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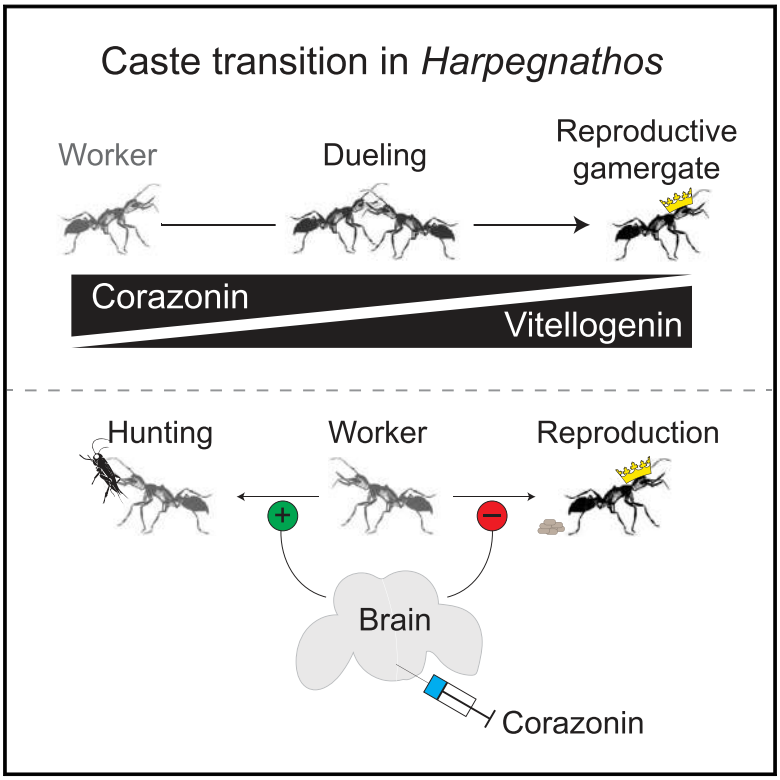
The Neuropeptide Corazonin Controls Social Behavior and Caste Identity in Ants

Graphical Abstract



Authors

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In Brief

Corazonin controls behavioral transitions between ant workers and pseudo queens.

Highlights

- Corazonin is a neuropeptide preferentially expressed by workers in social insects
- *Harpegnathos* workers that convert into reproductive gamergates downregulate corazonin
- Head injections of corazonin stimulate hunting behavior
- Corazonin inhibits vitellogenin expression and egg-laying in *Harpegnathos* and *Drosophila*

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The Neuropeptide Corazonin Controls Social Behavior and Caste Identity in Ants

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SUMMARY

Social insects are emerging models to study how gene regulation affects behavior because their colonies comprise individuals with the same genomes but greatly different behavioral repertoires. To investigate the molecular mechanisms that activate distinct behaviors in different castes, we exploit a natural behavioral plasticity in *Harpegnathos saltator*, where adult workers can transition to a reproductive, queen-like state called gamergate. Analysis of brain transcriptomes during the transition reveals that corazonin, a neuropeptide homologous to the vertebrate gonadotropin-releasing hormone, is downregulated as workers become gamergates. Corazonin is also preferentially expressed in workers and/or foragers from other social insect species. Injection of corazonin in transitioning *Harpegnathos* individuals suppresses expression of vitellogenin in the brain and stimulates worker-like hunting behaviors, while inhibiting gamergate behaviors, such as dueling and egg deposition. We propose that corazonin is a central regulator of caste identity and behavior in social insects.

INTRODUCTION

Genes shape brain circuitry during and after development, but the extent to which gene regulation and epigenetic pathways contribute to the behavioral repertoire of animals remains the subject of intense investigation. Social insects are emerging models to study the role of gene regulation in behavior because their distinct castes are typically derived from the same genome (Robinson et al., 2008). Despite their genetic similarity, different individuals perform specialized tasks within the colony by accessing distinct stereotypical behavioral repertoires. For example, workers are generally responsible for foraging, whereas the reproductively active queen never leaves the nest and mostly occupies herself with egg-laying (Hölldobler and Wilson, 1990). The molecular and neural mechanisms that activate different sets of behaviors in different social castes are largely unknown, although a recent study indicated the importance of chromatin modification pathways in this process (Simola et al., 2016).

Because of its unusual reproductive flexibility, the ponerine ant *Harpegnathos saltator* (Jerdon, 1851) offers unique opportunities for the molecular dissection of caste-specific behaviors (Bonasio, 2012; Yan et al., 2014). Typical *Harpegnathos* colonies comprise a single bona fide queen and many non-reproductive workers. True queens and workers are born as such and display pronounced differences in morphology, reproductive physiology, lifespan, and behavior (Gronenberg and Liebig, 1999; Peeters et al., 2000). Unlike most other ant species, when a *Harpegnathos* queen dies or becomes too old to maintain her reproductive output, some of the workers rise to replace her and take over reproductive duties, thus prolonging the life of the colony (Peeters and Hölldobler, 1995). The selection of the queen's replacements occurs by a ritualized "tournament," during which workers engage in policing, biting, and dueling behaviors, in the attempt to re-establish a social hierarchy (Peeters et al., 2000; Penick et al., 2014; Sasaki et al., 2016). Victorious individuals activate their dormant ovaries, stop performing worker tasks, and start laying fertilized eggs. These converted workers are called "gamergates" from the Greek "gámos ergátēs," which means "married workers" (Peeters and Crewe, 1984).

The transition from worker to gamergate is accompanied by dramatic changes in behavior. Gamergates cease to perform

worker duties, such as food provisioning and colony defense, live ~5 times longer, and assume behaviors typical of the former queen, including a dominant posture toward non-reproductive workers, an aversion to leave the secure nest, and a tendency to spend time near the brood (Penick et al., 2014). Because this *Harpegnathos* caste transition occurs in fully developed adult individuals, it offers a unique experimental paradigm to explore the role of gene regulation and epigenetic pathways in brain function and behavioral plasticity.

We recapitulated worker–gamergate transitions in a laboratory setting and identified hundreds of genes differentially regulated in the brain during behavioral reprogramming, including several neuronal genes implicated in synaptic plasticity and function. We focused on corazonin, a gene that became repressed in individuals that abandoned worker duties to pursue gamergate status, because it encodes a poorly understood neuropeptide widely conserved in arthropods (Predel et al., 2007). In *Drosophila melanogaster*, corazonin-expressing neurons play a role in regulating metabolism, stress response, and some aspects of male reproduction (Kapan et al., 2012; McClure and Heberlein, 2013; Tayler et al., 2012), but its role in social insects has never been investigated. Here, we show that injections of synthetic corazonin in the heads of *Harpegnathos* ants at early stages of the reproductive transition stimulated hunting, a typical worker behavior. Comparative transcriptomics revealed consistent trends in differential expression of corazonin in other social insect species, including wasps, bees, and termites. In addition to stimulating hunting behavior, corazonin inhibited ovary activation, suggesting long-term effects on caste identity. Based on our results, we propose that corazonin is a key regulator of worker behavior and worker caste identity in ants and other social insects.

RESULTS

Gene Regulation during a Behavioral Switch

We recapitulated the natural caste transition process of *Harpegnathos* in a controlled laboratory environment. Cohorts of young *Harpegnathos* workers from the same genetic background were separated from their original colony and transferred to a new nest in the absence of a dominant reproductive (Figure 1A). Dueling between aspiring dominant individuals typically began three days after colony reconstitution (Movie S1), peaked during days 5–8 with ~60% of the transferred workers engaging in dueling behavior, and began to subside after 2 weeks (Figure 1B). Besides a slight delay in the onset and peaking of dueling, these kinetics were similar to those observed upon gamergate removal from larger colonies containing workers of different ages (Sasaki et al., 2016).

