magnitude (Fig. 4B). Among them, we focused on the insect hormone biosynthesis pathway for further analysis and found that several enzyme genes responsible for biosynthesis of juvenile hormone and ecdysone were significantly differentially expressed at 30 h after ecdysis to L4 and by 20E (Fig. 4C). Among them, only spook and juvenile hormone esterase (JHE) exhibited significant up-regulation in both groups, with spook showing the highest fold change.

Since spook serves as the rate-limiting step in ecdysone production among steroidogenic enzymes in other insects[45,47–49], we investigated whether the expression of spook correlated with the onset of pupation initiated by the threshold level of 20E. We first examined the temporal expression of genes coding for ecdysteroidogenic enzymes during the development of *Ae. albopictus* L4. Notably, the expression of spook was low until 18 h after ecdysis. Subsequently, its expression rapidly increased to over 100-fold within the next 12 hours, followed by a decrease to about 15-fold at 42 h after ecdysis, and then remained at low levels (Fig. 4D). The expression of other enzyme genes either slightly decreased or had no apparent changes during the L4 development. The pulse-like expression of spook appeared exactly at the same time as the timing of the threshold 20E level for CW (Fig. 3A). To investigate the potential that the pulse-like expression of spook was induced by the threshold 20E, we examined the relationship between

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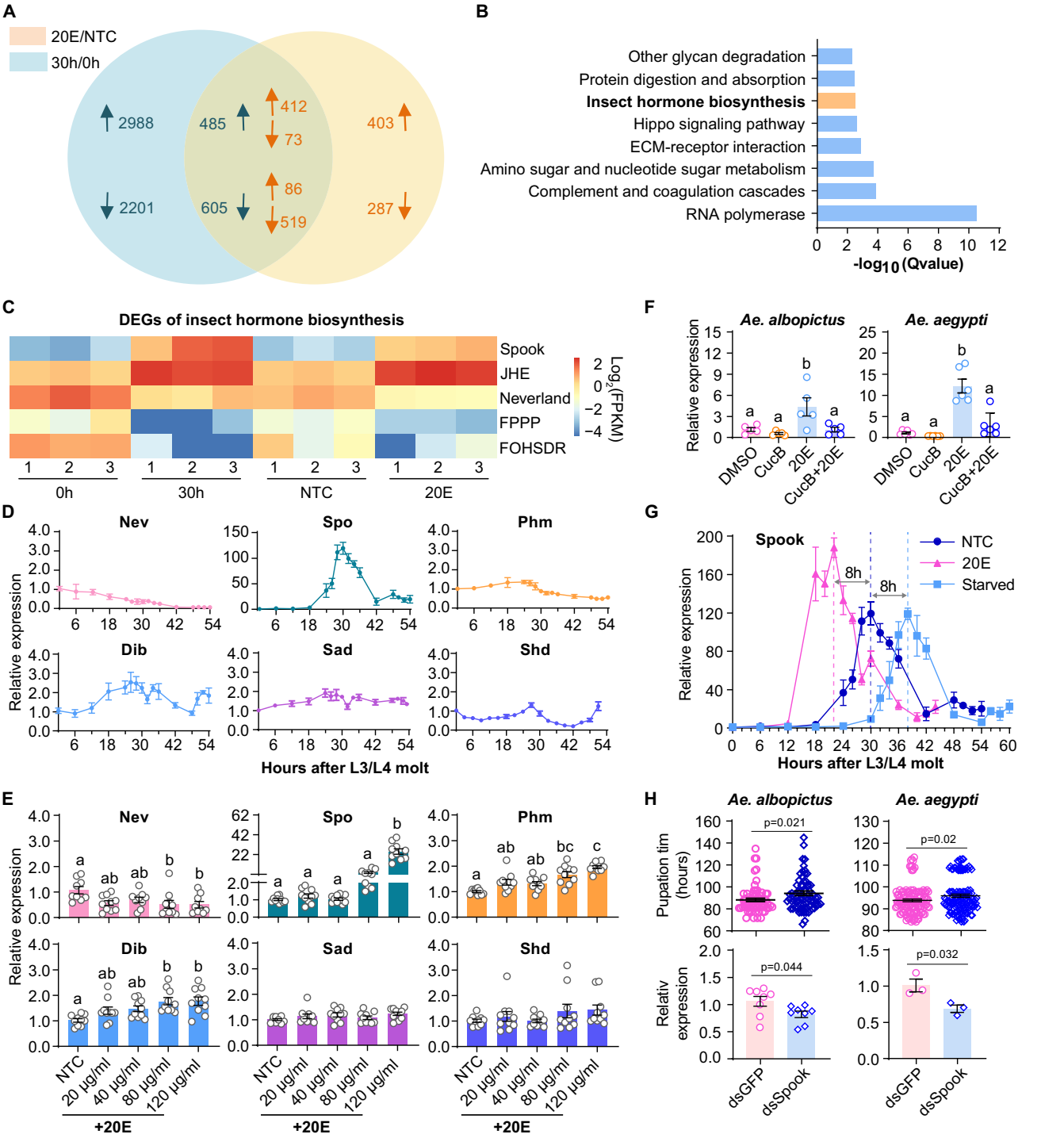

20E levels and the expression of spook and other enzyme genes through in vitro 20E supplementation within the first 12 h after the L3/L4 molt. We observed no changes in the expression of spook when the supplementation of 20E was below 80 μg/mL. However, the expression rapidly increased, ranging from 3.75− to 25−fold of the normal control, as 20E concentrations increased from 80 μg/mL to 120 μg/mL (Fig. 4E). The expression of other enzyme genes either decreased or only had slight changes after treatment with 20E. To further confirm the role of 20E signaling in the expression of spook, we disrupted 20E signaling in L4 larvae with EcR antagonist (CucB)[50–52] and measured its effect on 20E-induced spook expression in both *Ae. albopictus* and *Ae. aegypti*. We found that CucB pre-treatment could completely inhibit the upregulation of spook expression induced by 20E in both mosquito species (Fig. 4F). Collectively, these results are consistent with the predication that the pulse-like expression of spook is induced only when 20E reaches a sufficiently high level, supporting the presence of a positive feedback between spook expression and 20E production for triggering subsequent metamorphosis.

To confirm the correlation between reaching the threshold 20E level and the peak expression of spook, we assessed the temporal expression profile of spook following 20E supplementation or starvation during the early developmental stage of L4. We observed that modulating basal 20E levels through 20E supplementation or starvation either accelerated or delayed the attainment of the pulse-like expression of spook by 8 hours, respectively (Fig. 4G), mirroring their impact on the timing of threshold 20E levels (Fig. 3B). These findings

