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Early behavioral and molecular events leading to caste switching in the ant *Harpegnathos*

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Ant societies show a division of labor in which a queen is in charge of reproduction while nonreproductive workers maintain the colony. In *Harpegnathos saltator*, workers retain reproductive ability, inhibited by the queen pheromones. Following the queen loss, the colony undergoes social unrest with an antennal dueling tournament. Most workers quickly abandon the tournament while a few workers continue the dueling for months and become gamergates (pseudoqueens). However, the temporal dynamics of the social behavior and molecular mechanisms underlining the caste transition and social dominance remain unclear. By tracking behaviors, we show that the gamergate fate is accurately determined 3 d after initiation of the tournament. To identify genetic factors responsible for this commitment, we compared transcriptomes of different tissues between dueling and nondueling workers. We found that juvenile hormone is globally repressed, whereas ecdysone biosynthesis in the ovary is increased in gamergates. We show that molecular changes in the brain serve as earliest caste predictors compared with other tissues. Thus, behavioral and molecular data indicate that despite the prolonged social upheaval, the gamergate fate is rapidly established, suggesting a robust re-establishment of social structure.

[*Keywords*: antennal dueling; caste transition; social behavior; social insect; juvenile hormone]

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Phenotypic plasticity constitutes the capacity of an animal to undergo dramatic changes in its physiology and behavior under different environmental conditions. While model organisms are very useful in advancing our understanding of early developmental plasticity, they often do not show robust and dramatic phenotypic changes at the adult stage. Social insects, on the other hand, show phenotypic plasticity throughout their lives (Calabi and Traniello 1989; Gordon 1989; Penick et al. 2011; Herb et al. 2012; Yan et al. 2014; Simola et al. 2016; Gospocic et al. 2017; Opachaloemphan et al. 2018; Glastad et al. 2020), being able to change caste and dramatically alter their behavior to support the needs of the colony.

The division of labor among castes is a hallmark of nearly all ant societies in which queens reproduce while workers partake in age-dependent behaviors (polyethism): Young workers take care of the brood, while old workers forage for food and defend and maintain the nest. The age-based adult polyethism is plastic as the nurse-to-forager transition is reversible (Gordon 1989; Herb et al. 2012; Yan et al. 2014; Simola et al. 2016; Gospocic et al. 2017; Glastad et al. 2020). This behavioral plasticity in adult workers accommodates changes in a colony when nursing or foraging tasks are needed, thus enhancing colony fitness (Calabi and Traniello 1989; Gordon 1989; Herb et al. 2012; Yan et al. 2014). Although reproduction in social insect colonies is restricted to a dominant reproductive caste, functionally sterile workers in some ant species can differentiate into reproductive pseudoqueens, taking over and continuing reproductive roles in a queenless colony. This reproductive caste transition occurs naturally and is remarkably plastic in the Indian jumping ant *Harpegnathos saltator*, which shows fascinating superorganism resilience, allowing the colony to tolerate the loss of its germline; i.e., the reproductive individuals (Peeters

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et al. 2000; Hölldobler and Wilson 2008; Straub et al. 2015).

In social insect societies, all colony members share the same reproductive interest; i.e., to maintain the reproductive output of the colony. Selfish behavior is therefore unusual. However, conflicts periodically arise following disruption of the dominance hierarchy in these societies (Heinze et al. 1994; Frank 1995; Hölldobler and Wilson 2008). The production of new queens or the absence of reproductive females leads to intra-colony conflicts among newborn reproductive females. In monogyne (single-queen) colonies, new queens engage in a deadly fight or lead the colony to separate into subcolonies, each with a single queen (Heinze et al. 1994; Ross and Keller 1998). In the ant *H. saltator*, each newly fertilized queen founds a new colony and gives rise to all workers in the colony, as well as seasonal males and virgin queens (Frank 1995; Peeters et al. 2000). The innate ability of *H. saltator* workers to reproduce is suppressed by queen pheromones, and those workers that escape such pheromone regulation are severely policed upon attempting to lay eggs (Sasaki et al. 2016; Opachaloemphan et al. 2018). However, upon death or waning reproductive output of the queen, *H. saltator* workers quickly become involved in a dueling tournament to determine which workers will become the reproductives of the colony (Liebig et al. 1999). *H. saltator* workers duel with each other by trading strikes with their antennae (antennal dueling) (Fig. 1A; Supplemental Movie S1), and the tournament is often performed for months. Individuals with high dueling activities eventually become gamergates or pseudoqueens, as it takes several gamergates that share social dominance hierarchies to replace the larger senescent/deceased queen (Peeters et al. 2000; Sasaki et al. 2016). Gamergates show extensive adult

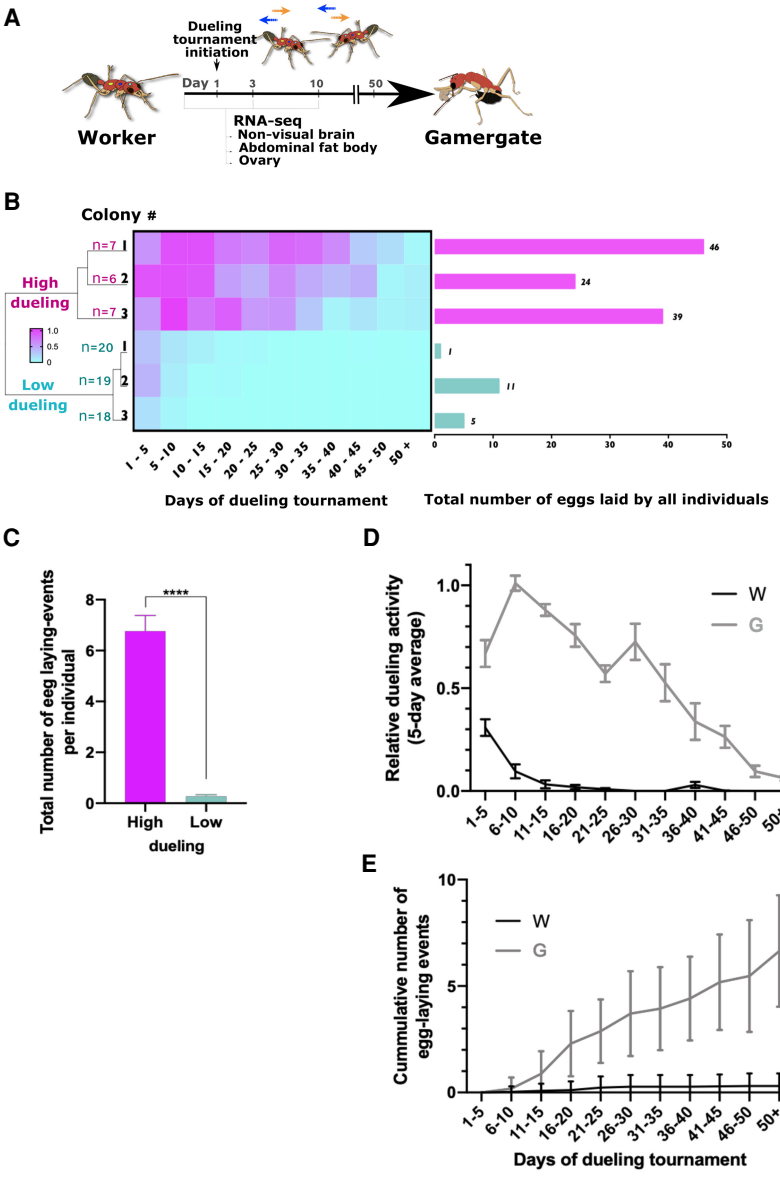

Figure 1. Persistent antennal dueling behavior results in social dominance and reproductive succession in *Harpegnathos saltator* workers. (*A*) Schematic of the worker-to-gamergate transition and tissue sampling at different time points. In a queenless colony, nonreproductive workers labeled with a unique color code initiate an antennal dueling battle among each other. The duelers eventually display queenlike phenotypes (egg-laying and nonworker behaviors) after 50 d of the dueling tournament. Three different tissues, including the nonvisual brain, abdominal fat body, and ovary, were collected for RNA-seq experiments at three different time points: prior to the transition setup, and on day 3 and day 10 after the beginning of the dueling tournament. (*B*) Two groups of workers were classified based on the antennal dueling activity during the worker-to-gamergate transition. Hierarchical clustering analysis of the dueling activity over 50 d after initiation of the dueling tournament classifies workers into high- and low-dueling clusters. A heat map displays average dueling activity of each 5-d window for over a 50-d period. Total numbers of egg-laying events observed in high- and low-dueling individuals from three independent colonies are shown in the *right* panel. The number of high- and low-dueling individuals (*n*) are represented in magenta and cyan, respectively. (*C*) Average numbers of egg-laying events per individual in the high- and low-dueling groups during the 50-d period. (Magenta) High-dueling; (cyan) low-dueling. (****) $P < 0.0001$, Mann–Whitney test. $N = 20$, 57, respectively, Bars and errors represent mean ± SEM. (*D*) Differential patterns of antennal dueling activity and egg-laying events in worker and gamergate castes. Worker (W) and gamergate (G) fates were behaviorally and physiologically determined after the 50 d of the dueling tournament. Antennal dueling activities of W and G (5-d average) over the 50-d period. (*E*) Cumulative numbers of the observed egg-laying events in workers (W) and gamergates (G) over the 50-d period. (W) Black; (G) gray. $N = 3$ colonies. Bars and errors represent mean ± SEM.