Four months after the start of dueling the newly formed colonies displayed a stable social hierarchy with approximately one in four individuals serving as gamergates (Figure 1C), as determined by the presence of fully mature oocytes in their activated ovaries (Figure 1C, right; Figure S1A). The remaining 75% of individuals in each colony comprised old workers that had failed to establish dominance during the original tournament as well as the offspring of the transitioned gamergates that never experienced the absence of dominant reproductives and, therefore, behaved as workers (Figure 1C). We observed no obvious morphological differences between ants that successfully converted to gamergates and those that did not (Figures S1B and S1C).

We compared brain transcriptomes between mature gamergates harvested 120 days after the start of the transition and individuals that were transferred at the same time but failed to achieve dominance. We identified 427 differentially expressed ($p < 0.01$) genes, 112 of which passed a false discovery rate (FDR) cutoff of 10% (Figures 1D and S1D; Table S1). The top two gene ontology (GO) terms for biological processes enriched in these differentially expressed genes were "synaptic target inhibition" and "oviduct morphogenesis" (Figure 1E; Table S2), consistent with the notion that transcriptional reprogramming of reproductive and neuronal function accompanies the worker–gamergate behavioral transition. Genes previously reported to be caste-specific in other social insects were also differentially expressed in *Harpegnathos* workers and gamergates, including several components of the insulin pathway (Daugherty et al., 2011; Libbrecht et al., 2013; Wang et al., 2013) (*Ilp1*, *Ilp2*, *InR1*, *chico*, and *hopscotch*) and energy storage proteins (Martins et al., 2010, 2011; von Wyschetzki et al., 2015) (*Hex70a*, *Hex70b*, and *NLaz*) (Table S1). Genes involved in neurotransmission, such as those encoding GABA receptors *Grik1* and *Grik2*, as well as genes implicated in dendrite and synapse remodeling, such as *Fd3F* (Parrish et al., 2006), *Sulf1* (Dani et al., 2012), and *Daw* (Serpe and O'Connor, 2006), were also differentially expressed after the behavioral transition.

The gene encoding the neuropeptide corazonin was the top most consistently worker-biased gene (Figures 1D and S1D) and caught our attention because of its homology with the human gonadotropin-releasing hormone (Tian et al., 2016), which regulates the reproductive cycle (Herbison, 2016). Corazonin expression in *Harpegnathos* was mostly confined to the central brain (Figure S1E), in keeping with its role as a neuropeptide and expression patterns in *Drosophila* (Choi et al., 2005) as well as carpenter ants (Schmitt et al., 2015).

Corazonin is an undecapeptide conserved in most arthropods that has been implicated in the modulation of the stress response (Veenstra, 2009; Zhao et al., 2010). *Harpegnathos* workers that witness dominance tournaments but do not acquire reproductive status might reasonably experience social stress, which could confound our analysis; however, in workers collected from unperturbed colonies, corazonin was expressed at similarly higher levels compared to gamergates (Figure 1F), showing that its expression tracked worker identity per se rather than colony context or life history.

Corazonin Is Upregulated in Foraging Workers across Social Insects

Caste-specific regulation of corazonin was not exclusive to *Harpegnathos* and its uniquely flexible social system. Analysis of newly generated and previously published RNA sequencing (RNA-seq) from *Camponotus floridanus* (Bonasio et al., 2010, 2012), a carpenter ant with a fixed social system, revealed 8-fold higher levels of corazonin transcript in the whole bodies of workers compared to reproductive queens ($p < 0.05$; Figure 2A). Confirming its potential for dynamic regulation, corazonin

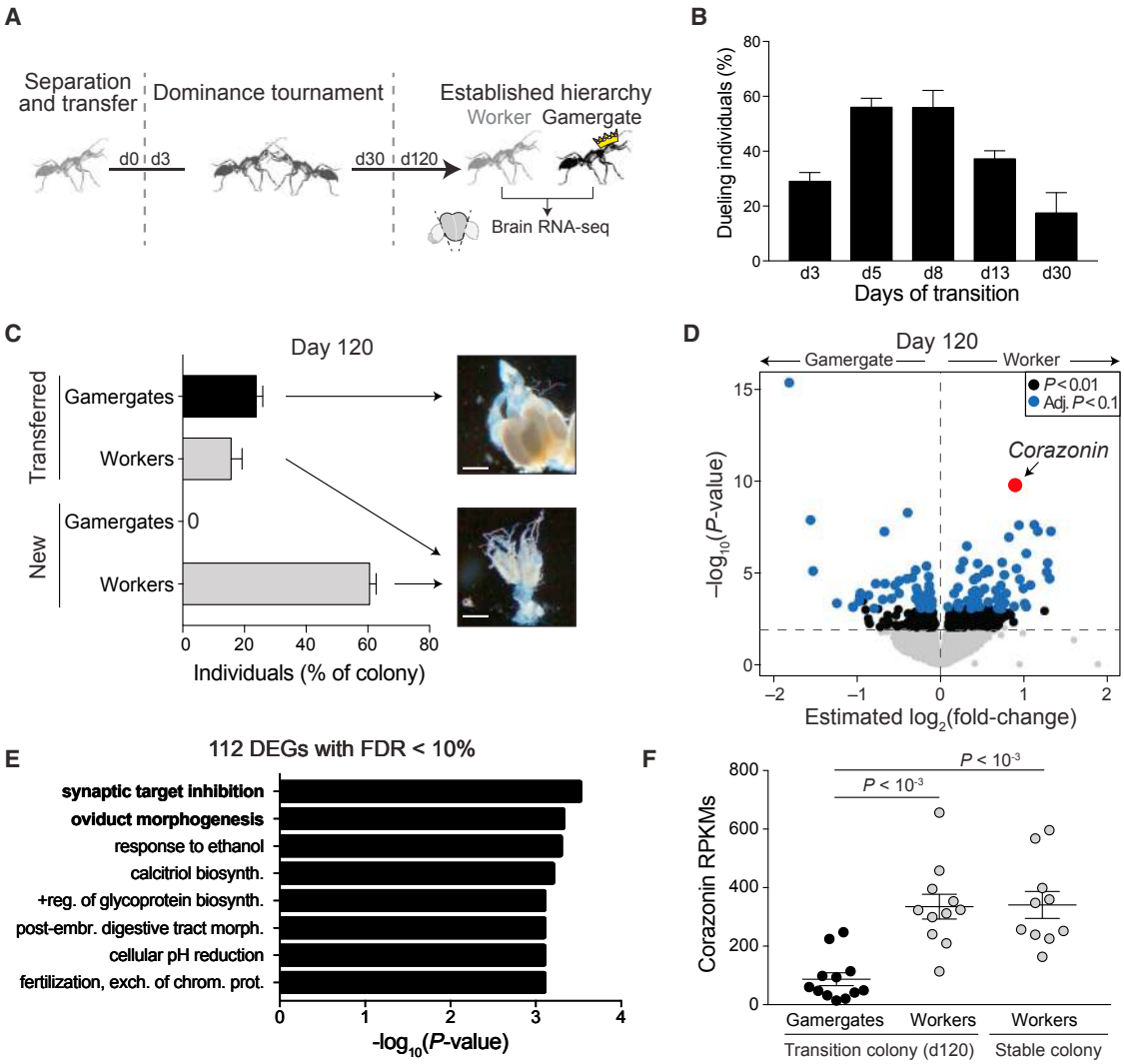

Figure 1. Phenotypic and Molecular Changes during the Worker–Gamergate Transition

(A) On day 0, young workers were transferred from stable colonies to new boxes without dominant reproductives. Tournaments typically began 3 days later and lasted for 3–4 weeks. Four months (d120) after transfer, brains (minus optic lobes) were processed for RNA-seq.