Fig. 4 | A pulse-like expression of spook induced by the threshold 20E might be crucial for determining the pupation time. A Venn diagrams showed overlapping expression of genes induced by 20E and modulated at the attainment of threshold 20E (n = 3 biological replicates with 10 larvae/replicate for each group). Up arrows: the number of up-regulated DEGs; down-arrow: the number of down-regulated DEGs. The number beside the blue arrows in the shared part means the shared DEGs and the number beside the orange arrows in the shared part mean the shared DEGs of 30 h/0 h that were up- or down-regulated in 20E/NTC. **B** Representative enriched KEGG pathways of co-regulated genes from (**A**), with the insect hormone biosynthesis pathway highlighted in bold. **C** Differentially expressed genes of enriched KEGG pathway of insect hormone biosynthesis from (**B**). **D** Temporal expression of genes coding for ecdysteroidogenic enzyme after L3/L4 molt till pupation. Samples used were from Fig. 3B, except Shd which were analyzed with samples from Fig. 3A. **E** The expression of genes coding for ecdysteroidogenic enzyme after treatment with different concentration of 20E for 12 h after L3/L4 molt ($n = 10$ biological replicates with 5 larvae/replicate). **F** The role of 20E signaling in the expression of spook. The newly emerged L4 larvae were treated with DMSO or 200 μg/mL of CucB for the first 12 h and then treated with DMSO or 80 μg/mL of 20E for the next 12 h. The expression of spook was determined by qRT-PCR ($n = 5$ biological replicates with 6 larvae/replicate for *Ae. albopictus*, $n = 6$ biological replicates with 5 larvae/replicate for *Ae. aegypti*). **G** Temporal expression of spook mRNA after 20E supplementation and starvation for 12 h. Data are generated from the samples of Fig. 3B ($n = 6$ pools for 0 h, $n = 4$ pools for others; the data of NTC was the same as Spo of Fig. 4D). **H** Pupation time of L4 after spook RNAi. Upper: The impact of Spook RNAi on pupation time (for *Ae. albopictus*, $n = 68$ and 72 for dsGFP and dsSpook, respectively; for *Ae. aegypti*, $n = 124$ and 132 for dsGFP and dsSpook, respectively.); lower: The knockdown efficiency of Spook RNAi ($n = 8$ biological replicates with 3 larvae/replicate for *Ae. albopictus*; $n = 3$ biological replicates with 8 larvae/replicate for *Ae. aegypti*). Statistic: (**E**, **F**) one-way ANOVA with Bonferroni's multiple comparison, bars with different letters are significantly different from each other, the exact *p*-value was provided in Source Data; (**H**) two-tailed Student's *t*-test. Data shown are means ± SEM. Source data are provided as a Source Data file.

indicate a synchronization between the peak expression of spook and the attainment of the threshold 20E level for CW. To investigate spook's role in pupation, we conducted RNA interference (RNAi) by soaking larvae with dsRNA to silence spook and measured its effect on pupation time. Our observations revealed that silencing spook significantly delayed pupation in both *Ae. albopictus* and *Ae. aegypti* (Fig. 4H). These results suggest that the pulse-like expression of spook may play a crucial role in regulating pupation timing.

Sex differences in basal 20E levels determine sexual dimorphism in pupation time

Sexual dimorphism in developmental time is a common phenomenon in the animal kingdom, with males often exhibiting faster development than females across various insect orders[53,54]. To characterize sexual dimorphism in mosquito larval development, we first determined that males pupated approximately 10 to 23 h earlier than females across four mosquito species: *Ae. albopictus, Ae. aegypti* (AFM and Aaeg-M strains), *Culex quinquefasciatus*, and *Anopheles stephensi* (Fig. 5A). To further understand the stage-specific differences in development between sexes, we individually reared newly emerged larvae of *Ae. aegypti* (AFM) and compared their growth at each larval instar. The results showed that the male larvae consistently developed significantly faster than the female larvae in all instars, except for a slight trend in quick growth during the L3 stage (Fig. 5B). Because the accumulation in time difference through L1 to L4 was almost the same as AFM of Fig. 5A, the ultimate difference in pupation time between sexes was attributed to the cumulative time differences observed in the development of each larval instar (Fig. 5C). To explore whether the basal 20E level contributed to this sexual dimorphism in pupation, we examined the expression of E75 during the early developmental stages of L4 in both *Ae. aegypti* and *Ae. albopictus* to assess their 20E levels. The results revealed that E75 expression levels were consistently higher in males than in females in both *Aedes* mosquitoes (Fig. 5D, left and right, 5E, left). We further directly measured the ecdysteroid titer in the whole body of male and female L4 larvae at 12 and 24 hrs after the L3/L4 molt in *Ae. albopictus* and *Ae. aegypti*, respectively. The results showed that ecdysteroid titer were significantly higher in males than in females in both mosquito species (Fig, 5D, middle, 5E, right). These findings suggest that elevated basal 20E levels may promote faster development in male mosquito larvae compared to females.

To investigate the factors contributing to faster pupation in male L4 larvae, we compared the timing in the attainment of CW and temporal profiles of 20E levels between males and females during L4 development in *Ae. albopictus*. Firstly, newly emerged L4 larvae were collected and starved at different times after ecdysis to L4. Pupation time were compared between the groups of starved and fed at each time point. This result showed males attain CW at 27 h post-molt, which is 3 h earlier than females, who attain CW at 30 h post-molt (Fig. 5F). Subsequently, newly emerged L4 larvae were collected and reared under normal conditions. Thirty larvae were sampled individually every 3 h till pupation and the sex was determined by the existence of Nix. We found that E75 expression attained a small peak several hours earlier in males than in females (Supplementary Fig. 3A). Because the threshold 20E for CW could induce a pulse-like expression of spook, we also detected the expression of spook in male and female L4 larvae. The results indicated that the pulse-like expression of spook in males occurred 3 hrs earlier than in females (Supplementary Fig. 3B). Finally, we directly measured ecdysteroid titers during the development of *Ae. albopictus* male and female L4 larvae. Once again, the results showed that the ecdysteroid titers reached a small peak about 3 hrs earlier in males than in females (Fig. 5G). After normalizing ecdysteroid titers at each time point to the titer in larvae collected at 0 hrs post-L3/L4 molt, the results demonstrated the relative ecdysteroid titer reaching ~ 3-fold at the small peak compared to the titer at 0 hrs (Supplementary Fig. 3C). Altogether, these results suggested that male L4 larvae pupated earlier than females due to the earlier attainment of the threshold 20E for CW.

A decrease in basal 20E levels at late instar delays pupation more significantly in females than in males

We have shown that the pupation time is modulated by basal 20E levels at the L4 stage of mosquito larvae. To investigate the impact of 20E levels on the stage-specific development of mosquito larvae, we treated mosquito larvae with exogenous 20E and its antagonist CucB and measured their development time in each stage. We observed that treatment with 20E or CucB significantly accelerated or delayed, respectively, the development time of each larval instar and of both sexes at L4 (Fig. 6A). To investigate how the change in basal 20E levels at each larval stage will influence sexual dimorphism in pupation time, *Ae. aegypti* (AFM) larvae at each of four stages from L1 to L4 were treated with CucB to weaken 20E signaling, pupation rates and times were compared between treatment and control and between sexes. Consistently, CucB treatment at all four stages delayed the pupation. The decrease in 20E signaling at L4 delayed pupation significantly more in females than in males [generalized linear model (GLM), Sex: Treatment interaction, $P = 0.011$], although this sex-specific effect was not observed in other stages (Fig. 6B–E). A stronger effect on delaying pupation in females compared to males was also observed in *Ae. albopictus* at the L4 stage (GLM, Sex: Treatment interaction, $P = 4.9E\text{-}10$) (Fig. 6F). By knocking down spook at the L4 stage using dsRNA-mediated gene silencing to reduce the endogenous 20E production, we also observed that delay in pupation occurred more significantly in females than in males for both *Ae. aegypti* (GLM, Sex: Treatment interaction, $P = 0.001$) and *Ae. albopictus* (GLM, Sex:-Treatment interaction, $P = 0.036$) (Fig. 6G, H). These results suggest that