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plasticity: Their social behavior changes, their ovaries are activated, and their life span increases dramatically, allowing these former workers to share the reproductive output normally assigned to the larger queen. Death or senescence of the gamergates normally reinitiates a dueling tournament, thus fostering the maintenance of a healthy queenless colony (Liebig et al. 1999; Peeters et al. 2000; Hölldobler and Wilson 2008). These remarkable changes during caste transition to gamergate in *H. saltator*, as well as the ability to manipulate this transition, make *H. saltator* an attractive model for studying the molecular basis of social behaviors, colony dynamics, and phenotypic plasticity (Peeters et al. 2000; Opachaloemphan et al. 2018).

Antennal dueling showed during the worker-to-gamergate transition is crucial for the formation of dominance hierarchies in *H. saltator*, as a "winner–winner" interaction promotes the shared hierarchy in dueling workers (Sasaki et al. 2016). However, before sustained dueling occurs among gamergates, most workers attempt to enter dueling but quickly abandon this pursuit and return to their duties in what is a "winner–loser" competition. The temporal dynamics of this social behavior and the molecular mechanisms that underlie the caste transition and acquisition of social dominance by some of the workers remain unclear and understudied. Here, we tracked the dueling behavior of workers over 50 d after the loss of the queen/gamergate. We found that individuals that eventually become gamergates show distinct behaviors as early as 3 d into the dueling process. This predictability of a future gamergate prompted us to determine the molecular changes associated with the worker-to-gamergate transition: We collected the nonvisual brain, abdominal fat body, and ovary from individuals at early time points (days 3 and 10) of the dueling tournament for transcriptomic analyses. These three tissues constitute a circuit that regulates plasticity of behavior and reproduction (Corona et al. 2007; Arrese and Soulages 2010; Libbrecht et al. 2013; Gospocic et al. 2017; Chandra et al. 2018). Our results indicate that the brain serves as a key tissue for caste differentiation and triggers hormone-associated changes in peripheral tissues. Collectively, the new social dominance hierarchy is behaviorally and molecularly determined at very early stages of the dueling tournament following the disturbance of social dominance structures, pointing to an effective restoration of colony structure in *H. saltator*.

Results

Antennal dueling frequency determines the transition to gamergate fate

To determine how antennal dueling dynamics correlates with gamergate establishment, we set up three transition colonies with 30 age-matched (~14-d-old posteclosion) young workers and 10 (>90-d-old) older workers in the absence of queen or gamergates. The older workers, which forage and perform other colony maintenance tasks, were introduced to maintain the colony health and were not analyzed as they did not contribute significantly to dueling. All workers were uniquely painted with a three-color-code system, allowing us to distinguish each individual and trace its activity (Fig. 1A; see the Materials and Methods). During an adaptation period of 5–7 d following the colony setup, workers huddled in the colony nest and groomed each other. This period of peace was disrupted when groups of workers began to duel. The first observation of dueling was denoted as day 1 in order to keep the time scales consistent between colonies given the variability in the onset of dueling. The antennal dueling activity of each individual (see the Materials and Methods) was monitored by video camera over 50 d of the dueling tournament; dueling occurrences were scored in 20-min windows every 45 min every day. We measured the frequency at which individuals displayed dueling behavior per day, and the egg-laying events of all individuals in the three different colonies for 50 d. Hierarchical clustering of the dueling activities over this 50-d period from the three colonies showed that there are two clearly distinct groups of workers: high- and low-dueling individuals. High-dueling workers actively and constantly duel (trading strikes with their antennae) during the caste switch, while individuals with no or only transient dueling are considered as low-dueling workers (Fig. 1B). Colony 1 consisted of seven high-dueling and 20 low-dueling individuals, colony 2 had six high and 19 low duelers, and colony 3 had seven high duelers and 18 low duelers (Fig. 1B); few animals (three, five, and five, respectively) died during the tournament in the three colonies. The total number of egg-laying events was significantly higher in the high-dueling group compared with that of the low-dueling group (Fig. 1B,C). Each high-dueling individual laid ~6.8 eggs in total, while the low duelers laid 0.3 egg on average during the 50-d period (Fig. 1C). These findings highlight that the well-known association between antennal dueling behavior and reproductive potential in *H. saltator* is very robust (Hölldobler and Wilson 2008; Sasaki et al. 2016).

After observing the behavior of the ants for the 50 d of the tournament, we were able to define the behavioral features of individuals that would remain workers versus those that would become gamergates: Workers performed nest cleaning and cricket hunting/foraging while gamergates continued antennal dueling and kept laying eggs. In addition, gamergates showed slow locomotion inside the nest and often held an upright posture on a brood pile, whereas workers were outside of the nest and displayed submissive posture near gamergates (Supplemental Fig. S1A). We then quantified the daily antennal dueling activity from day 1 to 50: The overall dueling activity of gamergates was dramatically higher than that of workers (Fig. 1D), consistent with the dueling classification obtained from the hierarchical clustering analysis (Fig. 1B). During days 1–5 of the dueling tournament, members of both castes joined the dueling, but the gamergates showed higher dueling activity relative to workers. Individuals that would become gamergates increased their dueling activity and reached the peak during the day 6–10 period, whereas the dueling activity of workers

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decreased (Fig. 1D). Later on, gamergates showed intensive dueling activity that persisted for at least another 45 d, whereas workers rapidly diminished their dueling activity after 5 d and subsequently rarely joined the battle (Fig. 1D). After 10 d, gamergates often laid eggs, but it was a rare event in workers (Fig. 1E). In summary, our behavioral observations confirmed that initial antennal dueling is a robust behavioral marker of the gamergate fate and that the tournament peaks as early as 5–10 d after its onset.

Early antennal dueling behavior determines gamergate identity

Although several studies have shown an overall correlation between dueling behavior and gamergate development (Peeters et al. 2000; Sasaki et al. 2016), the temporal dynamics of the dueling battle had remained unexplored. According to our behavioral observations, the peak of the dueling tournament arises at its very early stages (Fig. 1B,D). We therefore examined the early dueling dynamics of the worker-to-gamergate transition. First, we performed clustering analysis using the dueling data on days 1–7 in each colony with two main clusters of high- and low-dueling individuals (Fig. 2A; Supplemental Fig. S1B). Because of the dynamics and the colony instability in the early stages of colony re-establishment, some true workers (as defined after day 50) initially displayed high dueling activity. For one of the colonies (colony 3), this unsupervised learning method classified seven individuals as high duelers after 7 d postinitiation of the tournament, while the remaining 18 were categorized as low duelers. Six out of the seven high duelers subsequently became true gamergates, while all of the low duelers remained workers (Fig. 2A). The results in the two other colonies showed similar early divergence between high- and low-dueling patterns (Supplemental Fig. S1B). We also checked cumulative early dueling data over the first 7 d and performed statistical analysis to determine the earliest time we could identify significant differences between the dueling activities of workers and gamergates: Gamergates showed a significant increase in cumulative dueling activity within the first 5 d and consistently showed greater differences at later time points (Fig. 2B). These data suggest that the dueling divergence starts 2 d postinitiation of the tournament and should allow us to predict worker from gamergate fates at the very beginning of the dueling tournament.

To determine how early we could confidently predict the caste fate based on behavioral data, we performed receiver operating characteristic (ROC) analysis to define the dueling percentage value that distinguishes gamergates from workers over the initial 7 d of dueling (Fig. 2C; see the Materials and Methods; Supplemental Fig. S1C; Supplemental Table S1). A cutoff dueling percentage (recordings of dueling in each day/total recording in each day) that maximized gamergate and worker identification based on dueling was calculated from day 1 through day 7 (AUC = 0.9333, $P < 0.005$) revealing a cutoff value of 31.57% (LR = 5.6) (Supplemental Table S1). The cut-off dueling percentage was applied each day, and four values were calculated: positive predictive value (PPV), representing the ratio of true gamergates to all individuals in the colony; negative predictive value (NPV), a similar ratio involving true workers; sensitivity, the ratio of true