(B) Fraction of individuals engaged in dueling interactions as percentage of total individuals in the colony. Bars represent the mean of ≥5 colony replicates + SEM.

(C) Colony composition after tournaments. The reproductive status of the originally transferred (top) and newly eclosed (bottom) individuals was determined by the presence (upper image) or absence (lower image) of mature oocytes in their dissected ovaries. Bars represent the mean of 5 colony replicates + SEM. Scale bars in the microphotographs represent 0.5 mm.

(D) Volcano plot of RNA-seq from brains of workers and gamergates after 120 days of transition. Each circle represents a protein-coding gene. Differential genes with a FDR <0.1 are highlighted in blue. Data are from 11 worker and 12 gamergate brain replicates.

(E) Biological process gene ontology (GO) terms significantly (FDR <10%) enriched in the 112 differentially expressed genes (DEGs) in the worker–gamergate comparison from (D). Terms discussed in the text are in bold.

(F) Reads per kilobase per million (RPKMs) for corazonin RNA from brains of gamergates and workers 120 days after transition, as well as age-matched workers from stable colonies. Each circle represents a single brain. Means ± SEM. are shown. p values are from one-way ANOVA ($F[2, 16] = 15.56$, $p = 0.0002$) and a Holm-Sidak test.

See also Figure S1, Tables S1, S2, and S5, and Movie S1.

was upregulated during the worker phase and silenced during the reproductive phase in *Ooceraea biroi* (Figure S2A), a queenless ant species in which the same individuals perform worker and queen duties in alternating cycles that affect the whole colony (Oxley et al., 2014). Caste-specific differences in corazonin RNA levels were not restricted to ants; workers of *Polistes canadensis*, a primitively social wasp (Patalano et al., 2015), expressed more corazonin than reproductive queens (Figure 2B). We also observed statistically not-significant but suggestive trends in differential expression of corazonin between non-reproductive and reproductive female castes in termites (Figure S2B), which belong to a different insect order (Blattodea)

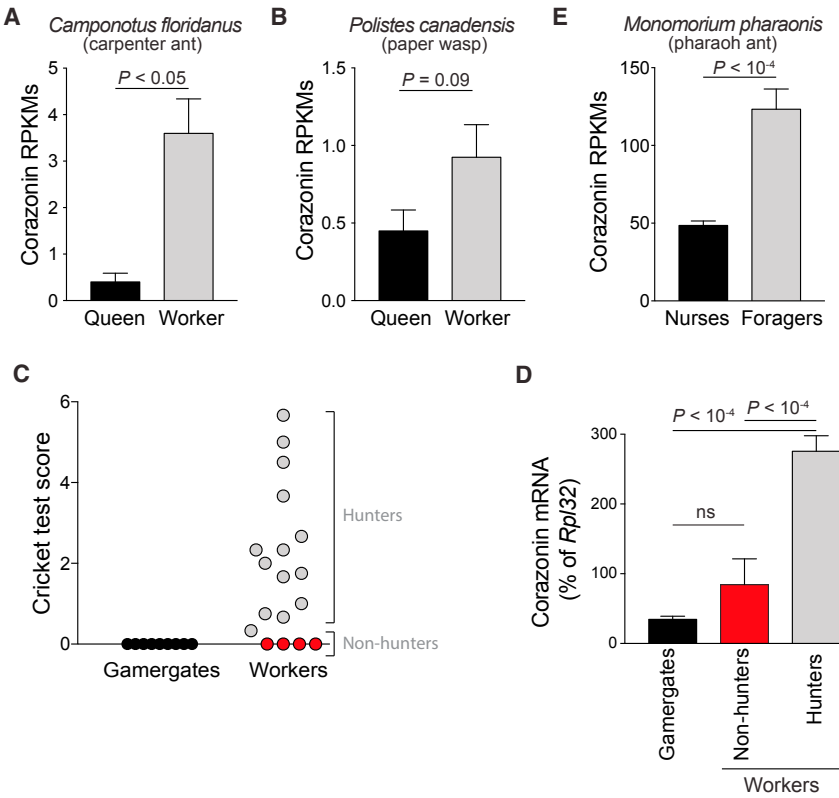

Figure 2. Corazonin Is a Conserved Marker of Foraging Activity

(A) RNA-seq levels of corazonin in the whole bodies of mated queens and combined major and minor workers of *Camponotus floridanus*. Data are from two (queens) or four (workers) biological replicates. p value is from a two-sided t test. Bars represent mean + SEM.

(B) RNA-seq levels of corazonin from brains of workers and reproductive queens of the paper wasp *Polistes canadensis* (Patalano et al., 2015). Data are from four (queens) or six (workers) replicates. p value is from a two-sided t test. Bars represent mean + SEM.

(C) Cricket test score in stable *Harpegnathos* colonies for gamergates and workers. Each circle is an individual ant. The experiment was performed in four separate colonies.

(D) qRT-PCR for corazonin in the brain of individuals tested for hunting activity in (C). Workers were stratified into non-hunter (cricket test score = 0; red bar) and hunter (cricket test score > 0; gray bar) groups. Bars represent mean + SEM. p values are from one-way ANOVA ($F[2, 31] = 63.26$, $p < 0.0001$) and a Holm-Sidak test.

(E) RNA-seq levels of corazonin in heads of *Monomorium pharaonis* workers engaged in nursing or foraging behaviors. Data are from ≥9 biological replicates per group. p value is from a two-sided t test. Bars represent mean + SEM.

See also Figure S2.

that diverged from Hymenoptera ~350 million years ago (Terrapon et al., 2014).

We used qRT-PCR to measure corazonin RNA levels in *Harpegnathos* individuals from stable colonies (>120 days old), while observing their foraging behavior. *Harpegnathos* is an obligate predator and its workers hunt for live prey (Jerdon, 1851). We set up a hunting arena where live crickets were trapped in a transparent tube sealed with a cotton plug impregnated with the cricket odor to provide both visual and olfactory stimuli (Movie S2). The arena was connected to the test colony and the interactions of each individual with the trapped crickets were captured on video (Movies S2 and S3) and quantified to yield a "cricket test score." As expected, gamergates did not hunt for crickets, whereas most workers carried out multiple hunting attempts per observation window (Figure 2C). Consistent with the results from the RNA-seq analysis above, corazonin RNA was abundant in the brains of hunting workers but scarce in gamergates (Figure 2D). Some workers displayed no interactions with the cricket tube (Figure 2C; "non-hunters"), despite having inactive ovaries, as verified by dissection at the end of the experiment. These non-hunting workers expressed low levels of corazonin (Figure 2D), comparable to those in gamergates, suggesting that increased levels of corazonin are associated with hunting (i.e., foraging) behavior rather than reproductive caste identity.