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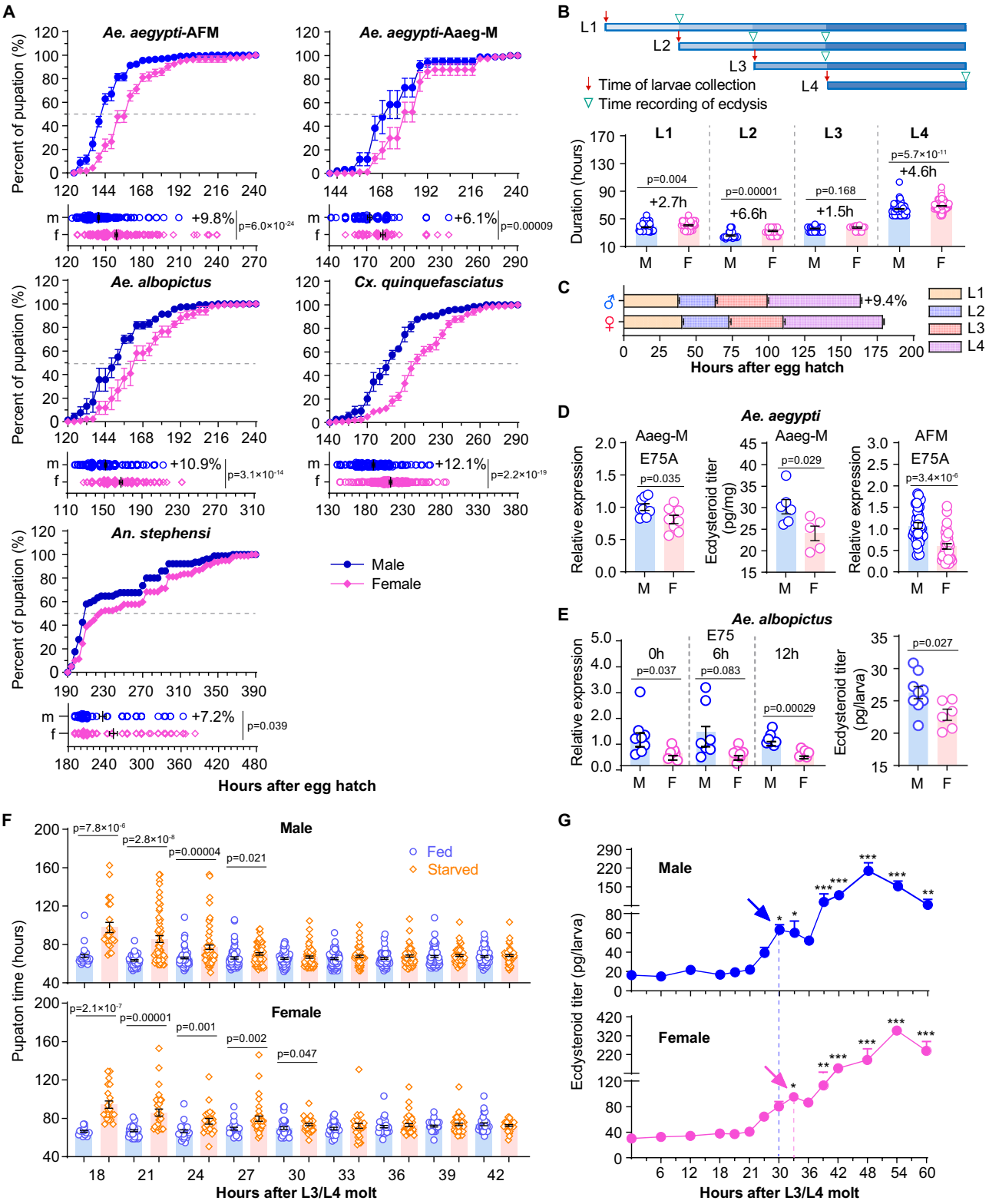

the effect of decreased 20E levels on sexual dimorphism in pupation is both conserved across different mosquito species, and stage-specific, with females more sensitive than males at L4.

Supplementation of 20E at late instar results in female-specific lethality and strong male-bias

The observation of a high lethality to larvae when 20E supplementation near the pupation (Supplementary Fig. 4A) motivated us to investigate any difference in this effect between sexes. Firstly, we wanted to know whether male and female larvae have the same susceptibility to the high lethality induced by 20E supplementation. Newly emerged L4 larvae of *Ae. albopictus* were collected and supplemented with 20E about 12 h before the beginning of pupation. The results demonstrated that both male and female have the same susceptibility to 20E at this time point (Supplementary Fig. 4B). To further define the time window that 20E supplementation will cause failure in late

Fig. 5 | The higher basal 20E levels are associated with the faster larvae development in male than in female. A Male larvae pupated earlier than female. Top: the percentage of pupae by the time. Bottom: Time difference in pupation between sexes, with the percentage indicating an increase in pupation time in female relative to male (n = 299 and 285 for male and female of *Ae. aegypti*-AFM, respectively; n = 87 and 76 for male and female of *Ae. aegypti*-Aaeg-M, respectively; n = 239 and 168 for male female of *Ae. albopictus*, respectively; n = 204 and 213 for male and female of *Cx. quinquefasciatus*, respectively; n = 73 and 85 for male and female of *An. stephensi*, respectively.). **B** Male larvae molted earlier than female at each instar. The numbers above the top of columns indicate the time female took more than male (n = 83 and 65 for male and female of L1, respectively; n = 33 and 25 for male and female of L2, respectively; n = 45 and 15 for male and female of L3, respectively; n = 164 and 157 for male and female of L4, respectively. **C** The difference in pupation time between sexes was a sum of variation in development time of each instar (data was deduced from Fig. 5B). **D** Left and right: The expression of E75A in Aaeg-M and AFM strains of *Ae. aegypti* was higher in male than in female at 24 hrs after L3/L4 molt (n = 8 biological replicates with 3 larvae/replicate for Aaeg-M strain; n = 31 larvae for each sex for AFM strain); middle: the ecdysteroid titer in Aaeg-M strain of *Ae. aegypti* was also higher in male than in female at 24 h after L3/L4 molt (n = 6 and 5 biological replicates with 3 larvae/replicate for male and female, respectively). For Aaeg-M strain, sex was determined by male-specific expression of GFP protein and for AFM strain, the sex was determined by the expression of Nix gene. **E** Left: The expression of E75 of *Ae. albopictus* was higher in male than in female at 0 (n = 8 for male; n = 7 for female), 6 (n = 7 for both sexes), and 12 h (n = 8 for male; n = 6 for female) afterL3/L4 molt; right: the ecdysteroid titer of *Ae. albopictus* was also higher in male than in female after normalizing with body size at 12 h after L3/L4 molt (n = 9 for male; n = 6 for female). Larvae were collected individually, and the sex was determined by the expression of Nix gene. **F** Determination of the timing of critical weight for *Ae. albopictus* male and female L4 larvae. Newly emerged L4 larvae were collected, starved at different times after L3/L4 molt. Pupation times were compared between the groups of fed and starved (n = 95, 186, 196, 195, 203, 200, 203, 203 and 197 for group of 18, 21, 24, 27, 30, 33, 36, 39 and 42 hrs after L3/L4 molt). **G** The profiles of ecdysteroid titers during the development of *Ae. albopictus* male and female L4 larvae. Larvae were collected individually, and sex was determined by the existence of Nix in head and ecdysteroid titer were assayed in carcass. Three carcasses were pooled, and 4 pools were set for each time point of each sex. The blue and purple vertical dashed lines indicate the small 20E peak for male and female, respectively. Statistics: (**A**–**F**) two-tailed Student's t-test; (**G**) One-way ANOVA with LSD multiple comparison, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs 0 h, the exact p-value was provided in Source Data. Data shown are means ± SEM. Source data are provided as a Source Data file.

development, *Ae. aegypti* (AFM) larvae at the stages L3 and L4 were treated with 20E every 12 h after ecdysis. Larvae treated near the time of ecdysis, 24–36 h after L2/L3 ecdysis and 0–12 after L3/L4 ecdysis, and near the pupation time, 48-60 hrs after L3/L4 ecdysis, resulted in 84%, 97.6% and 52.4% of lethality, respectively (Fig. 7A). A mild lethality, with 33.3% of larvae killed, was also observed for the treatment during 12–24 h after L3/L4 ecdysis. No impacts of 20E supplement on larval survivorship were observed for the treatment during the other time periods (Fig. 7A). These results are consistent with the previous reports that a transient pulse production and subsequent rapid decline of 20E are both necessary for successful larval-to-larval molt and metamorphosis[44] (Fig. 7B, left).