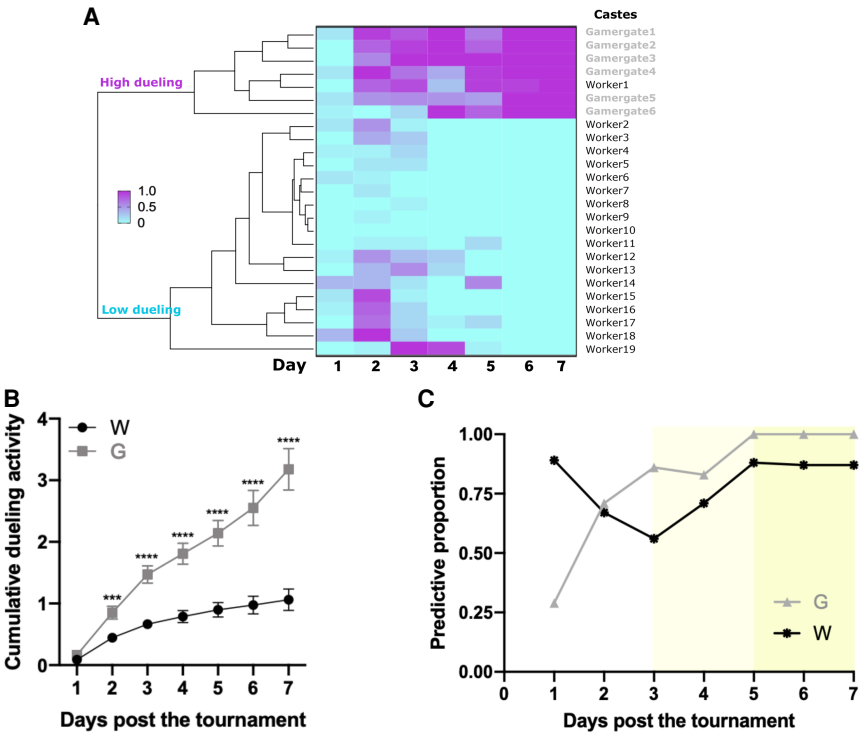

Figure 2. Antennal dueling behavior rapidly determines the gamergate (pseudoqueen) fate within the first week of the dueling tournament. (*A*) Hierarchical clustering of the early dueling activity from day 1 to 7 from a representative colony differentiates all ants into two main clusters (high-dueling [magenta] and low-dueling [cyan] clusters). Each row represents a single individual. Six out of seven high-dueling individuals in the upper cluster are true gamergates. The low-dueling individuals are all true workers. True caste fates were determined after the tournament and are indicated at the *right*. (*B*) Cumulative dueling activity during the first week of the dueling tournament in workers and gamergates (black and gray, respectively). $N = 3$. (***) $P < 0.001$, (****) $P < 0.0001$, multiple *t*-test. Dot and errors represent mean ± SEM. (*C*) Accuracy of the worker and gamergate fate prediction based on the early dueling activity on each day. On day 3 postinitiation of the tournament, the ratio of true gamergates to predicted gamergates or sensitivity is 0.86. The ratio of true workers to predicted workers or specificity is 0.56. The highest ratio of true gamergates to predicted gamergates begins on day 5 postinitiation of the tournament. (W) Black; (G) gray.

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to predicted gamergates; and specificity, the ratio of true to predicted workers (Fig. 2C; Supplemental Table S1; Supplemental Fig. S1C). We found that based on the first week of dueling data, the unsupervised clustering identified future gamergates with 86% accuracy on day 3 postinitiation of the dueling tournament (Fig. 2C). The predictability of gamergates (sensitivity) and workers (specificity) increased every day and reached its peak on day 5 (Fig. 2C). Thus, worker and gamergate fates can be accurately predicted based on their dueling activity after the first 3 d of the dueling tournament. Furthermore, the response to the disrupted colony structure rapidly leads to the specification of gamergates likely to assure a rapid resolution of the disordered social dominance structure.

Early molecular changes in nondueling and dueling individuals

Early dueling behavior and its maintenance are a clear indication of gamergate determination that must be reflected by physiological and molecular changes in the dueling workers. We therefore set up new colonies for gene profiling. We collected three different tissues, the nonvisual brain, the abdominal fat body, and the ovary, of duelers and nonduelers at different time points postinitiation of dueling and acquired their tissue-specific transcriptomes through mRNA sequencing. These tissues have been shown to contribute to a circuit involved in plasticity (Corona et al. 2007). We sampled five biological replicates of both duelers and nonduelers at 3 and 10 d postinitiation of the dueling tournament (from independent colonies to avoid disturbing the colony), as well as five individuals prior to the colony transition as a baseline (presetup). While after day 5, our worker and gamergate average prediction efficiencies were 81% and 95% respectively, this was not the same at day 3. To ensure that we select accurately prospective workers and prospective gamergates at day 3, we selected individuals that either were showing almost no dueling activity (presumptive workers) or were showing constant and high dueling activity for the entire 3-d period from day 1 to day 3 (presumptive gamergates).

Principal component analysis (PCA) showed that the tissue-specific transcriptomes were clustered tightly by tissue type but not by dueling activity (Supplemental Fig. S2A), which is expected given that these tissues are quite distinct. The first two principal components did not distinguish the transcriptomes of the brain or fat body between the dueling and nondueling workers; however, the ovarian transcriptomes of day 10 duelers were well separated from earlier ovarian gene profiles (Supplemental Fig. S2A, light green triangle). This is likely due to the rapid and extensive morphological changes accompanying the activation of the ovary and maturation of oocytes in the day 10 duelers (Supplemental Fig. S2B). Such dramatic changes were not observed in the brain and fat body of immature gamergates. Next, we performed differential gene expression analysis between duelers and nonduelers in the three tissues. While we did not find any differentially expressed genes (DEGs) in the brain and ovaries at day 3, we identified 22 DEGs in the fat body (Fig. 3A, top; Supplemental Table S2). At day 10, we found 60 DEGs in the brain, 531 DEGs in the fat body, and 3740 DEGs in the ovary (adjusted P-value < 0.05) (Fig. 3A; Supplemental Table S2). The latter number again likely reflects activation of the ovary at day 10.

To test whether day 10 DEGs between duelers and nonduelers showed any changes in each tissue at day 3, we performed logistic regression for each tissue to train a classifier using the expression profiles of day 10 DEGs at day 3 as predictors of caste fate (either worker or gamergate). We used eight of the 10 replicates (four worker and four gamergate data sets) to train the algorithm, and the remaining two replicates served as a test data set (see the Materials and Methods). Interestingly, day 10 DEGs of the brain did serve as an efficient classifier of workers and gamergates by day 3, despite not being significantly differentially expressed at this earlier time (Fig. 3B). In contrast, the 531 DEGs identified at day 10 in the fat body were not able to classify castes at day 3 (~50% accuracy for two possible outcomes) (Fig. 3B′), and reciprocally, the 22 DEGs of the day 3 fat body failed to classify castes at day 10 (Supplemental Fig. S2C). Although the fat body was the only tissue with day 3 DEGs, their early divergence could not effectively predict the gamergate fate. Only three of these genes were still differentially expressed at day 10 (Supplemental Fig. S2D). Last, although the ovary had the highest number of DEGs at day 10, they were not able to classify castes based on ovarian expression profiles at day 3 (Fig. 3A″,B″). These data suggest that there are not as yet any significant molecular changes that can be detected in the day 3 ovary or fat body. Collectively, these data reveal the significance of the early molecular differences (day 3) in the dueling brain, suggesting that the brain is responsible for the initial caste determination and behavioral differences.

Early molecular brain markers associated with antennal dueling and caste determination

As indicated by the molecular changes identified at day 3, the brain might be responsible for triggering antennal dueling in gamergate-destined workers. However, likely due to the extensive cell type heterogeneity of the brain, we could not find any significant changes at day 3 (Fig. 3A), although the logistic regression suggested that some of the genes that were differentially expressed at day 10 were already different at day 3. To find which genes in the brain are possibly related to caste specification at day 3, we examined early expression patterns of day 10 brain DEGs (Supplemental Table S2). We found two different expression patterns during days 3–10: (1) early differences at day 3 that increased through day 10, and (2) no differences at day 3 that only appeared at day 10 (Fig. 4A; Supplemental Table S2).

Worker-biased brain DEGs One of the early response worker-biased DEGs is *Gp-9-like pheromone-binding protein*, which was slightly lower at day 3 but drastically down-regulated at day 10 in the brain of dueling animals (Fig. 4A). The worker-biased brain genes that were only