Corazonin levels were also significantly ($p < 10^{-4}$) higher in the heads of *Monomorium pharaonis* workers caught in the act of foraging when compared to workers that cared for the brood ("nurses"; Figure 2E). As both foragers and nurses in *Monomorium* are sterile, the differences in corazonin expression do not reflect different reproductive potentials. Consistent with these observations, corazonin also showed a trend ($p = 0.12$) toward higher levels in honeybee foragers compared to nurses (Figure S2C).

Thus, robust expression of corazonin is a conserved feature of worker brains in social insects and is associated with foraging activity.

Corazonin Stimulates Hunting and Inhibits Dueling

We determined the kinetics of corazonin expression by analyzing a different dataset, for which, in addition to mature gamergates, we had also collected dueling individuals at intermediate time points. The decrease in corazonin RNA was evident as early as day 6 after separation and had reached gamergate levels at day 13 (Figure 3A). Thus, we reasoned that injecting corazonin in early transitioning individuals might restore some of the worker behaviors.

To analyze whether differences in the abundance of corazonin RNA were reflected on peptide levels, we measured the concentration of the corazonin peptide using quantitative mass spectrometry with a calibrated curve and an isotope-labeled spike-in control (Figure S3A) and determined that workers had, on average, 5.4 ng of corazonin per brain (Figure S3B), or ~2.5-fold more than gamergates, which is consistent with our previous mRNA quantifications.

To determine whether changes in corazonin were sufficient to alter hunting behavior, we injected early transitioning ants (day 5

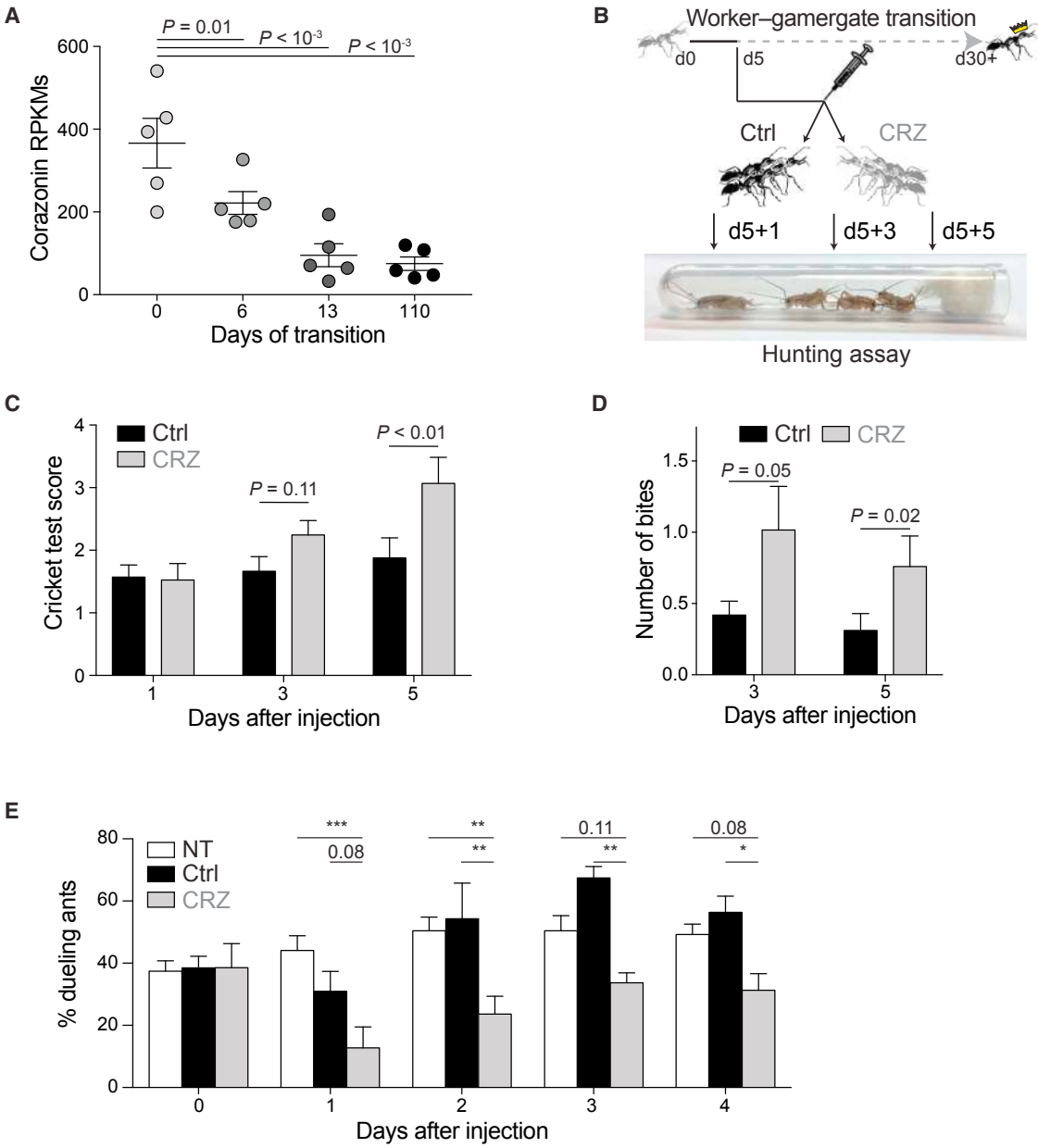

Figure 3. Corazonin Stimulates Hunting and Inhibits Dueling

(A) RNA-seq levels for corazonin at different time points (days) of transition. Three whole brains (including optic lobes) per time point were pooled and sequenced from five independent colonies (individual circles). Horizontal bars represent mean ± SEM. p values are from one-way ANOVA and a Holm-Sidak test.

(B) Dueling individuals at day 5 of the transition were injected with corazonin (CRZ) or scrambled control (ctrl). Hunting behavior was quantified 1, 3, and 5 days after injection using the cricket-in-the-tube test.

(C) Cricket test scores for corazonin- and control-injected ants. Each micro-colony contained three individuals injected with corazonin peptide and three injected with a control scrambled peptide. Bars represent the mean scores of >40 biological replicates (micro-colonies of 3 + 3 ants) + SEM. p values are from two-sided Wilcoxon tests.

(D) Same as in (C), except only biting events were scored.

(E) In transitioning colonies of 30 individuals, half of the ants were injected with corazonin peptide (CRZ) and half with control scrambled peptide (Ctrl). As an additional control, some colonies were left untreated (NT). Bars represent the mean of the percentage of ants observed to duel in each colony + SEM. Data are from ≥4 biological replicates (colonies); i.e., ≥ 120 injected individuals. p values are from ANOVA and a Holm-Sidak tests.

See also Figures S3 and S4, Table S5, and Movies S2–S6.

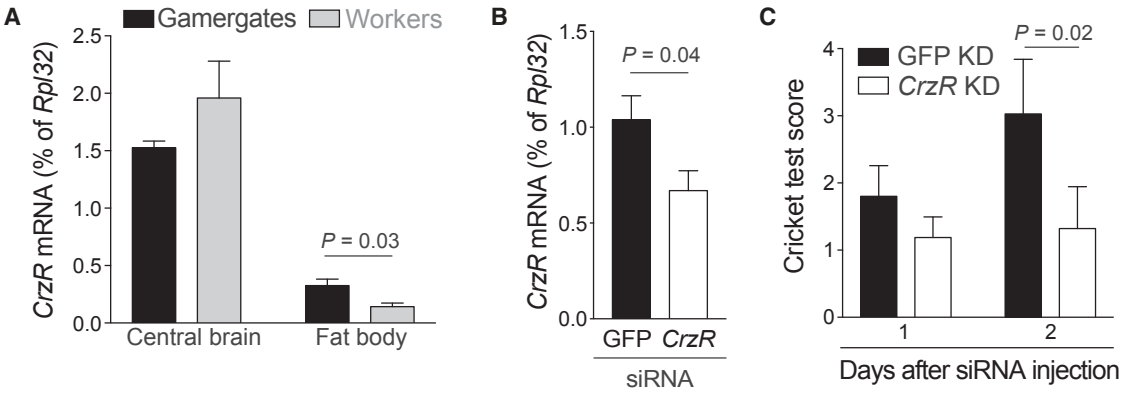

Figure 4. Corazonin Receptor Knockdown Decreases Hunting

(A) qRT-PCR for the corazonin receptor in the brains and fat bodies of workers and gamergates from stable colonies. Bars represent the mean from four biological replicates (individual ants) + SEM. Differences in the brain are not significant. p value is from a two-sided t test.