Given that males develop faster than females at each larvae stage, we proposed to induce female-specific lethality by supplementing 20E when most male larvae have already past the 20E pulse and the early period of L4 but the majority of female are just near the pulse when they are still highly sensitive to 20E treatment. To determine the best time window for this experiment, we chose four different time points at 25%, 50%, 75% and 90% of L3/L4 ecdysis for 20E treatment (Fig. 7B, right). The results showed that 20E supplementation only at 50% and 75% of L3/L4 ecdysis resulted in a significant female-specific lethality, resulting in 81.2% and 71.7% of male-bias, respectively (Fig. 7C). We thus selected the time point of 50% of L3/L4 ecdysis to confirm the potential of 20E supplement in inducing female-specific death for separating males from females. Remarkedly, we observed that 20E supplement resulted in 90.4% male-bias, significantly higher than the control groups with either no treatment (NTC) or treatment with its solvent DMSO (Fig. 7D). Moreover, all these male larvae treated with 20E pupated within 12 h and their pupation time was about 19 h and 23 h earlier than those from the control groups of DMSO and NTC, respectively (Fig. 7E). These results support the potential to develop a sex separation strategy by utilizing the sex differences in basal 20E during the larvae development.

Discussion

In this study, we have explored the role of 20E in assessing CW attainment in both *Ae. albopictus* and *Ae. aegypti* mosquitoes. Our results have revealed that 20E was used to assess the attainment of CW in these two mosquitoes. After L3/L4 molt, the basal 20E gradually accumulates to a threshold over time, followed by a constant period until the production of 20E pulse, which signals the onset of pupation. Based on RNA-seq and in vitro 20E supplementation, we have showed that the threshold 20E induces a pulse-like expression of spook. Given the essential roles of spook in 20E biosynthesis, these results indicate triggering the positive feedback loop between spook and 20E results in surpassing the critical threshold 20E. Furthermore, we have demonstrated that differences in basal 20E levels between males and females lead to earlier pupation in male mosquitoes. By precisely manipulating 20E levels within a specific timeframe, we have achieved a > 90% male bias in the mosquito population.

The basal 20E level plays a critical role as a timing signal for development

Critical weight (CW), which marks the transition from larval development to metamorphosis, has been extensively studied in two model species: *Manduca sexta* (Lepidoptera)[26,55,56] and *Drosophila melanogaster* (Diptera)[16,18,31,57]. In both species, a response to starvation was observed before reaching CW, leading to a delay in metamorphosis[17,27,31,57]. This is consistent with our observation that starvation significantly delays pupation in *Ae. albopictus* before reaching CW (Fig. 1A, B). However, while in *M. sexta*, the delay in metamorphosis was attributed to elevated levels of Juvenile Hormone (JH)[27], in both *Ae. albopictus* in this study and *D. melanogaster*[31], the delay resulted from suppressed production of 20E. We also observed that in *Ae. albopictus* and *Ae. aegypti* L4 larvae, the first 12 h after L3/L4 ecdysis was the most critical period affected by starvation, 20E supplementation, or CucB treatment, indicating that basal 20E levels, especially during early development, determine pupation time and the timing of the pulsatile release of 20E responsible for larval-pupal ecdysis through modulating the timing of threshold 20E for CW. Conversely, in *D. melanogaster*, basal 20E levels before reaching CW were not linked to pupation timing, and starvation delayed pupation by affecting the production of the CW ecdysone peak − the first of three minor peaks of 20E in the final instar of *Drosophila* − rather than basal 20E levels[15,16,28]. This suggests differential regulation of basal and pulse 20E synthesis in *Drosophila*. The developmental duration of the preceding three larval instars in *Ae. albopictus*, similar to the L4 stage, could also be influenced by basal 20E levels (Fig. 6A), indicating that basal 20E is a vital temporal cue governing the entire larval development, susceptible to modulation by environmental factors. While environmental conditions, such as nutrition, can affect CW timing in the final larval instar, the intrinsic factors involved may vary among different insect species. In *Aedes* mosquitoes, our results show that it is mediated through the modulation of basal 20E levels.

Critical weight is assessed by a threshold 20E level

Our results demonstrate that in *Ae. albopictus* L4 larvae, 20E levels gradually increased until they reached a small peak, precisely

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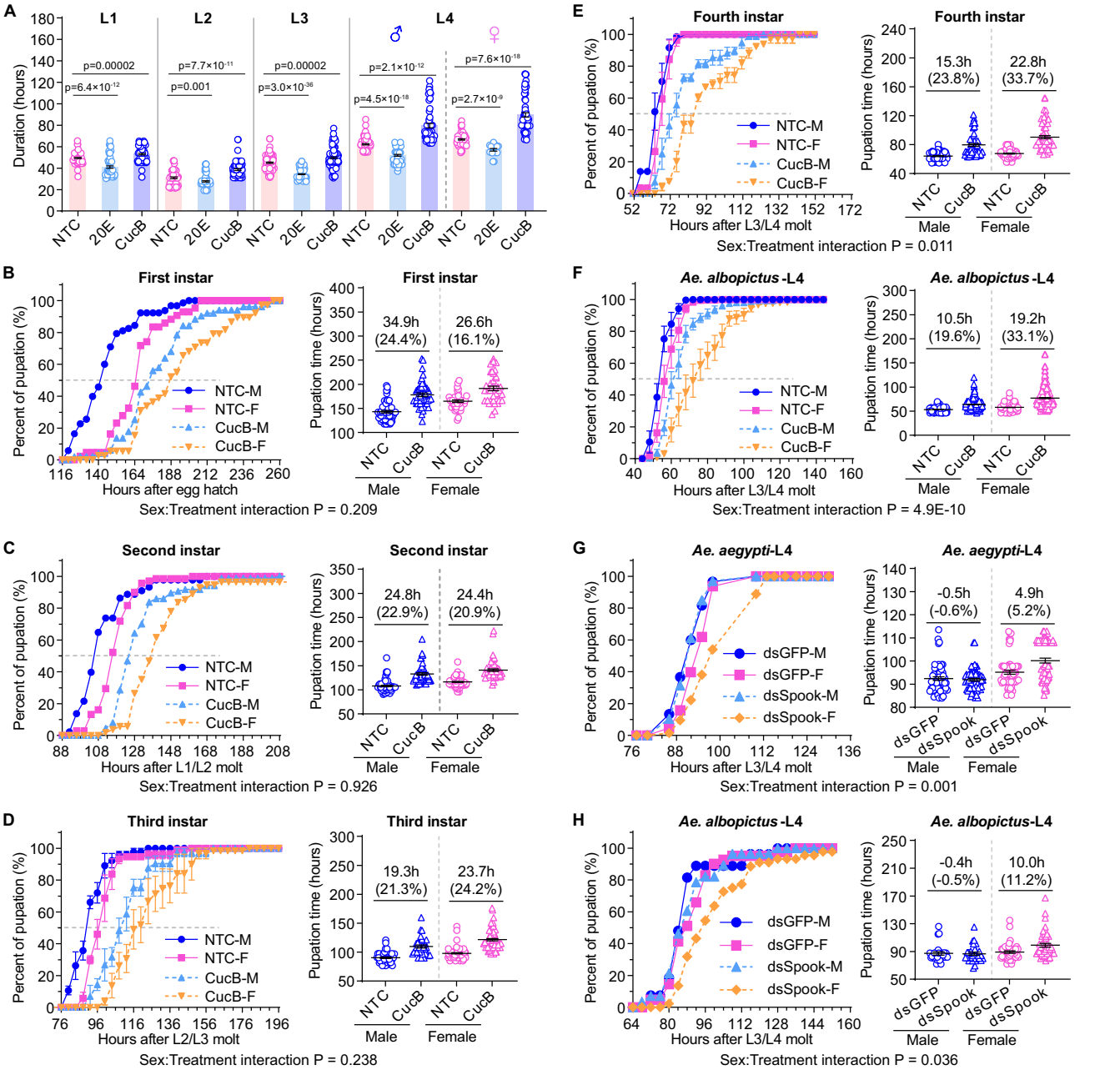