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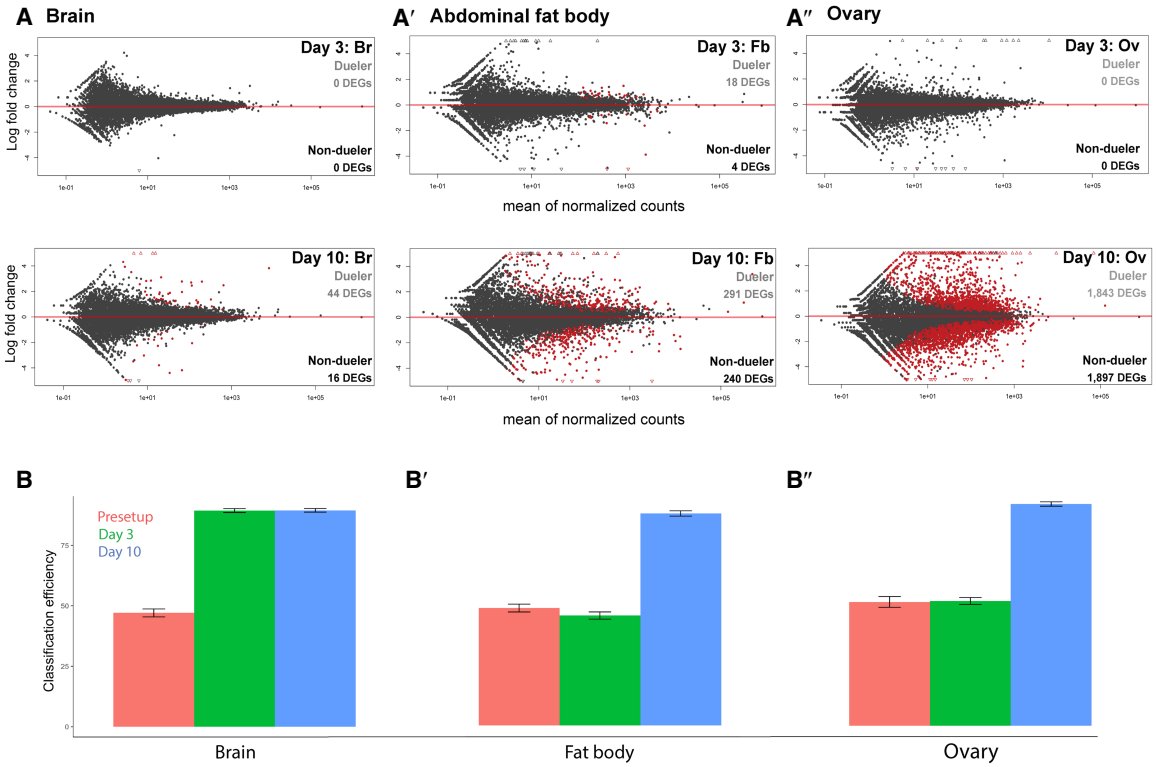

Figure 3. Early molecular changes in brain, fat body, and ovaries show distinct dynamics and capacities to classify caste fate. (*A*–*A*″) Differential expression analyses in three different tissues, collected from two time points (day 3 and 10 postinitiation of the dueling tournament). MA plots show the number of differentially expressed genes (DEGs) in the nonvisual brain (central brain; *A*), abdominal fat body (*A*′), and ovary (*A*″) on days 3 and 10 (*top* and *bottom* panels, respectively). Red dots represent a single DEG (adjusted *P*-value < 0.05), and triangles represent points where the log fold change is >5 or less than −5. DEGs located on the upper part of MA plots are biased to a dueling or a gamergate-destined worker. (*B*–*B*″) Plots of the caste classification efficiency using DEGs identified from each tissue on day 10 using linear regression modeling. (*B*) A model derived from changes in the brain DEGs on day 10 classifies caste fate based on the brain transcriptomic profiles on precolony setup (red), on day 3 (green), and on day 10 (blue). The day 10 brain DEGs can classify caste from day 3 with an accuracy >80%. There are only two possible outcomes from the trained model (worker or gamergate: random coin flip efficiency ~50%). (*B*′,*B*″) Contrarily, linear models trained on day 10 fat body and ovary DEGs cannot classify castes from day 3.

differentially repressed in gamergates at day 10 but not at day 3 include *Krüppel homolog 1* (*Kr-h1*), the neuropeptide *corazonin* (encoding the Crz foraging-related neuropeptide), *insulin-like growth factor* (*IGF*), *neuroparsin*, and *neprilysin-2* (*Nep-2*) (see below; Fig. 4A; Supplemental Fig. S3A,C). *Kr-h1* is transcriptionally induced by juvenile hormone (JH) (Kang et al. 2017); its low levels in the dueling brain indicate a low titer of JH, consistent with a previous study in *H. saltator*, showing that the whole-body JH titer is high in foraging workers but low in queens and gamergates (Penick et al. 2011). Corazonin, as well as JH, are responsible for foraging behavior in workers (Penick et al. 2011; Gospocic et al. 2017; Nagel et al. 2020). JH titer and *IGF* expression in the brain are positively correlated in the harvester ants and honeybees. JH treatment induces *IGF* expression in their brain, consistent with brain *IGF* and JH jointly suppressing *vitellogenin* expression in workers and thus oogenesis (Corona et al. 2007; Libbrecht et al. 2013; Wang et al. 2013b). Furthermore, the higher expression of both *corazonin* and *neuroparsin* in workers is consistent with their antigonadotropic role (Gospocic et al. 2017). Overall, the genes that we found down-regulated in the brain of duelers at day 10 appear to stimulate foraging behavior and to inhibit reproduction.

Gamergate-biased brain DEGs Three genes showed increased expression both early and late in the brain of future gamergates. *vitellogenin* (*Vg*), *membrane metalloendopeptidase-like 1* (*MMEL1*), and *kunitz-type protease inhibitor* (*HCRG1*) were increased in the dueling brain at day 3 and further up-regulated at day 10 (Supplemental Fig. S3A′). Although vitellogenin, which is an egg yolk protein, is mainly produced by the fat body (Arrese and Soulages 2010) and transferred to the oocyte (Raikhel and Dhadialla 1992), expression of vitellogenin mRNA and protein is widely observed in the adult brain of reproductive female *Hymenoptera* such as honeybees, bumblebees, and several ant species, in which it might serve as a circulating signaling molecule (Münch et al. 2015; Lockett et al. 2016; Gospocic et al. 2017; Kohlmeier et al. 2018). The early role of MMEL1 and HCRG1 in the gamergate brain remains to be explored; however, proteases and protease inhibitors have been shown to affect synaptic

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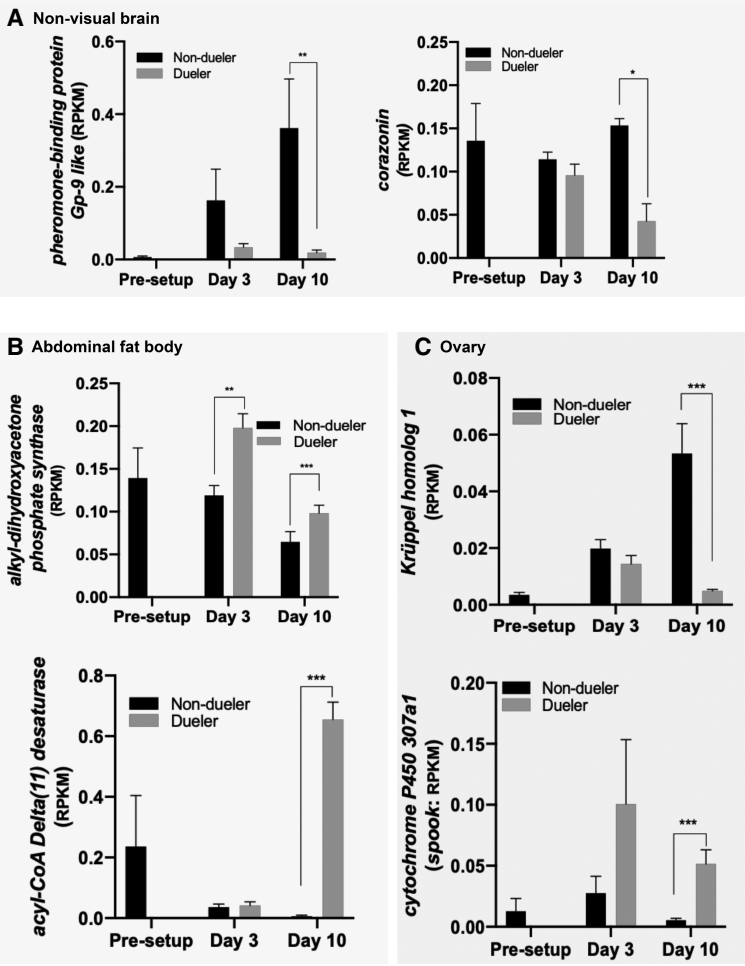

Figure 4. Differentially expressed genes (DEGs) identified in three different tissues during the early dueling tournament. Expression levels (reads per kilobase of transcript [RPKM]) of representative DEGs from the tissue-specific transcriptomes of the nonvisual brain (*A*), abdominal fat body (*B*), and ovary (*C*) are compared between dueling and nondueling individuals at three time points (prior to the colony setup and at days 3 and 10 postinitiation of the dueling tournament). $N = 5$. (*) adjusted P-value < 0.05, (**) adjusted P-value < 0.01, (***) adjusted P-value < 0.001. (*A*) The pheromone-binding protein Gp-9-like is enriched in destined worker brains at day 10 but has already started being expressed more highly in their brain, as early as day 3. In contrast, *corazonin* is up-regulated in destined worker brains only at day 10. (*B*) The fat body displayed differential dynamics in gene expression at days 3 and 10. Only three genes that were differentially expressed on day 3 were still differentially expressed on day 10. One such example is *alkyl-dihydroxyacetone phosphate synthase.* Most other genes, such as *acyl-coA Δ (11) desaturase*, display differential expression between workers and gamergates on day 10 but have no difference at all on day 3. (*C*) Similar to the brain, the ovary had no DEGs on day 3. However, the genes that were differentially expressed on day 10, such as *Kr-h1*, showed no difference on day 3, with few exceptions (e.g., *spook*).

plasticity and behavior in the vertebrate brain (Almonte and Sweatt 2011) and could be triggering widespread changes in the gamergate-destined brain.