(B) qRT-PCR for the corazonin receptor in the brains of ants injected with siRNAs against the corazonin receptor or control siRNAs against GFP. Bars represent the mean from ≥7 biological replicates + SEM. p value is from a two-sided t test.

(C) Cricket test scores after corazonin receptor knock-down. Bars represent means from ≥12 biological replicates (micro-colonies) per group per time point + SEM. p value is from a two-sided Wilcoxon test.

See also Figure S5 and Table S5.

after separation) with 100 ng of synthetic corazonin peptide, reasoning that we needed to administer more than the endogenous amounts to reach similar effective concentrations at the active sites. Thirty minutes after injection, levels of corazonin peptide in the head had increased ~7-fold (Figure S3C). We monitored behavior in > 40 micro-colonies composed of six young dueling ants each, three injected with corazonin and three with scrambled control peptide and measured their hunting activity at different time points (Figure 3B). Because all injected individuals were dueling in the transitioning colony of origin, before being transferred to the micro-colony, they were assumed to be at comparable stages in their transition to gamergates.

Five days after corazonin injections, the interactions of treated individuals with crickets increased significantly ($p < 0.01$) and a trend ($p = 0.11$) was observed as early as three days after injections (Figure 3C). Attempts at biting the crickets were more frequent in ants treated with corazonin at both time points (Figure 3D; Movie S4). Mortality rates were comparable between treatment and control (Figure S4A) and no difference was observed in locomotory activity (Figure S4B; Movie S5) or in the low baseline number of interactions with similarly prepared tubes but with no crickets inside (Figure S4C). The increased interactions with the cricket tube displayed by corazonin-injected individuals were not due to an overall heightened aggression, as the treatment did not alter the proportion of aggressive and non-aggressive reactions to an invasive stimulus simulated by a confrontation with metal forceps (Figures S4D–S4H; Movie S6). In contrast, injection of corazonin inhibited dueling behavior at various time points (Figure 3E), as compared to both untreated individuals (NT) and individuals injected with scrambled peptide as control.

To confirm the specificity of the observed behavioral effect with an independent approach, we performed small interfering RNA (siRNA)-mediated knockdown for the corazonin receptor (*CrzR*), which was expressed at comparable levels in the brains of both workers and gamergates (Figure 4A). We obtained a partial (~35%) but significant ($p = 0.04$) knockdown of *CrzR* mRNA (Figure 4B), which did not affect corazonin expression (Figure S5A). Individuals treated with corazonin receptor siRNAs were significantly ($p = 0.02$) less likely to hunt at day 2 post-injection than individuals treated with controls siRNAs against GFP (Figure 4C). A similar trend was observed at day 1 post-injection. No differences in survival rates between treatment and control were observed (Figure S5B).

We conclude that corazonin promotes hunting, a worker-specific behavior, and inhibits dueling, a behavior that accompanies the transition to gamergates.

Transcriptional Changes following the Activation and Inhibition of Corazonin Signaling

We studied the early transcriptional response to altered corazonin levels by performing RNA-seq in the brains of individuals 24 hr after corazonin or control injections (Figure 5A). We identified 76 differentially expressed genes ($p < 0.01$), of which 47 were activated and 29 repressed (Table S3). Genes affected by corazonin injections included *Sur*, which regulates hormone secretion from corpora cardiaca (Kim and Rulifson, 2004), *Teq*, implicated in long-term memory formation (Didelot et al., 2006), and *mav* and *gogo*, which regulate synaptic differentiation in *Drosophila* (Fuentes-Medel et al., 2012; Tomasi et al., 2008). Ranked lists of genes affected by corazonin injections and genes differentially expressed between workers and gamergates overlapped partially but significantly ($p < 10^{-4}$) (Figure 5B). Eight genes were affected at a p value cutoff of 0.01 by both treatments (overlap $p < 10^{-3}$, hypergeometric distribution), and were affected in a direction consistent with our model; that is, genes upregulated in workers compared to gamergates were also upregulated in response to corazonin injections and vice versa (Figure 5C).

Similarly, knockdown of the corazonin receptor by siRNAs in the brain resulted in an extensive transcriptional response

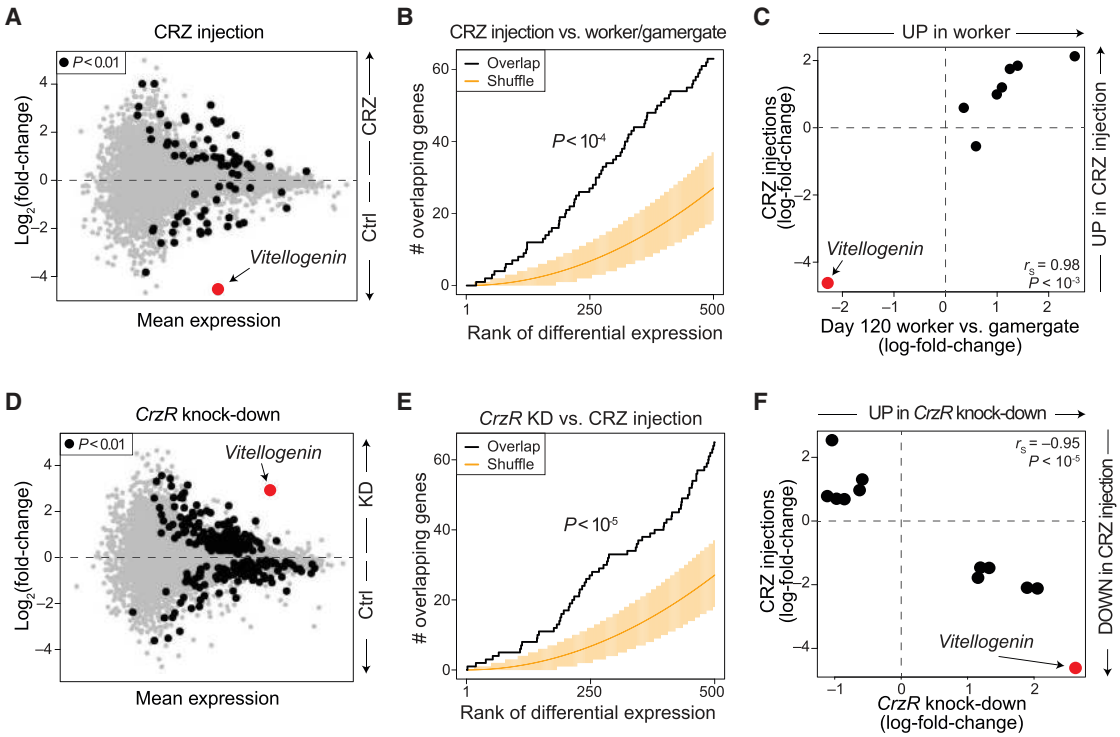

Figure 5. Changes in Brain Transcriptomes upon Corazonin Injections and Receptor Knockdown

(A) MA plot of RNA-seq data from brains of transitioning ants, 24 hr after injections with corazonin peptide (CRZ) or scrambled control (ctrl). Genes with $p < 0.01$ are highlighted in black. Data are from ≥ 10 biological replicates (individual ants) per condition.