Fig. 6 | Female larvae are more sensitive to decreases in basal 20E levels during late instars. A The developmental times of each instar were modulated by the change in 20E levels using either 20E or CucB. Newly hatched L1 and newly emerged L2 to L4 larvae were collected and treated in 6-well plate with 80 μg/ml of 20E or 200 μg/ml of CucB for 12 hrs, and then reared under normal condition (n = 118, 89 and 117 for DMSO, 20E and CucB group of L1, respectively; n = 74, 101 and 88 for DMSO, 20E and CucB group of L2, respectively; n = 130, 136 and 138 for DMSO, 20E and CucB group of L3, respectively; n = 109, 73 and 62 for DMSO, 20E and CucB group of L4 male, respectively; n = 95, 19 and 69 for DMSO, 20E and CucB group of L4 female, respectively). **B**–**E** The impact of CucB supplementation during early development of each instar on pupation time of *Ae. aegypti*. Newly hatched L1 and newly emerged L2 to L4 were collected and treated with 200 μg/ml (L1 and L2) or 400 μg/ml (L3 and L4) of CucB for 12 h and then reared under normal condition. For **B**, n = 62 and 51 for NTC and CucB male, respectively; n = 43 and 38 for NTC and CucB female, respectively. For **C**, n = 66 and 54 for NTC and CucB male, respectively; n = 70 and 54 for NTC and CucB female, respectively. For **D**, n = 45 and 34 for NTC and CucB male, respectively; n = 82 and 74 for NTC and CucB female, respectively. For **E**, n = 65 and 62 for NTC and CucB male, respectively; n = 77 and 70 for NTC and CucB female, respectively. **F** The impact of CucB supplementation during early development of *Ae. albopictus* L4 on pupation time. Newly emerged L4 were collected and treated with 400 μg/ml of CucB as above. n = 246 and 231 for NTC and CucB male, respectively; n = 283 and 270 for NTC and CucB female, respectively. Left: Percent of pupae by the time. Right: Difference in pupation time between different treatment and sexes, with the percentage mean percent of increase in pupation time in treatment relative to control. The pupation time was the period from the time point of larval collection to pupation. **G, H** The impact of Spook RNAi during early development of L4 on sex difference in pupation time of *Ae. aegypti* (**G**) and *Ae. albopictus* (**H**), data were retrieved from Fig. 4H. Statistics: (**A**) two-tailed Student's *t*-test; (**B**–**H**) generalized linear model (GLM) analysis. Data shown are means ± SEM. Source data are provided as a Source Data file.

coinciding with CW attainment and the expression of E75 at this peak was consistently about 6-fold higher than in newly emerged L4 larvae (Figs. 1A, B, 3A, B). When the timing of CW attainment and ecdysteroid titers were analyzed separately in male and female L4 larvae (rather than in mixed sexes), we found once again that a small peak in ecdysteroid titers coincided precisely with the timing of CW attainment in both sexes. In each case, this small peak in ecdysteroid titers was approximately 3-fold higher than in newly emerged L4 larvae

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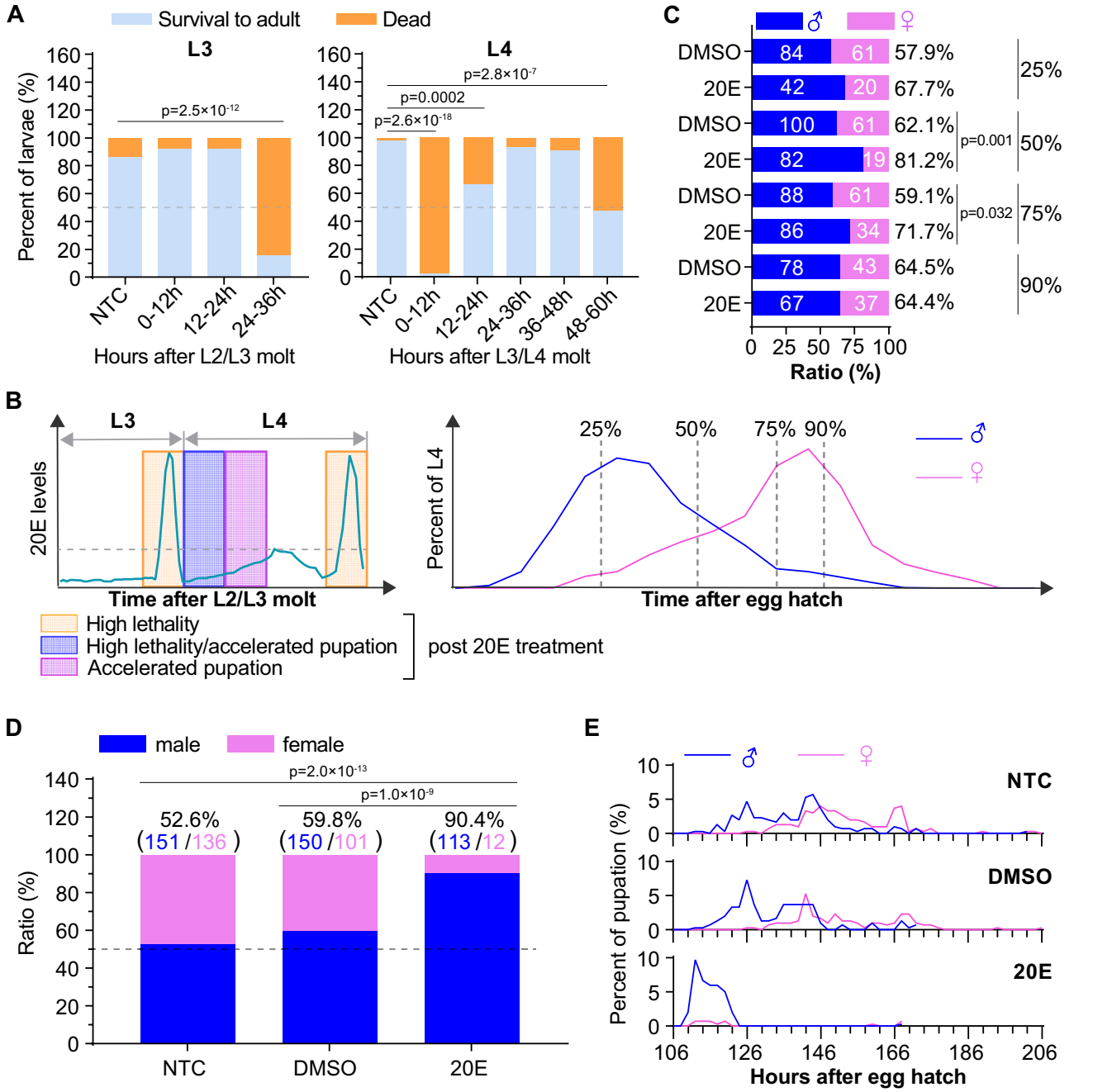

Fig. 7 | Supplementation of 20E induces female-specific lethality. A The impact of 20E treatment on larval survival to adults. Larvae were treated with 120 μg/mL 20E for 12 h at different time after ecdysis to L3 (Left) and at different time after ecdysis to L4. Groups of 21 larvae for L4 and 25 larvae for L3 were treated and reared in 6-well plate. Two biological replicates were conducted for each instar. **B** The models of 20E-induced female lethality and different development dynamics during L3/L4 molt between sexes. Left: 20E treatment near the time of L3/L4 molt and for newly emerged L4 is lethal, and 20E treatment from 12 h after L3/L4 molt and before the threshold 20E level for CW could significantly accelerate larval development. Right: There is sexual dimorphism in larval development time and male develops faster than female. The number of percent means percent of L3 that have already molted into L4. **C** The impact of 20E supplementation at different percent of L3/L4 molt on male ratio (the numbers inside the column mean the number of adults by sex). The impact of 20E supplementation at 50% of L3/L4 molt on male adult ratios (**D**) and pupation dynamics (**E**). Larvae were treated with 120 μg/mL 20E at 50% of ecdysis to L4 (**D** and **E**). The numbers above the column mean the number of adults by sex (blue: male; purple: female). NTC, larvae reared under normal condition; DMSO, larvae treated only with DMSO, the solvent of 20E. Statistic: (**A, C, D**) two-sided Chi-square test. Source data are provided as a Source Data file.