Most of the gamergate-biased genes, including *neprilysin-4* (*Nep-4*) and *Insulin* (*Ins*), were only activated at day 10 (Supplemental Fig. S3A″). This is in sharp contrast to their paralogs *Nep-2* and *IGF* that were decreased in the brain of gamergates and increased in workers (worker-biased brain genes) (see above; Supplemental Fig. S3A). Neprilysins are endopeptidases that degrade amyloid peptides and small peptides in the brain. Several neuropeptides, such as tachykinin and corazonin, are substrates of neprilysins. Moreover, *Nep-4* overexpression in *Drosophila* larvae reduces food intake and represses gene expression of *insulin-like peptides* (*Dilps: Dilp-1, Dilp-2, Dilp-3*, and *Dilp-5*) in the larval brain (Hallier et al. 2016). Similarly, *Nep-4* was up-regulated in the gamergate-destined brain and *IGF* was down-regulated, whereas *Nep-2* was down-regulated and *Ins* was highly expressed in the dueler brain. This suggests that *Nep-2* and *Nep-4* play opposite roles in the brain in transition and might act specifically on *IGF* or *Ins*.

In summary, several DEGs in the gamergate-destined brain, such as *Gp-9* (down-regulated) and *vitellogenin* and *HCRG1* (up-regulated), respond rapidly to the lack of queen pheromones. Subsequently, the down-regulation of *Kr-h1* and *Crz* and the up-regulation of *Ins* together presumably induce behavioral and physiological changes in the future gamergates (Gospocic et al. 2017; Chandra et al. 2018).

Highly dynamic molecular changes in the abdominal fat body

The insect fat body is distributed throughout the body cavity and serves as a primary source of energy storage, similar to the mammalian liver. The abdominal fat body in *H. saltator* consists of two different cell types: adipocytes that store energy as lipid droplets, and oenocytes responsible for energy metabolism and cuticular hydrocarbon/pheromone biosynthesis (Gutierrez et al. 2007; Krupp et al. 2008; Arrese and Soulages 2010). The fat body was the only tissue that showed a rapid response at day 3 (Fig. 3A′), although only three out of 22 day 3 DEGs were also identified as day 10 DEGs (Supplemental Fig. S2D). *Alkly-dihydroxy acetone phosphate synthase* and *FERM domain-containing protein 5* were up-regulated in both day 3 and day 10 duelers (Fig. 4B; Supplemental Fig. S3B), whereas *cardioacceleratory peptide receptor* (*CCAPR*) was up-regulated in day 3 duelers but down-

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regulated at day 10 (Supplemental Fig. S3B). The majority of DEGs at day 3 (19 out of 22) was temporarily differentially expressed at day 3 but not at day 10 (Supplemental Fig. S3B′). This transient differential expression of distinct genes suggests that the fat body plays different roles in early versus late stages of caste transition. Five of the transient gamergate-biased genes at day 3 (*ubiquitin-conjugating enzyme E2 R2* [*UBE2R2*], *cullin-2* [*CUL2*], *26S proteasome regulatory subunits 2* and *6A* [*PSMD2* and *PSMC3*], and *COP9 signalosome complex subunit 6* [*COPS6*]) are involved in ubiquitin-dependent proteolysis, suggesting a role of protein degradation during the early gamergate transition.

Many of the response genes in the fat body that were identified only at day 10 are involved in insect pheromone biosynthesis, hormone, and reproduction, which is consistent with the physiological changes in prospective gamergates (Fig. 4B; Supplemental Fig. S3B″, S3D): (1) As described above in the brain, *Kr-h1* was also transcriptionally repressed in the gamergate fat body. In the *Drosophila* fat body, *Kr-h1* represses lipolysis (Kang et al. 2017). (2) *Acyl-CoA desaturase* (*SCD*), which was up-regulated in the gamergate fat body, is necessary for fatty acid metabolism and insect pheromone biosynthesis (Knipple et al. 1998; Serra et al. 2007; Liénard et al. 2008, 2010), for instance, sex pheromones in the moth (Knipple et al. 1998; Moto et al. 2004; Serra et al. 2007; Liénard et al. 2008, 2010). Ablation of corpora allata, the JH-producing gland, in female *Drosophila* shows delayed mating and an increase of some female-specific cuticular hydrocarbons (7,11-C23, 7,11-C25, and C27 dienes). Application of a JH analog in the allatectomized females rescued the phenotypes, suggesting that JH is involved in sex pheromone production and might be negatively associated with a particular desaturase, increasing specific unsaturated hydrocarbons (Bilen et al. 2013). In *H. saltator*, the expression levels of multiple *desaturases* in workers are extremely low compared with that of reproductive castes, supporting an essential role for pheromone biosynthesis rather than metabolism (Helmkampf et al. 2015). The up-regulation of *acyl-CoA desaturase* (*SCD*) in the fat body of day 10 duelers may help immature gamergates develop a queenlike cuticular hydrocarbon profile for recognition by other nestmates. (3) Expression of *vitellogenin* was dramatically increased in gamergates at day 10, consistent with the production of eggs at this stage of the transition. Vitellogenin not only is a component of the oocyte but also serves as an antioxidant that promotes longevity and a negative regulator of foraging behavior in honeybees (Corona et al. 2007). Vitellogenin expression is inhibited by JH (Corona et al. 2007). Therefore, low levels of JH and *Kr-h1* mRNA correlate with the up-regulation of *vitellogenin* in gamergates.

In summary, the abdominal fat body is a versatile tissue, and its rapid molecular changes dynamically respond to the gamergate transition, causing different genes to be expressed differentially at early versus late stages of caste transition, likely changing the pheromone profile and providing components of the eggs.

Downstream responses of the ovary during the worker-to-gamergate transition

Egg laying is first observed at day 10 (Fig. 1E). In accordance, we identified thousands of ovarian DEGs at day 10 but none at day 3 when the ovary was still inactive (Fig. 3A″). Among the 3740 ovarian DEGs at day 10, approximately half (1897 DEGs) were down-regulated, and the rest (1843 DEGs) were up-regulated in the activated ovaries (Fig. 3A″, bottom).

As in the brain and fat body, *Kr-h1* was also down-regulated in the ovary of gamergates at day 10, presumably to prevent its inhibitory effects on ovary maturation (Fig. 4C; Supplemental Fig. S3A, S3B″), again suggestive of low JH levels. Reproductive females (queens or gamergates) have low JH and high ecdysone (Ec), whereas foraging workers have high JH and low Ec (Robinson et al. 1991; Penick et al. 2011). In accordance, the expression of genes involved in Ec biosynthesis was significantly elevated in the active ovary at day 10, including the cytochromes P450 *spook* (*spo*; *Cyp307a1*), *shadow* (*sad*; *Cyp315a1*), *shade* (*shd*; *Cyp314a1*), and *oxygenase neverland* (*nvd*) (Rewitz et al. 2006). Two Ec-responsive genes, *Hormone receptor 3* and *4* (*Hr3/4*), were also increased in ovaries of day 10 duelers (Fig. 4C; Supplemental Fig. S4A; Lam et al. 1997; King-Jones et al. 2005). This profile supports previous studies that JH is associated with foraging behavior in workers, while ecdysone has a positive effect on up-regulating reproduction in females (Robinson et al. 1991).

Both brain *Ins* and ovarian *IGF* were up-regulated in gamergates at day 10 (Supplemental Fig. S3A″, S4B,C). Up-regulation of *Ins* promotes ovary maturation (Chandra et al. 2018) and works along with Ec (and antagonistically to JH) to control ovary activation and vitellogenesis (Corona et al. 2007; Chandra et al. 2018). However, a function of *IGF* in the insect ovary is still unknown.

In conclusion, the gamergate ovary shows increased ecdysone and *IGF* during the early stages of ovary activation. These increases are consistent with the known interplay among JH, ecdysone, and insulin signaling pathways that regulates body size and developmental timing in many insects and is crucial for stimulating egg development (Wang et al. 2013b; Chandra et al. 2018). Importantly, the observed gene expression differences in the ovary occurred later than those in the brain during the caste transition process. This subsequence contradicts the ovarian/reproductive ground plan hypothesis in which ovaries are the key players in shaping caste traits in social insects (Pennisi 2009), and instead highlights the importance of the brain in this process.