(B) Genes affected by corazonin injections from (A) significantly overlap with genes differentially expressed in the worker–gamergate transition from Figure 1D. Genes were ranked by the product of the absolute log-fold-change multiplied by the log-converted p value. Overlap between the first 500 genes in each list is shown (black line) compared to 100,000 random permutations (orange line + confidence interval). p value was computed empirically based on the permutations.

(C) Comparison of log-fold-change for differentially expressed genes ($p < 0.01$) present in both the worker versus gamergate comparison (x axis) and the corazonin peptide versus control peptide injection comparison (y axis).

(D) MA plot of RNA-seq data from brains of transitioning ants, 24 hr after injections with siRNAs against the corazonin receptor (KD) or GFP (ctrl). Genes with $p < 0.01$ are highlighted in black. Data are from nine biological replicates (individual ants) per condition.

(E and F) Same as that in (B) and (C), but comparing genes ranked for their changes after corazonin peptide or *CrzR* siRNA injections.

See also Tables S3–S5.

involving 275 significantly ($p < 0.01$) affected genes (Figure 5D; Table S4). The ranked list of genes affected overlapped partially but significantly ($p < 10^{-5}$) with genes affected by corazonin injections (Figure 5E). Twelve genes were affected at a p value cutoff of 0.01 by both treatments (overlap $p < 10^{-9}$, hypergeometric distribution). Genes strongly upregulated after peptide injections were strongly downregulated following knockdown of the corazonin receptor (Figure 5F), demonstrating that the two treatments affected similar pathways but in opposite directions.

Vitellogenin Opposes Corazonin in Regulating Hunting and Reproduction

We noticed that the mRNA for vitellogenin was among the most consistently affected by the natural worker–gamergate transition (Table S1), corazonin injections (Figures 5A and 5C) and corazonin receptor knockdown (Figures 5D and 5F). These changes in response to corazonin were consistent with the timing of the natural upregulation of vitellogenin during the worker–gamergate transition (Figure 6A, compare to Figure 3A). Vitellogenin is also expressed in the brain of other Hymenoptera, such as honeybees and bumblebees, where it has been suggested to serve a neuroendocrine role (Lockett et al., 2016; Münch et al., 2015); however, the bulk of vitellogenin is synthesized in the fat body—the insect equivalent of the vertebrate liver and adipose tissue (Arrese and Soulages, 2010)—and is a trophic factor for egg development (Raikhel and Dhadialla, 1992).

We confirmed by qRT-PCR that injections of corazonin peptide and knockdown of the corazonin receptor caused a decrease and increase, respectively, of vitellogenin mRNA levels in the brain (Figures 6B and 6C). We injected siRNAs targeting vitellogenin into the heads of dueling workers, which resulted in a substantial (~73%) decrease in vitellogenin transcripts (Figure 6D) but did not affect the levels of corazonin mRNA (Figure 6E). Knockdown of vitellogenin stimulated hunting (Figure 6F), suggesting that the pro-hunting activity of corazonin might be, in part, due to its repression of vitellogenin in the brain.

Reproductive gamergates expressed much higher levels of vitellogenin in their fat bodies compared to workers (Figure 6G). According to the FlyAtlas (Chintapalli et al., 2007), *Drosophila* fat bodies express the corazonin receptor and we also detected

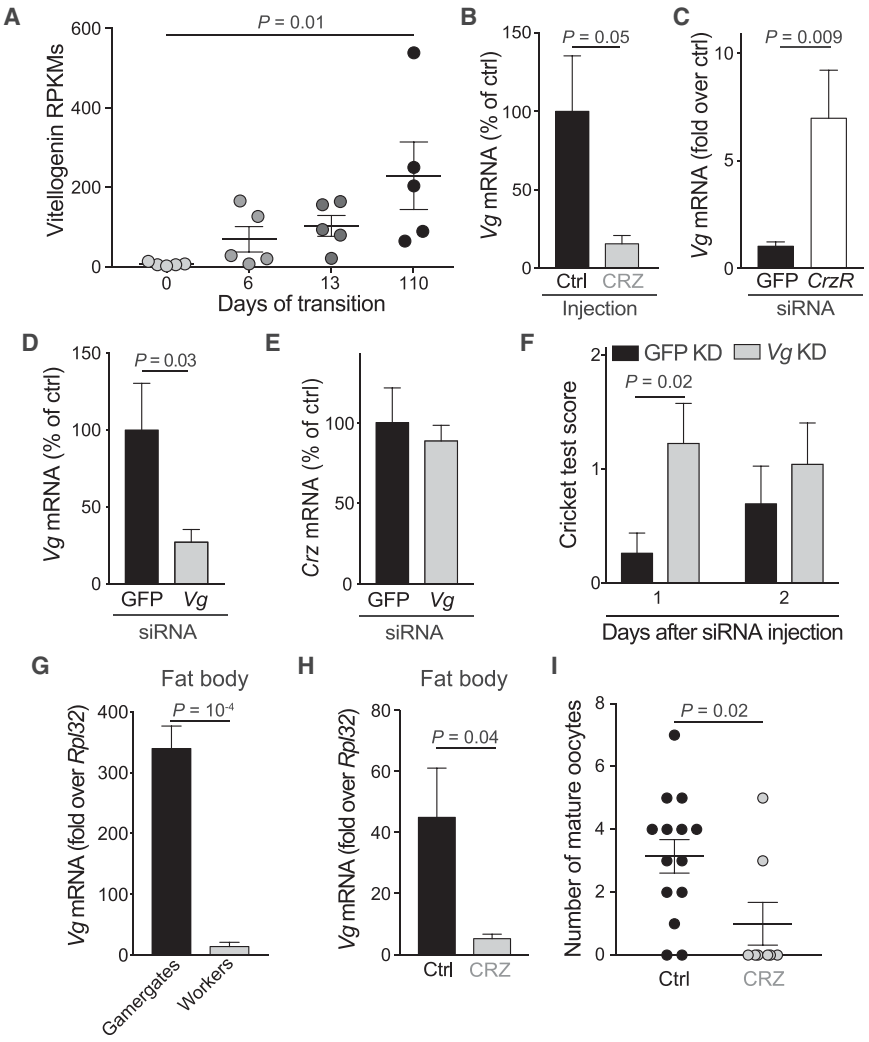

Figure 6. Vitellogenin Is a Target of Corazonin and Regulates Hunting and Reproductive Behavior

(A) RNA-seq levels of vitellogenin in whole brains (including optic lobes) during the transition. Values are from the same RNA-seq data set used in Figure 3A. Horizontal bars represent mean ± SEM. p values are from one-way ANOVA and a Holm-Sidak test.

(B and C) qRT-PCR of vitellogenin in ant brains 1 day after injection of corazonin and control peptides (B) or *CrzR* and GFP siRNAs (C) in the head. Data are expressed relative to control injections. Bars represent the mean from ≥7 biological replicates (individual ants) per group + SEM. p values are from two-sided t tests.