(Fig. 5G, Supplementary Fig. 3C). The influence of fluctuations in basal 20E levels on pupation time was most significant during early development, gradually diminishing with larval growth until CW was attained, at which point changes in 20E levels no longer affected timing (Fig. 3D, E). Similar to the effect of starvation on pupation time, which waned gradually until CW attainment, our findings indicate that CW coincides with a small peak in 20E levels which always reach a constant threshold level. Furthermore, we observed that L4 larvae, when supplemented with sufficient exogenous 20E after remaining fasted post L3/L4 ecdysis, could still pupate, and the percentage of pupation increased with higher 20E levels. This implies that 20E levels are crucial in determining whether and when a L4 larva will pupate. Since CW determines the duration of larval growth periods, and consequently the final adult body size[10,31], our findings also support that

body size in *Aedes* mosquitoes may be determined by basal 20E levels, with the final body size established when the threshold 20E level for CW is reached.

In other insect species, such as *Drosophila* and *Manduca*, CW is not a constant mass and can be influenced by various environmental conditions[56–58], suggesting the existence of intrinsic factors responsible for determining CW in these species. For example, in *Drosophila*, the CW ecdysone peak plays a crucial role in triggering CW attainment[25,31,59,60]. Unlike our findings, in *Drosophila*, the basal 20E levels before CW attainment either had no effect on pupation time[15,16,28] or higher basal 20E levels, induced by the deletion of the ban locus, delayed pupation[61]. In contrast, in Manduca, CW assessment is linked to oxygen limitation[56]. Given the impact of oxygen levels on basal ecdysone levels[62], it is possible that 20E is involved in regulating CW in Manduca as well.

In insects, the juvenile hormone (JH) is another important hormone that controls the timing of metamorphosis and, consequently, the duration of larval development[28]. In *Manduca*, JH has been recognized as a critical factor regulating the CW attainment in many studies. In the last instar larvae of *M. sexta*, CW attainment is followed by a decline in JH titer and an upregulation of JH esterase (JHE), a key enzyme that catabolizes JH, leading to its clearance of JH from the hemolymph. This elimination of JH disinhibits the secretion of PTTH and subsequent the PTTH-induced production of ecdysone[13,27,37]. Similarly, in this study, our RNA-seq analysis showed that JHE expression was significantly up-regulated, while the expression of two other enzyme genes, FPPP (farnesyl phosphatase) and FOHSDR (farnesol dehydrogenase), was significantly down-regulated at CW attainment; both these up-regulation and down-regulation were induced by 20E (Fig. 4C). These results suggest that although JH is involved in regulating CW attainment in both *Manduca* and *Aedes* mosquitoes, it operates in different ways. In *Manduca*, the clearance of JH leads to the final production of a 20E pulse, whereas in *Aedes* mosquitoes, the attainment of threshold 20E appears to lead to JH clearance, resulting in the final production of a 20E pulse. Nevertheless, the role of JH in regulating CW attainment and subsequent 20E pulse production remains unclear in mosquitoes, and further studies are needed to explore this issue.

In the insect world, cues for initiating metamorphosis are diverse among species. Apart from CW-induced metamorphosis, the absence of food has been shown to induce metamorphosis in other species like *Onthophagus Taurus*[63], *Psacothea hilaris*[64] and *Osmia lignaria*[10]. These findings indicate that insects have evolved distinct mechanisms to trigger metamorphosis and evaluate body size during development in response to varying environmental conditions and ecological niches.

A pulse-like expression of spook underlies onset of metamorphosis

Our findings in *Ae. albopictus* L4 revealed that the threshold 20E level for CW triggered a pulse-like expression of spook (Fig. 4D, E, G), and the timing of this peak expression showed a strong correlation with pupation timing (Figs. 3C, 4G). In addition, silencing spook using RNAi resulted in delayed pupation in both *Ae. albopictus* and *Ae. aegypti* (Fig. 4H). In insects, spook is one of Halloween family genes and a potential rate-limiting enzyme in the "Black Box" reactions in the upstream pathway for ecdysone biosynthesis[45,47–49]. Studies in *Manduca* and *Tribolium castaneum* have linked spook to ecdysone production stimulated by PTTH- and FoxO/insulin signaling[49,65]. In *D. melanogaster*, spookier, a paralogous gene of spook, plays a pivotal role during larval development as a target gene regulated by factors influencing ecdysone production[45,47,66]. Our results, combined with these previous findings, suggest that as a crucial target gene in the regulation of ecdysone biosynthesis, spook may play a significant role in the transition from larval development to larval-pupal metamorphosis upon achieving CW in *Aedes* mosquitoes. However, due to the low knockdown efficiency of RNAi through dsRNA soaking, spook RNAi in this study could only slightly but significantly delay the pupation time and did not have any detrimental effects on the pupation rate. In our mosquito system, it appears that the attainment of CW initiates a timer that induces pupation at a consistent time point. The threshold 20E level required for CW triggers a pulse-like expression of spook, acting as the starting signal for this timer. However, further research is necessary to elucidate the downstream components of this timer and its mechanisms.

Gender-based variations in 20E levels control sexual dimorphism in development

Male larvae typically exhibit faster development than their female counterparts in most insect species[53,54], a phenomenon also observed in the mosquito species as showed in this study (Fig. 5A). These sex-based differences in development time were consistent across all four larval instars in mosquitoes (Fig. 5B). We found that at the L4 stage, basal 20E levels were higher in males compared to females in both *Ae. aegypti* and *Ae. albopictus* (Fig. 5D, E). Given that the impact of fluctuations in basal 20E levels on development time during the three earlier larval instars mirrored that of L4 (Fig. 6A), it is a reasonable inference that sexual dimorphism in development time during these larval instars is also attributed to sexual differences in basal 20E levels. Previous studies have identified sexual differences in ecdysone levels in some other insects, primarily focusing on adults and finding that female adults often have much higher ecdysone titers than males, which is essential for female reproduction[67–69]. On the contrary, *Anopheles gambiae* exhibits sexual differences in ecdysone levels, with virgin males having higher ecdysone titers than virgin females; these ecdysone levels can be transferred to females during mating, likely influencing post-mating effects[36,70]. However, research on sexual differences in 20E levels outside the context of mating and reproduction has been relatively limited. A study in *Bicyclus anynana* provided conclusive evidence of sexual dimorphism in hormone titers driving the development of a sexual trait in insects for the first time[52]. They demonstrated that sexual differences in 20E levels during the wandering stage regulated sexual dimorphism in wing patterns of dry-season individuals. Our findings in this study indicate that sexual dimorphism in development time is driven by sex-based disparities in basal 20E levels, marking another sexual trait in insects regulated by a single steroid hormone. This suggests that sexual differences in 20E levels may be a more common phenomenon across insects than previously thought, playing a role in various aspects of development, maturation, and reproduction.