Juvenile hormone treatment promotes the worker fate while ecdysone induces gamergate fate

The global increased expression of *Kr-h1*, a known target of JH, in nondueling workers and the increased expression of ecdysone-regulated genes in the ovaries of duelers suggested a crucial role for these hormones for the specification of worker or gamergate fate, respectively. To verify whether the effects were directly due to regulation by

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the two hormones, we administered a JH analog (JHA; methoprene) and 20-hydroxyecdysone (20E) to young workers and probed the expression of selected genes in the nonvisual brain, ovary, and fat body. A single abdominal injection of JHA into young workers led to significantly increased levels of *Kr-h1* in the nonvisual brain, fat body, and ovary compared with the control/EtOH-injected workers. The strongest effects of JHA were observed at 12 h after injection but then slightly decreased after 24 h (Fig. 5A). Interestingly, we found that JHA induced the expression of *IGF* only in the brain; this agreed with the RNA-seq data in which *IGF* was found to be highly expressed in the brain of nonduelers (Fig. 5B; Supplemental Fig. S3A). In contrast, injection of 20E into the abdomen of young workers triggered the expression of *Hr4* in the brain 48 h after injection, the fat body after 24 h, and the ovary after 12 h (Fig. 5C). Expression levels of other caste-biased genes—such as *Gp-9*, *Crz*, *Ins*, *Vg*, and *HCRG1* in the brain; *Gp-9*, *Vg*, and *IGF* in the fat body; and *Gp-9*, *shade*, and *IGF* in the ovary—were also measured, but no transcriptional changes were detected (Supplemental Fig. S5). This suggests that juvenile hormone and ecdysone trigger several of the observed transcriptional changes that are associated with caste specification but that there are also other molecules (such as corazonin or insulin) that act in parallel to control different aspects of the transition.

We then asked which of the physiological and behavioral effects associated with caste specification are controlled by these two hormones in young workers (~2 wk old) and in immature dueling workers. Young workers received a single injection of 20E or JHA and were fed with 20E-injected and JHA-injected crickets for 7 d, respectively, in order to maintain high levels of the two hormones. 20E-treated workers showed an increased number of yolky oocytes with more active ovarioles compared with the control (10% EtOH-treated) young workers 7 d after the injection. In contrast, JHA-treated young workers had fewer

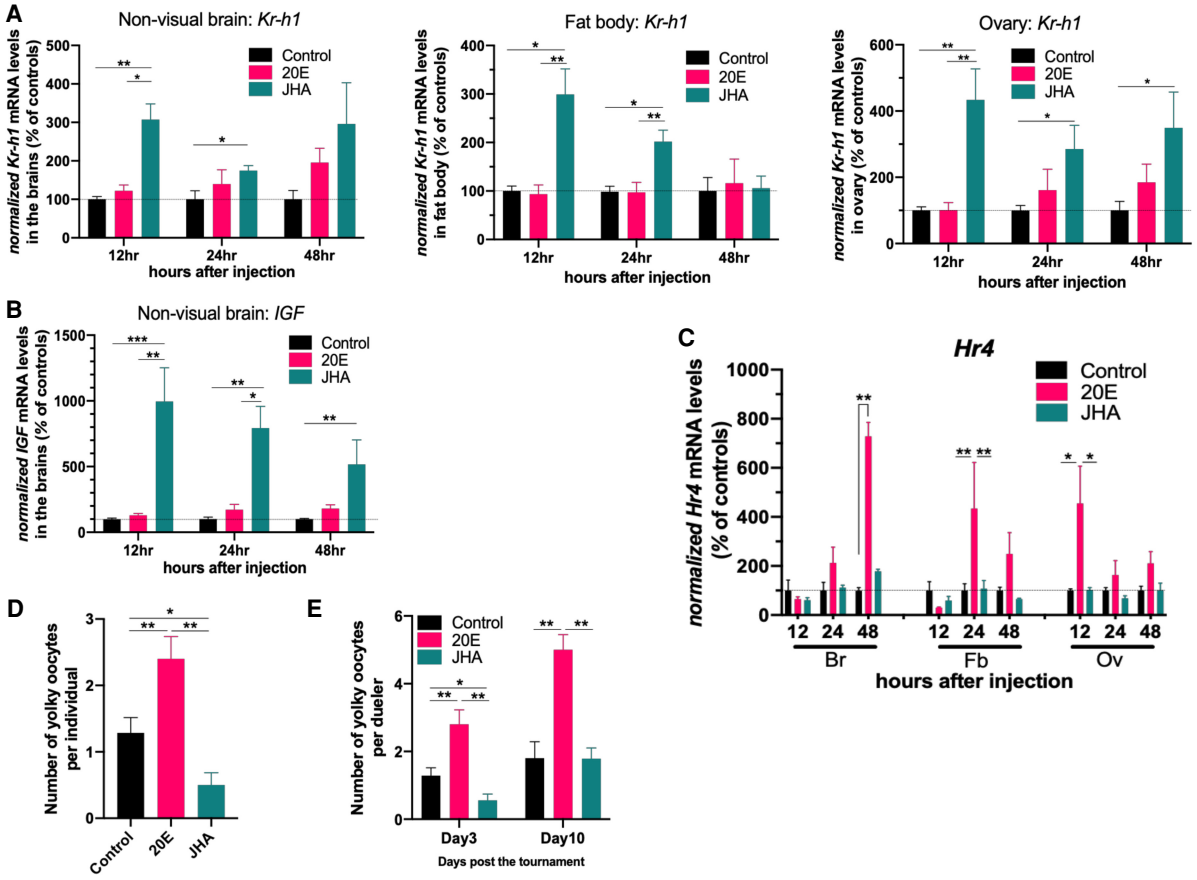

Figure 5. Juvenile hormone analog (JHA) promotes worker fate, while 20-hydroxyecdysone (20E) induces gamergate fate during the early caste transition. (*A–C*) RT-qPCR for *Kr-h1*, *IGF*, and *Hr4* from different tissues after JHA or 20E treatment at three time points. Abdominal injection of JHA (green) in young workers increases *Kr-h1* expressions across all three tissues (*A*) and triggers *IGF* expression in the brain (*B*). Abdominal injections of 20E in young workers increase *Hr4* expression in all three tissues (*C*). *Ribosomal protein 32* (*rpl32*) was used as a normalization control. Bars represent the means from four or more individuals ± SEM. (*D*,*E*) Effect of JHA or 20E on ovary maturation in queenless colonies. Ovaries of JHA-treated young workers (*D*, green) and day 3 duelers (*E*, green) contained fewer yolky oocytes compared with controls (black), while 20E-treated young workers (*D*, pink) and day 3/10 duelers (*E*, pink) developed ovaries with more yolk oocytes. Bars represent the means from ≥12 individuals ± SEM. *P*-values are from Mann–Whitney test. (*) *P*-value < 0.05, (**) *P*-value < 0.01, (***) *P*-value < 0.001.

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yolky oocytes and fewer active ovarioles compared with the controls 7 d after the injection (Fig. 5D; Supplemental Fig. S6A). Similarly, an increased number of yolky oocytes with more active ovarioles were observed 7 d after day 3 and day 10 duelers were injected with 20E and fed with 20E-injected crickets. JHA-treated day 3 duelers also had less developed ovaries compared with the controls. However, JHA had no significant effects on ovary maturation of day 10 duelers (Fig. 5E; Supplemental Fig. S6B). We also monitored the workers' dueling activities for 7 d after the treatment with the hormones. The dueling activities were drastically diminished after injection of either hormone, which we believe is due to an effect of the anesthesia and injection (Supplemental Fig. S6C). These experiments highlight the contribution of juvenile hormone for the determination of worker fate and of ecdysone for the future gamergate fate.

Discussion

Organized social structure and cooperation are fundamental for survival in a social setting. Social insects, and in particular the ant *H. saltator*, have fascinating superorganism resilience, allowing them to tolerate the loss of germ cells in its colony (gamergates and queens) (Hölldobler and Wilson 2008; Straub et al. 2015). We took advantage of the caste plasticity in *H. saltator* that possesses both rigid and flexible adult caste systems (Opachaloemphan et al. 2018) to study the molecular features that underlie the re-establishment of the social hierarchy structure. In response to the lack of queen pheromones, multiple *H. saltator* workers initiate aggressive antennal dueling to produce new reproductives (gamergates). Unlike other hostile competitions, the aggressiveness of antennal dueling has no apparent negative effects among the contestants (Sasaki et al. 2016). Instead, after a rapid exchange in which most workers return to work (winner–loser), dueling among gamergates appears to promote reproduction and social dominance hierarchy in a winner–winner configuration (Liebig et al. 1999; Sasaki et al. 2016). Other types of social interactions, such as biting, infrequently occur during the caste transition and strongly inhibit the targeted individuals from continuing to duel. However, bitten and policed workers were not included in this study.

By tracking the dueling behavior during the months-long caste transition, we were able to show that future gamergates are determined expediently within the first few days of the dueling tournament. Future gamergates show significantly increased dueling activity compared with the workers, allowing us to predict the gamergate fate with 86% accuracy at 3 d postinitiation of dueling (Fig. 2C). Activation of the ovary of the future gamergates is rapid, with egg laying being apparent at day 10 (Fig. 1E). The ovary consists of eight ovarioles in total. At day 3 post-dueling initiation, the dueler's ovary is partially activated and occasionally develops a yolky oocyte in one of the ovarioles. By day 10, prospective gamergates have developed yolky oocytes in approximately three of the eight ovaries (Supplemental Fig. S2B). Dueling activity peaks during days 6–10 (Fig. 1D), followed by a decrease that corresponds to an increase in egg-laying events after day 10 (Fig. 1E), implying that the cost of social dominance is swiftly reinvested in reproduction after the re-establishment of social structure. Nevertheless, dueling has not yet ended, and the prospective gamergates constantly duel to show and maintain their social dominance, suggesting a well-balanced energy investment between reproduction and dominance hierarchy. In contrast, workers with low or no dueling invest their energy in colony maintenance tasks, including foraging, brood caring, nest defending, and waste collecting, rather than in social competition and reproduction. Altogether, *H. saltator* rapidly senses and responds to the absence of a reproductive female: New gamergates are rapidly determined, emphasizing a remarkable resiliency in the wake of disturbance to the social hierarchy in *H. saltator*.