(D and E) qRT-PCR for vitellogenin (D) and corazonin (E) in the brains of ants injected with siRNAs against vitellogenin or GFP. Bars represent the mean from ≥ 7 biological replicates per condition + SEM. p value is from a two-sided t tests.

(F) Cricket test scores after vitellogenin knockdown. Bars represent means from ≥16 biological replicates (micro-colonies) per group per time point + SEM. p value is from a two-sided Wilcoxon tests.

(G) qRT-PCR for vitellogenin in the fat body of workers and gamergates. Bars represent the mean from four biological replicates (individual ants) per group + SEM. p value is from a two-sided t test.

(H) qRT-PCR for vitellogenin in the fat body of transitioning ants one day after injection of corazonin (CRZ) or scrambled control in the gaster. Bars represent the mean from ≥7 biological replicates (individual ants) + SEM. p value is from a two-sided t test.

(I) Number of mature oocytes in transitioning ants 10 days after injections of corazonin peptide (CRZ) or scrambled control. Each circle represents a biological replicate (individual ant). Horizontal bars represent the mean ± SEM. p value is from a Mann-Whitney test.

See also Figure S6 and Table S5.

its presence in fat bodies from both *Harpegnathos* gamergates and workers, albeit at lower levels than in the brain (Figure 4A, right). Based on these observations and on the fact that corazonin can act systemically in *Drosophila* (Kubrak et al., 2016), we hypothesized that the increased levels of corazonin in *Harpegnathos* workers could affect vitellogenin expression in the fat body, potentially linking the behavioral and reproductive changes observed during the worker–gamergate transition. Indeed, injections of corazonin peptide into the gasters of transitioning ants inhibited vitellogenin expression in the fat body (Figure 6H) and reduced ovary activation, as shown by a ~70% decrease in the number of mature oocytes (p = 0.02) 10 days after corazonin injections compared to control injections (Figure 6I).

The connection between corazonin activity, vitellogenin levels, and reproduction was not limited to *Harpegnathos*. Using an *elav*-GAL4 driver, we knocked down endogenous corazonin and overexpressed the *Harpegnathos* corazonin gene in *Drosophila* brains (Figures S6A and S6B). Consistent with our observations in ants, knockdown of corazonin in flies increased expression of the three vitellogenin homologs (*Yp1*, *Yp2*, and *Yp3*) and resulted in a higher egg-laying rate, whereas overexpression of corazonin caused the opposite effects (Figures S6C–S6E).

These data support the conclusion that corazonin promotes worker behavior at least in part by repressing vitellogenin expression in both the brain and the fat body. This and other downstream effects of corazonin activation result in the stimulation of worker behavior, such as hunting, and the inhibition of gamergate behaviors, such as dueling and egg-laying, thus controlling the balance between the two caste identities (Figure 7).

DISCUSSION

By measuring changes in gene expression in the brains of *Harpegnathos* individuals undergoing a behavioral and reproductive switch, we identified corazonin as a neuropeptide that promotes a worker-specific behavior—hunting—and reinforces worker caste identity at the expense of reproductive activity. To our knowledge, this is the first time that a neuropeptide is implicated in caste-specific behavior in ants.

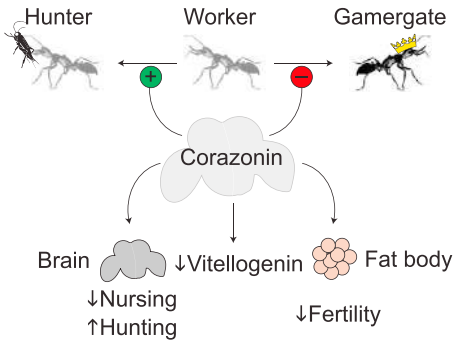

Figure 7. Model for the Action of Corazonin

High corazonin levels stabilize worker identity by stimulating hunting behavior and inhibiting the transition to gamergate. At the molecular level, this could be, in part, achieved by decreased levels of brain vitellogenin, which represses nursing and stimulate hunting, as well as decreased levels of fat body vitellogenin, which regulates fertility.

There is great interest in dissecting the genetic and epigenetic control of behavior in animals; yet, few genes have been directly linked to the regulation of complex social traits (Bendesky et al., 2017). Here, we showed that changes in social status within an ant colony result in a reprogramming of the transcriptional output in *Harpegnathos* brains (Figures 1D and S1D), and that the expression of one of those genes, corazonin, is causally linked to the performance of caste-specific behaviors. Although the present study focuses on corazonin, the other differentially expressed genes comprise promising candidates involved in transcriptional regulation and neuronal plasticity and previously unknown to play roles in social behavior (Table S1).

We observed a consistent trend for corazonin upregulation in foraging workers from multiple social insect species representing several independent origins of eusociality (wasps, bees, termites, and ants). This is consistent with the hypothesis that alternative, pre-existing genetic programs are responsible for differences in caste behavior. In support of this, effects of corazonin on life phase transitions have been observed in other, non-social insects, including the silkworm (*Bombyx mori*) (Tanaka et al., 2002), the hawk moth (*Manduca sexta*) (Kim et al., 2004), and, most notably, locusts (Maeno et al., 2004; Sugahara et al., 2015; Tawfik et al., 1999). Locusts can switch between two alternate phenotypes: a low-corazonin solitary form and a high-corazonin gregarious form that gives rise to the swarms that continue to be a major agricultural and social plague (Ernst et al., 2015; Sugahara et al., 2015). Although locusts do not display reproductive division of labor, the differences between the two phenotypes present intriguing analogies with those between ant castes: similar to gregarious locusts, ant workers have shorter lifespans and larger brains than queens (Durst et al., 1994; Gronenberg et al., 1996; Gronenberg and Liebig, 1999; Keller and Genoud, 1997), are much more active foragers, and, as we showed here, also express high levels of corazonin.

The existence of an ancient pathway connecting corazonin with reproduction is further supported by our observations that corazonin knockdown in *Drosophila* brains stimulated vitellogenin expression and egg deposition, whereas its overexpression had opposite effects (Figure S6). Previous studies reported that corazonin-expressing neurons regulate insulin production, stress response, and metabolism in *Drosophila* (Kapan et al., 2012; Varga et al., 2016; Zhao et al., 2010). In our ant system, natural and artificial changes in corazonin signaling were not followed by changes in the expression of stress-related genes, although we did observe changes in the insulin pathway (Tables S1, S3, and S4). More specifically, it was proposed that corazonin might act as a mediator of nutritional stress in several insects (Veenstra, 2009), which is consistent with the observation that corazonin-producing neurons also express a fructose receptor, *Gr43a,* that endows them with the ability to respond to nutrient levels in the hemolymph (Mishra et al., 2013; Miyamoto et al., 2012). It is tempting to speculate that an ancient nutrient-sensing pathway that regulated reproduction was coopted during the evolution of eusociality in order to force the worker caste into a persistent food-seeking state that would benefit the colony, while repressing the workers' reproductive potential.