Currently, the mechanism underlying the induction of these sex differences in 20E levels remains unknown. Recently, differences in development time between sexes were often considered merely as a by-product of evolutionary adaptations towards sexual size dimorphism (SSD)[54]. SSD is prevalent across the animal kingdom, yet the underlying mechanisms remain a mystery[71]. Notably, in *Drosophila*, it is well-established that basal 20E levels exert negative control over animal growth, and elevated basal 20E levels can lead to reduced adult body size[15,46,72,73]. Male mosquitoes are notably smaller than their female counterparts. This SSD might extend throughout mosquito larval development, similar to observations in *Drosophila*[71]. Indeed, for at least the L4 stage in both *Aedes* species studied here, newly emerged males were smaller than females after the L3/L4 ecdysis. Consequently, this SSD in mosquitoes aligns with the negative influence of elevated basal 20E levels on animal growth in *Drosophila*, suggesting that the smaller body size of male *Aedes* mosquitoes may result from higher basal 20E levels in male larvae. In many animals, the development of sexual traits is typically controlled by sex determination pathways. The expression of sex determination genes such as transformer (tra) and sex-lethal (sxl) has been shown to play a role in driving larger female body size[71,74,75]. Thus, it is plausible that these sex determination genes

may influence body size by modulating the synthesis or release of 20E levels. Evidence of interactions between the doublesex gene and juvenile hormone signaling in the regulation of sex-specific mandible growth has been reported in the stag beetle *Cyclommatus metallifer*[76]. Collectively, the influence of basal 20E levels on mosquito larval growth and potential interactions between the expression of sex determination genes and 20E levels warrant further examination in future studies.

Sexual dimorphism in development is modulated for mosquito sex-separation

Both the sterile insect technique (SIT) and *Wolbachia*-based incompatible insect technique (IIT) have demonstrated their effectiveness in mosquito population suppression. However, one common challenge with both methods is the necessity to separate females from males before release as female mosquitoes are capable of causing nuisance biting and transmitting diseases[77]. Recent progress in sex separation techniques include leveraging size differences between male and female pupae, followed by pupal irradiation to sterilize any remaining contaminated females or using industrial vision systems and machine learning classifiers to further remove female adults[33,78,79]. However, these approaches are often time-consuming, require complex and costly machinery, or involve the use of transgenic mosquitoes, which often raise public concerns[77]. We have showed that supplementing 20E at 50% of L3/L4 ecdysis can result in a male-biased population of over 90%, with these males pupating approximately one day earlier than control groups (Fig. 7D, E). This innovative approach potentially introduces a novel concept for mosquito sex separation by modulating 20E levels during immature development, although the impact of early pupation on male competitiveness warrants further studies. While it may not entirely eliminate females, its effectiveness can be further enhanced by combining it with other methods, such as existing mechanical sorters, or its concept can be further improved by gaining a deeper understanding of the mechanisms governing sexual dimorphism in pupation.

Nutritional signaling is involved in threshold 20E-mediated onset of metamorphosis

The development of insect larval stages, including the rate and duration of growth, is vulnerable to changes in environmental factors like temperature and nutrition[80,81]. Similar to what occurs in Drosophila and Manduca, poor nutrition during the early development of the last instar larvae of *Ae. albopictus* retards the timing of pupation by inhibiting the gene expression of ecdysteroidogenic enzymes, thereby decreasing ecdysone production[18,82]. In *Aedes* mosquitoes, the timing of the 20E threshold for CW is regulated by the basal 20E levels during early development of L4. This means that poor nutrition affects the timing of the threshold 20E and thus the developmental time by decreasing the production of basal 20E levels. The conserved nutrient-sensitive signaling pathways, especially insulin/insulin-like growth factor (IIS) signaling pathways and Target of Rapamycin (TOR), respond to variations in diet quantity and quality to adjust growth rate and duration[81]. Studies have shown that, in *Drosophila*, the nutritional conditions of larvae are detected by the fat body via TOR and unknown pathways. Several peptide signals are then released from the fat body and target the insulin-producing cells in the brain, activating the IIS signaling pathway in PGs to modulate developmental time[83–85]. In *Aedes* mosquitoes, at least *Ae. aegypti*, ecdysone is synthesized in the thorax and abdomen, but not in PGs[86]. Therefore, it is highly possible that the regulation of developmental time by poor nutrition differs between mosquitoes and other insects. Since the tissues responsible for detecting nutrient levels and ecdysone production in *Aedes* mosquitoes might be the same, poor nutrition conditions could be communicated to the tissues responsible for ecdysone production immediately. Further work needs to be done to investigate the underlying mechanisms through which poor nutrition delays the developmental time of L4 larvae of *Aedes* mosquitoes.

In summary, our findings highlight that CW assessment in both *Aedes* mosquito species relies on a specific threshold level of 20E and a positive feedback loop between spook and 20E may be a critical factor in achieving CW. Our results also underscore the significant role of basal 20E levels as a key developmental timing signal in *Aedes* mosquitoes and as a determinant of sexual dimorphism in development time. Given that the impact of 20E on molting and metamorphosis is evolutionarily conserved within the Arthropoda, it's plausible that the regulatory mechanisms involving basal 20E levels in sexual dimorphism and the use of threshold 20E for CW assessment might also apply to other insect species. Moreover, we have utilized this knowledge to develop a potentially novel concept for sex separation, which holds promise not only for mosquito disease vectors but also for agriculturally important pests to facilitate the development of SIT and IIT-based population control strategies.

Methods

Mosquito strain and rearing

Ae. albopictus with Guangzhou origin, *Ae. aegypti* (AFM) with Mexican origin, and *Ae. aegypti* Aaeg-M strain with male-specific GFP expression, a kind gift of Dr. Eric Marois (INSERM, France)[87], were all maintained in a climate-controlled room at 27 ± 1 °C and 80 ± 10% relative humidity with a 12:12 h (light: dark) photoperiod. Adult mosquitoes were provided with 10% glucose solution. For egg production, adult mosquitoes that have starved for 24 h were blood-fed on commercial human blood (ZenBio, SER-WB) for 30 min and were allowed to oviposit on wet filter paper 3 days later.

Larval rearing and starvation

Larvae were reared in the above climate-controlled room at about a density of 1 larva/mL in de-chlorinated water or RO water. For *Ae. albopictus*, larval diet (6.0%) composed of bovine liver powder, shrimp powder and yeast powder[88] and provided as: 0.21, 0.45 and 0.64 mg/larva from day 1 to 3, respectively, and 1.0 mg/larva in the next days. For *Ae. aegypti*, larval diet composed of 6% bovine liver powder and provided as: 0.1, 0.2, 0.3 and 0.6 mg/larva from L1 to L4, respectively. Under this rearing regime, the L3/L4 molt usually happened on day 4 and the L4 began to pupate usually at day 6. In this study, the larvae with white heads were collected as newly emerged larvae when needed in all of our analysis. To determine the timing of the attainment of CW for *Ae. albopictus*, newly emerged L4 were reared individually in 24-well plates containing 2 mL water per well to prevent cannibalism and starved by changing rearing water with fresh water without food at required time points. The critical time point for CW was determined according to the published methods with a minor modification[89].

20E and CucB treatment

The 20-hydroxyecdysone (Selleck) and its antagonist Cucurbitacin B (CucB) (Cayman)[52] were used to modulate 20E signaling during mosquito immature development. Both chemicals were dissolved in DMSO as a 40 mg/mL for stock. For treatments, groups of newly hatched or emerged larvae were collected and maintained in 6-well plates with 10 mL fresh water per well that added with larval diet and different concentrations of these two drugs for 12 h. The control groups were treated in the same way with DMSO only. After treatments, larvae were transferred to disposable plastic bowls or cups and reared under normal rearing condition. For the experiment of 20E induced male-bias, the eggs of *Ae. aegypti* were hatched by vacuum for 30 min and reared in groups of 1000 to 2000 larvae per liter of RO water. About 30 to 50 larvae were transferred to a disposable plastic cup with 90 mL RO water at about 12 h before the beginning of L3/L4 ecdysis. Ecdysis was checked every 1 to 2 h after the beginning of L3/L4 ecdysis to determine the time point for 20E treatment. The larvae were treated with

20E as above and transferred to plastic cups after treatment and reared under normal condition.