Recent modeling of *H. saltator* during the worker-to-gamergate transition has underscored the importance of dueling in maintaining shared dominance amongst gamergates (Sasaki et al. 2016). The prolonged dueling among gamergates after their fate is established is a "winner–winner" scenario in which individuals that duel mutually benefit, resulting in the production of several gamergates in a colony, likely necessary for colony maintenance given that the gamergates retain the smaller worker body and cannot produce as many eggs as actual queens (Peeters et al. 2000). Our observations during the early stages of the dueling tournament indicate that most, but not all, individuals that duel were destined to become actual gamergates. Some early duelers gave up on the dueling tournament and eventually remained as workers, suggesting that the antennal dueling is not always a winner–winner scenario, at least during the unstable social structure in the early transition. Once duelers (immature gamergates) reach a mature stage and fully develop their production of queenlike pheromones, dueling activity considerably decreases (Fig. 1D). The other workers (subordinates) in the colony recognize the mature gamergates as the reproductives and do not challenge them (Liebig et al. 1999, 2000), and their reproductive capacity is repressed by the pheromones of the mature gamergates (Ghaninia et al. 2017). Other social interactions (for example, dominant biting) influence the gamergate determination as bitten individuals are excluded from the dueling tournament, although not all of the transient dueling behavior ceases because of biting (Liebig et al. 1999; Sasaki et al. 2016). Therefore, the transient dueling behavior in some workers during the early period ensures that no cheaters threaten the upcoming social hierarchy. Once the gamergate fate is established, the sustained dueling interactions reinforce the developmental plasticity as a positive-feedback loop that positively influences the shared dominance among duelers.

Because we could predict future gamergates as early as at 3 d, we could identify multiple molecular markers associated with the dueling behavior and the transition to gamergate, as evidenced by the tissue-specific gene expression profiles from dueling versus nondueling workers.

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We observed both differential behavioral dynamics and differential molecular gene expression in distinct tissues, indicating physiological changes during the early worker-to-gamergate transition.

Each tested tissue showed different responsiveness to the absence of the queen pheromones. The brain of nonduelers and duelers starts diverging as early as day 3 postdueling initiation, and these differences become more apparent at day 10. On the other hand, the abdominal fat body has an initial rapid response at day 3, but most of the early response gene differences between duelers and nonduelers disappear by day 10. Several of the late response genes, involved in pheromone production and vitellogenesis processes, may be subsequently induced by changes in the brain. Vitellogenin is transported to the ovary and supports oogenesis. Therefore, the ovary appears to act as a downstream tissue receiving signals from the other tissues and responding to them extensively after day 3 and until day 10 (Supplemental Fig. S7).

We propose a model whereby the brain serves as a primary tissue driving the activation of other peripheral tissues during the antennal dueling tournament. Expression levels of several genes in the brain show a rapid divergence between prospective gamergates and workers and serve as early molecular markers of gamergate fate.

Gp-9-like pheromone-binding protein (PBP) is down-regulated in the brain, fat bodies, and ovaries of prospective gamergates (Fig. 4A; Supplemental Fig. S3B″, S4B). In Lepidoptera, expression of PBPs is very high in antennae (Mao et al. 2016) in which the proteins facilitate pheromone perception by olfactory receptors (Chang et al. 2015). However, in the red fire ants (*Solenopsis invictia*), expression of many PBPs, including Gp-9, is less abundant in the antenna compared with the rest of the head and the thorax (Zhang et al. 2016), and Gp-9 was proposed to function as a transporter of chemical compounds that acts beyond olfaction. It could also serve as a protein circulating in the hemolymph that functions as a carrier of specific hormones or signaling molecules (Wang et al. 2013a) that has an inhibitory effect on the queenlike phenotypes.

Vitellogenin expression is increased in the brain of prospective gamergates. The expression of *vitellogenin* in the fat body is known to be crucial for oogenesis and is repressed by corazonin, neuroparsin, and JH (Corona et al. 2007; Libbrecht et al. 2013; Yang et al. 2014; Gospocic et al. 2017), but its expression in the brain is not well understood. *Vitellogenin* expression is found in the red fire ant workers, which have no ovaries, suggesting additional roles of vitellogenin besides being an egg yolk protein in ants (Wurm et al. 2011). Several studies in ants and honeybees propose that the vitellogenin precursor may mediate responsiveness to social cues such as broods and food and consequently affect social behaviors by increasing parental care (Roy-Zokan et al. 2015; Kohlmeier et al. 2018).

Membrane metallo-endopeptidase-like 1 (*MMEL1*) and *kunitz-type protease inhibitor* (*HCRG1*) are up-regulated in the early gamergate-destined brain; however, their roles in the insect brain are unknown. On the other hand, proteases and serine protease inhibitors are widely reported in the vertebrate brain to influence inflammation, synaptic plasticity, and behavior (Almonte and Sweatt 2011) by processing extracellular matrix molecules that contribute to neuronal outgrowth (Rivera et al. 2010). Their up-regulation in the dueling brain may facilitate structural changes in the brain that allow neuronal growth or migration during caste establishment.

Following the initial response of the gamergate brain, we observed a cascade of downstream events in the brain, fat body, and ovary that appear to promote queenlike and suppress worker behaviors in the prospective gamergates (Fig. 6). Down-regulation of *corazonin* in the brain suppresses foraging behavior and derepresses the expression of *vitellogenin* in the fat body and, hence, oocyte maturation (Gospocic et al. 2017). The reduction of *IGF* in the brain results from the lowering of JH levels, likely mediated by the reduction of *Kr-h1* (Fig. 5B). This may subsequently contribute to the increase of *vitellogenin* expression. Global reduction of *Kr-h1* leads to up-regulation of Ec biosynthesis in the ovary (Zhang et al. 2018). Similar to other social insects (Robinson et al. 1992; Pamminger et al. 2016; Norman et al. 2019), JHA treatment shows negative effects on ovary growth in *H. saltator* workers, whereas 20E treatment promotes ovary maturation in workers. As the JHA or 20E treatments do not affect *Vg* expression in the brain and fat body of workers (Supplemental Fig. S5A, A′), this suggests that vitellogenesis in *H. saltator* is controlled by other signaling (Gospocic et al. 2017), as observed in the black garden ant (Pamminger et al. 2016). Finally, we observed an increase in the expression levels of brain *Ins* and ovarian *IGF* in prospective gamergates. However, although brain *IGF* in workers is induced by JH treatment, both gamergate-biased genes (brain *Ins* and ovarian *IGF*) are not affected by JH or by 20E. The function of worker-brain and gamergate-ovarian *IGFs* is unclear, but brain injection of *Ins* in clonal raider ants stimulates oocyte maturation (Chandra et al. 2018). In summary, we showed here that high JH in nonduelers induces brain *IGF* expression and promotes the worker fate while high ecdysone in duelers promotes ovary maturation (Fig. 6).

In conclusion, we monitored the behavior and gene expression profiles at the very beginning of caste transition. The absence of queen pheromones rapidly initiates behavioral changes (antennal dueling) in workers with changes in gene expression in the brain of prospective gamergates. We discovered that the gamergate fate is rapidly determined and can be efficiently predicted at 3 d after dueling initiation, suggesting a rapid restoration of social structure in *H. saltator*. Furthermore, altered gene expression in the brain appears to be the primary genetic factor for caste switching. These early molecular alterations in the brain are likely due to a response to the absence of inhibitory queen pheromones and to the antennal dueling behavior, which subsequently lead to other systemic effects in peripheral tissues that might be mediated by JH signaling that appears to play a crucial role in the maintenance of the worker fate while ecdysone (Ec) promotes reproduction in gamergates (Fig. 6).

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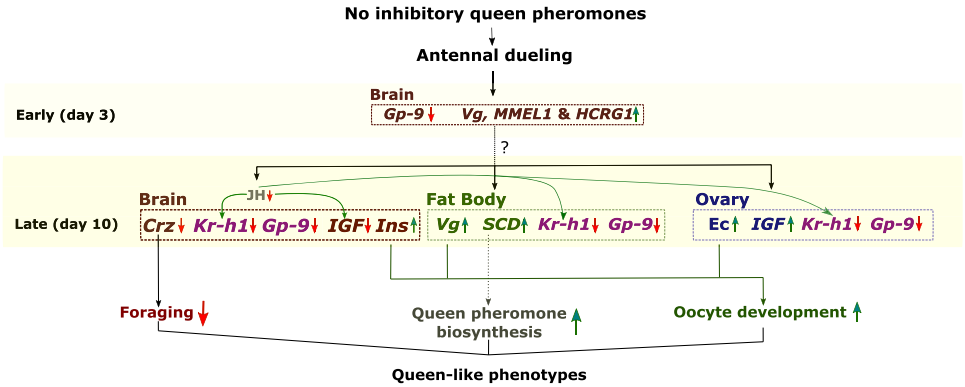

Figure 6. A hypothetical pathway during the early caste transition. The absence of inhibitory queen pheromones unleashes the antennal dueling in workers and subsequent molecular changes in the different tissues. We propose that initial gene expression changes in the brain, such as down-regulation of *Gp-9-like pheromone-binding protein* and up-regulation of *vitellogenin* in the destined gamergates, trigger a cascade of gene expression changes in the brain, fat body, and ovary that promote queenlike phenotypes, such as queen pheromone biosynthesis and oocyte development, and suppress worker-like phenotypes, such as foraging. High juvenile hormone (JH) enables the worker fate, while increased ecdysone (Ec) promotes reproduction in duelers or prospective gamergates. *Ins* is the homolog of human *insulin*, whereas *IGF* is the homolog of human *IGF1*. *Ins* was previously referred to as *Ilp1* and *IGF* to *Ilp2* in *H. saltator* (Bonasio et al. 2010; Gospocic et al. 2017), while the *Ins* homolog in honeybees and clonal raider ants was designated as *Ilp2* (Corona et al. 2007; Wang et al. 2013b; Chandra et al. 2018). Genes down-regulated in the dueling ants are indicated by red arrows, whereas up-regulated genes are indicated by green arrows. Solid lines indicate a known functional role of a gene. Broken lines represent hypothetical relationships.

Materials and methods

Worker-to-gamergate transition setup and maintenance

Forty workers, consisting of 30 younger workers (~2 wk after adult emergence) and 10 older workers (at least 3 mo after emergence), were uniquely labeled with Uni-paint markers and placed into a dental plaster-floored container with an underground arena covered by glasses (Darby Dental 849-1560). The container was a clear plastic box with lid (Pioneer Plastics 079C). Distilled water was constantly added to the plaster to provide moisture. The colony was fed three times a week with prestung paralyzed crickets. The transition colony was maintained in the containment room with a controlled temperature of 25°C with 12 h of light–dark cycle. Behavioral and molecular experiments were independently conducted on different colonies.

Monitoring antennal dueling behavior

Activities inside the transition colonies were monitored under a surveillance camera for 24 h over a 50-d period using the Logitech C920 web cam. The recording was performed at 1280 × 720-pixel resolution and 30 frames/sec. The dueling frequency was scored every 45 min during the 12 h of light cycle (a total of 16 times a day). The scoring approach is based on the presence or absence of dueling behavior within each 20-min time window. After the period of observation of over 50 d, we determined the fate of each individual based on its dueling activity and egg laying. Any high-dueling individual that laid eggs at least twice was classified as a gamergate. Low-dueling ants with less than two egg-laying events were considered as workers. Other cast-specific behaviors such as cricket hunting, trash collection, and dominant behavior (Supplemental Fig. S1A) were also observed and considered for further validation by gene expression. The egg-laying events were scored together with the dueling behavior and only observed within a specific time window during the daytime in each video. Not all the events were identified.

Tissue collection and RNA extraction

Brain dissection Dissections were performed on a clear rubber pad with fine forceps (FST Dumont 5) and scissors (FST 15004-08) in 1× phosphate-buffered saline (PBS). Brains from workers and gamergates were harvested on ice by first removing the head capsule using fine forceps. The brain, including the optic lobes, was detached from the retina by sliding the forceps under each optic lobe and then gently pried away from the retina. All connective tissue was removed, and the brain was kept in TriPure isolation reagent (Sigma 11667157001).

Ovary and fat body dissection The thorax was removed from the pediole/gaster. The content of the gaster (i.e., gut, fat body, ovaries, etc.) were removed by holding the pediole and gently inserting the tip of the forceps under the postpediole and gently prying away muscle from tergite and sternite from the gaster. Removal of the postpediole was performed by subsequent removal of sternites and tergites. Ovaries were freed from the gut and fat body by gripping the large intestine and gently pulling away from the posterior end of the gaster. Fat bodies were gently pried away and stored in ice-cold TriPure. Tissues were kept frozen at −80°C.

Total RNA extraction

For extracting RNA, a pipette was used to gently aspirate and agitate the tissue. After agitation, TriPure was added to bring the volume to 1 mL. The mixture was placed into a new 1.5-mL microcentrifuge tube and 200 µL of chloroform per 1 mL of TriPure was added. The tube was inverted repeatedly for 30 sec and left for 5 min at room temperature. The TriPure mixture was centrifuged at 12,000*g* for 15 min at 4°C. The top clear aqueous phase (~500 µL) was transferred to a new microcentrifuge tube containing 1 µL of Glycoblue (Thermo Fisher AM9515) and 1× volume of isopropanol. The RNA/isopropanol/Glycoblue mix was then centrifuged at 20,000*g* for 30 min at 4°C. The supernatant was removed, and the pellet was washed twice with 70% and 80% ethanol, respectively, and subsequently centrifuged at

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20,000*g* for 10 min at 4°C. The pellet was air-dried for 5 min before being dissolved in BTE buffer (10 mM Bis-Tris at pH 6.8, 1 mM EDTA). RNA samples were treated by DNase I (Ambion 2239), and DNase I was removed by phenol/chloroform extraction and isopropanol precipitation.

RNA-seq and data analyses

For library preparation, polyA+ RNA was isolated from ~500 ng of total RNA using NEBNext poly(A) mRNA magnetic isolation (E7490). Libraries were prepared using a NEBNext Ultra II RNA library preparation kit (E7770, E7335, and E7500), quantified before and after amplification using a KAPA SYBR Fast universal qPCR kit (KK4854) and sequenced on an Illumina Hiseq 2500.

The reads were trimmed for quality and adapter sequence using Trimmomatic (leading: 3; trailing: 3; slidingwindow: 4:15; minlen: 36) (Bolger et al. 2014). Reads from both sample sets were mapped with STAR (v.2.5.3a) (Dobin et al. 2013) on the *Harpegnathos* genome version 8.5 (Shields et al. 2018) and counted using HT-Seq (Anders et al. 2015). On average, we sequenced 3.1 million reads per sample, 3 million of which were uniquely mapped on the genome (~95% mapping efficiency). Of these reads, 1.6 million were mapped to a specific gene (~55% using the union mode in ht-seq).

Differential expression analysis was performed using DESeq2 (Love et al. 2014). An adjusted *P*-value of <0.05 was considered significant. RNA-seq data are available on GEO under accession number GSE162969. For all genes discussed in this study, gene symbols, IDs and identifiers are reported in Supplemental Table S3.

Linear regression modeling

To perform linear regression, we used the *glmnet* package in R. For each of the tissues, we generated a linear model using the differentially expressed genes at day 10 as classifiers to predict the outcome (gamergate or not) on day 3. We randomly selected four out of the five future gamergates and workers as a training set and the remaining two ants (one gamergate and one worker) as a test set and enumerated the number of correct predictions of the outcome based on the expression levels of these genes on day 3. We iterated this process 50 times and calculated mean and standard error of the classification efficiency.

Hormone treatments

Both juvenile hormone analog (methoprene, Sigma 33375) and 20-hydroxyecdysone (Sigma H5142) were dissolved in EtOH. Each ant was injected into an opening between the abdominal tergites with a quartz capillary needle (Sutter Instrument QF100-50-7.5) pulled using a micropipette puller (Sutter Instrument, model P-200, program 46) and an Eppendorf FemtoJet injector (5427, Pi = 100hPa, Ti = 1.0s, Pc = 0hPa). Hormone-treated ants were injected with 0.5 µg of 20E or 0.5 µg of JHA diluted in Hank's balanced salt solution (HBSS; Gibco 14025076). The control ants were injected with ~1 µL of 10% EtOH in HBSS.

To determine the transcriptional changes mediated by JHA or 20E, we injected ~2-wk-old workers and dissected the nonvisual brain, abdominal fat body, and ovary at 12, 24, and 48 h postinjection. Tissues were subjected to RNA extraction and cDNA synthesis using a QuantiTect reverse transcription kit (Qiagen 205314). Individuals that had died before the collected time points were excluded. Primer sequences for RT-qPCR are reported in Supplemental Table S4.

For the physiological assays, we established a satellite colony (Pinoneer Plastics 028C) consisting of 20 young workers with a nest arena (2.5 in × 2.0 in × 0.5 in). Two colonies were designated for each treatment. After 3 or 10 d of the dueling tournament, we anesthetized ants with CO_2 and injected both duelers and nonduelers in a colony. Each colony was daily fed with one EtOH/JHA/20E-treated cricket for 7 d. Dueling behavior and egg laying were scored five times a day (20-min window). Ovaries from injected duelers were dissected and scored for ovary maturation 7 d postinjection. The number of yolky oocytes was counted in all eight ovarioles. An active ovariole was defined as having at least one yolky oocyte. All individuals that died before 7 d postinjection were excluded.

Competing interest statement

The authors declare no competing interests.

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Early behavioral and molecular events leading to caste switching in the ant *Harpegnathos*

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