Pupation time determination

To determine the pupation time, the L4 was checked for pupation every 3 to 4 h after the first pupa emerged. The earliest time of pupation was launched at about 2 days after L3/L4 molt under normal rearing condition, whereas the L4 reared with 120 μg/mL 20E pupated usually at 36 h after L3/L4 molt. The pupation time of a L4 larva was defined as the duration of L4 development and the mean pupation times were calculated and compared between different groups. To determine when the CW was attained, we reared newly emerged L4 with food, and then starved them after different periods of feeding and pupation times were recorded at 3 hrs interval after the beginning of pupation. To compare the timing in the attainment of CW between male and female, newly emerged *Ae. albopictus* L4 larvae were collected and reared under normal condition, and then were divided into the groups of feeding and starvation at 18 to 42 hrs after L3/L4 molt at 3 h interval. Pupation time was recorded at 2-3 h interval after the beginning of pupation.

RNA-seq analysis

For groups reared under normal condition, *Ae. albopictus* larvae were collected at 0 and 30 h after L3/L4 ecdysis, and for 20E treatment, newly emerged L4 were soaked in water with 80 μg/mL 20E for 12 h and collected in Trizol solution immediately after soaking. Total RNA was extracted from three biological replicates of the above samples using Trizol (Invitrogen) according to manual instruction. The final RNA pellet was dissolved in Nuclease-free water. The total RNA was qualified and quantified using Agilent 2100 bioanalyzer (Thermo Fisher Scientific). cDNA synthesis, library construction, and RNA sequencing was performed on BGIseq-500 platform (BGI-Shenzhen, China). The clean reads were mapped to the reference genome of *Ae. albopictus* (GCF_001876365.2_canu_80X_arrow2.2) using Bowtie2. The gene expression levels were normalized to FPKM using RSEM. Differentially expressed genes were identified by DEGSeq2 and genes with ≥ 2-fold change and Q-value ≤ 0.001 were considered to have significant difference.

dsRNA synthesis

For dsRNA synthesis, cDNA from L4 was used as template for PCR. A T7 promoter sequence was added to the 5′ end of primers (Supplementary Table 1). PCR product was purified using NucleoSpin Gel and PCR Clean-up (TaKaRa) after cutting from gel analysis. dsRNA was synthesized by using T7 RiboMAX^{tm} Express RNAi System (Promega). dsGFP was also synthesized for larval RNAi as control.

Larval RNAi

For silencing spook using RNAi, groups of 25 newly emerged L4 of *Ae. albopictus* and *Ae. aegypti*, with 3 to 6 replicates for each group, were collected and transferred into a 1.5 mL centrifuge tube. The larvae were washed twice with 500 μL Nuclease-free water and then 600 μL Nuclease-free water with 1 μg/μL dsSpook or dsGFP was added. The larvae were soaked with the dsRNA for 15 h, then transferred into fresh water and reared with food for 4 h. After this short period of feeding, larvae were soaked with 1 μg/μL dsRNA for another 15 h. Subsequently, larvae were transferred into fresh water and reared under normal conditions until pupation. Pupation time of these larvae were determined and compared between different groups. For RNAi efficiency determination, groups of 25 newly emerged L4 were treated as above. Larvae were collected in Trizol solution at 20 h after soaking with 3 larvae/replicate and 8 biological replicates per group for *Ae. albopictus*, and 8 larvae/replicate with 3 biological replicates per group for *Ae. aegypti*. Total RNA was extracted, cDNA was synthesized and Real-time PCR was conducted.

Real-time PCR

For determining the expression profiles of E75, about 10 L4 were sampled individually at 3 h intervals. For analyzing the impact of starvation, 20E and CucB on the expression of E75, EcR and Usp, groups of larvae were sampled for each treatment group. For determining the impacts of 20E and starvation on expression profiles of E75 and Spook, 4 or 6 pools of 5 L4 were sampled at 2 to 6 h intervals. For determining the E75 expression between sexes, about 30 larvae were sampled individually in Trizol. Sex was determined by the expression of Nix. When the *Ae. aegypti* Aaeg-M strain was used to analyze the expression of E75 between sexes, 8 biological replicates with 3 larvae per replicate were sampled for each sex, which was determined by the presence or absence of GFP expression. The E75 transcripts used in this study are a common region for *Ae. albopictus* and E75A for *Ae. aegypti*, respectively. The total RNA was extracted from the above samples using TRIzol (Invitrogen) and cDNA was synthesized using HiScript II Q SuperMix (+ dDNA wiper) (Vazyme) following the manufacturer's protocols. Quantitative real-time PCR (qPCR) was performed using LightCycler96 (Roche) and TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa). Primers were listed in Supplementary Table 1. The relative expression levels of target genes were normalized to rpS7 for *Ae. albopictus*[90] or rpS6 for *Ae. aegypti*[91] based on the $2^{-\Delta\Delta CT}$ calculation method[92].

Quantification of 20E titer in hemolymph and whole body of L4 larvae

Hemolymph at 24 h after starvation of *Ae. albopictus* L4 larvae was collected as previously described[93]. In brief, the decapitated L4 larvae were transferred to a 0.5 mL centrifuge tube with several holes at the bottom punctured with a 1 mL syringe and the tube was placed into a 1.5 mL centrifuge tube with 0.5 μL of 30 mg/mL phenylthiourea at the bottom. Hemolymph was collected at the bottom of the outer tube after centrifuging at 375 × *g* for 10 min at 4 °C. Then the same volume of hemolymph was used for ecdysone extraction with methanol. To compare 20E titer in whole body between sexes, newly emerged L4 larvae were collected at 12 hrs and 24 hrs after L3/L4 molt for *Ae. albopictus* and *Ae. aegypti* (Aaeg-M), respectively. For the Aaeg-M strain of *Ae. aegypti*, the sex of larvae was determined by the expression of GFP. Larvae were collected in pools of 3, with 5 to 6 pools for each sex. For *Ae. albopictus* L4 larvae, larvae were collected individually, and the sex was determined by the existence of Nix in head and the carcass without head was used for ecdysone extraction. To determine and compare the temporal profiles of ecdysteroid titers between male and female larvae of *Ae. albopictus*, newly emerged L4 larvae were collected, and 30 larvae were sampled individually at 3 to 6 h interval till pupation. For each larva, head was used for determining the sex and the carcass without head were pooled for ecdysone extraction as 3 larvae per pool and 4 pools were used for each time point of both sexes. For ecdysone extraction, samples of whole body or carcasses without head were homogenized in 200 to 300 μL of methanol and hemolymph was also mixed with 200 μL of methanol by thoroughly vortexing. Next, the samples were centrifuged at 13,000 × *g* for 10 min at 4 °C and the supernatant was transferred to another 1.5 mL centrifuge tube. Ecdysone was extracted by lyophilized with vacuum and was dissolved with EIA buffer. 20E titer was measured by using ELISA kits (Cayman Chemical and mlbio) according to manufacturer's instructions.

Statistical analysis

SPSS statistics 19.0 was used for carrying out statistical analyses. The data shown are mean ± SEM. One-way analysis of variance (ANOVA) with Bonferroni's multiple comparison and LSD multiple comparison tests were used to compare between multiple groups and Student's *t* test was used to compare other groups with control. Chi-square test was used to analyze the data of percentage.

Reporting summary
Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability
The raw data of RNA-seq generated in this study have been deposited in the NCBI database under accession code PRJNA1196416. All data needed to evaluate the conclusions are present in the paper or the Supplementary Materials. Source data are provided with this paper.

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Author contributions

M.Z. and Z.X. designed all experiments. M.Z., Q.S., D.Z. Y.L., H.W. and A.X. performed all experiments. M.Z. analyzed the data. J.B. contributed material. M.Z. and Z.X. provided funding. Z.X., X.Z., Y.W. and J.C. provided supervision. M.Z. and Z.X. wrote the manuscript with input from all other authors, and all authors contributed to the submitted version.

Competing interests

Z.X. is a co-founder and shareholder of Guangzhou Wolbaki Biotech Co., Ltd and a member of its scientific advisory board. The other authors declare no competing interests.

Additional information

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