Based on the downregulation of vitellogenin in response to corazonin expression, we propose a corazonin-vitellogenin axis as a key regulator of caste-specific behavior and identity in *Harpegnathos* (Figure 7) and, possibly, other social insects. Most vitellogenin is synthesized in the fat body and delivered to developing eggs where it serves as food source (Raikhel and Dhadialla, 1992). During the evolution of formicoid ants the vitellogenin gene was duplicated and some paralogs acquired worker-specific expression (Corona et al., 2013). This is consistent with the reproductive ground plan hypothesis, which states that the evolution of eusociality involved the genetic uncoupling of maternal care behaviors, such as foraging, from reproduction (Amdam et al., 2004; West-Eberhard, 1987). However, honeybees and ponerine ants, such as *Harpegnathos*, only have one vitellogenin, suggesting that the same gene might perform both reproductive (queen-specific) and behavioral (worker-specific) functions (Ihle et al., 2010), possibly dependent on context and site of expression. Therefore, both the behavioral and reproductive effects of corazonin might be in part mediated by its negative regulation of vitellogenin in brain and fat body, respectively (Figure 7). It would be interesting to know which vitellogenin responds to corazonin in extant ant species with multiple paralogs and whether different vitellogenins are controlled by corazonin in the brain compared to the fat body.

Although corazonin inhibits vitellogenin expression in *Harpegnathos*, lack of corazonin is not sufficient for the activation of reproductive behavior, as demonstrated by the fact that non-hunting workers do not activate their ovaries despite low corazonin levels (Figure 2D) and that *Monomorium* nurses express low levels of corazonin but remain obligately sterile (Figure 2E). It is likely that the upregulation of vitellogenin and ensuing activation of reproductive status require more than one signal, with corazonin only providing one of the many checks and balances.

Signs of conservation for a social function of corazonin can also be found in vertebrates. Social status in the fish *Astatotilapia burtoni* correlates with changes in the expression levels of gonadotropin releasing hormone (White et al., 2002), the only corazonin homolog in vertebrates, suggesting that a role for

corazonin in regulating reproduction and behavior in response to social cues might even predate the split between arthropods and chordates.

Based on these considerations and our results, we propose that in addition to its potential role in the stress pathway, corazonin is a regulator of reproduction that was co-opted during the evolution of caste-specific physiology and behavior in social insects.

In conclusion, the present study further validates ants as a model organism approach to the study of social behavior at a molecular level (Bonasio, 2012; Yan et al., 2014). Future studies on corazonin and other genes differentially expressed between *Harpegnathos* workers and gamergates should reveal how social cues, such as the absence of a queen, can give rise to the epigenetic reprogramming of an entire organism to a different physiological and behavioral phenotype.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, five tables, and six movies and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2017.07.014.

AUTHOR CONTRIBUTIONS

Caste-specific expression of corazonin was discovered by R.B. as a postdoc in D.R.'s laboratory using samples from a transition experiment designed and performed by C.A.P and J. L., with help from H.Y. (Figures 3A and 6A). Y.L. and B.A.G. performed mass spectrometry. T.A.L. and A.S.M. provided the unpublished *Monomorium* data. All remaining experiments were performed by J.G. in R.B.'s laboratory with help from K.M.G. and support from S.L.B. E.J.S. and R.B. performed bioinformatic analyses. R.B. wrote the manuscript with input from all authors.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Corazonin (pETFQYSRGWTN-NH2)	Abbiotec	#350130
Scrambled control (QRETYNSWFTG-NH2)	Abbiotec	NA
Critical Commercial Assays		
Power SYBR Green RNA-to-CT 1-Step Kit	Thermo Fisher	#4389986
Power SYBR Green PCR Master Mix	Thermo Fisher	#4367659
QuantiTect Reverse Transcription Kit	QIAGEN	#205313
TURBO DNase (2 U/μL)	Invitrogen	#AM2239
NextSeq 500/550 High Output Kit v2 (75 cycles)	Illumina	#FC-404-2005
NEBNext Library Quant Kit for Illumina	NEB	#E7630
Deposited Data		
C. floridanus, two workers, whole body RNA-seq (Figure 2A)	Bonasio et al., 2010	GEO: GSE22680
C. floridanus, one queen, whole body RNA-seq (Figure 2A)	Bonasio et al., 2012	GEO: GSE31346
P. Canadensis, brain RNA-seq (Figure 2B)	Patalano et al., 2015	GEO: GSE59525
O. biroi, brain RNA-seq (Figure S2A)	Oxley et al., 2014	SRA: SRP066896
Z. nevadensis, whole body RNA-seq (Figure S2B)	Terrapon et al., 2014	SRA: SRP022929
A. mellifera, brain CAGE-scan (Figure S2C)	Khamis et al., 2015	GEO: GSE64315
Harpegnathos, d120 worker versus gamergate, brain minus optic lobes RNA-seq (Figures 1D–1F)	This paper	GEO: GSE83798
C. floridanus, two workers and one queen, whole body RNA-seq (Figure 2A)	This paper	GEO: GSE83799
M. pharaonis, head RNA-seq (Figure 2E)	This paper	GEO: GSE83803
Harpegnathos, transition time points, whole brain RNA-seq (Figures 3A and 6A)	This paper	GEO: GSE83804
Harpegnathos, corazonin injections, brain minus optic lobes RNA-seq (Figures 5A–5C)	This paper	GEO: GSE83806
Harpegnathos, corazonin receptor knock-down, brain minus optic lobes RNA-seq (Figures 5D–5F)	This paper	GEO: GSE99130
Experimental Models: Organisms/Strains		
Harpegnathos saltator: Wild type	This paper	N/A
Monomorium pharaonis: Wild type	Warner et al., 2017	N/A
Drosophila: Wild type: w1118	Bloomington Drosophila stock center (BDSC)	#5905
Drosophila: Wild type: OreR	BDSC	#5
Drosophila: RNAi of Crz: y1v1; P{TRiP.JF02023}attP2	BDSC	#25999
Drosophila: UAS-nGFP: w1118; P{UAS-GFP.nls}14	BDSC	#4775
Drosophila: elav-GAL4: w*; P{GAL4-elav.L}3	BDSC	#8760
Drosophila: UAS-hsalCrz: y1w*; P{UAS-hsalCrz}attP/CyO	This paper	N/A
Drosophila: w*/y1v1; +/+; P{GAL4-elav.L}3/ P{TRiP.JF02023}attP2	This paper	N/A
Drosophila: w*/w1118; P{UAS-GFP.nls}14/+; P{GAL4-elav.L}3/+	This paper	N/A
Drosophila: w*/y1w*; P{UAS-hsalCrz}attP/+; P{GAL4-elav.L}3/+	This paper	N/A
Oligonucleotides		
qPCR primer, *CrzR* F: GGCTCGACGATCTACACGCTGC	This paper	N/A
qPCR primer, *CrzR* R: GTTACCCCACAACCAGGCGACG	This paper	N/A
qPCR primer, *Crz* F: CGCGGATGGACCAACGGAAAAAG	This paper	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
qPCR primer, *Crz* R: GCAGCGGTTGTTCATCGACA	This paper	N/A
qPCR primer, *Vg* F: CACCTTGACACAACTAGATATAC	This paper	N/A
qPCR primer, *Vg* R: GTCGGAAGCCTTGATTTG	This paper	N/A
qPCR primer, *Rpl32* F: CGTAGGCGATTTAAGGGTCA	This paper	N/A
qPCR primer, *Rpl32* R: TTTCGGAAGCCAGTTGGTAG	This paper	N/A
DsiRNA, *CrzR* top: rGrUrGrArArArArArCrArUrArArArGrArArGr UrArUrCrATT	This paper	N/A
DsiRNA, *CrzR* bottom: rArArUrGrArUrArCrUrUrCrUrUrUrArUr GrUrUrUrUrUrCrArCrCrU	This paper	N/A
DsiRNA, *Vg* top: rGrArUrArGrUrUrUrCrUrUrCrArArUrArArAr CrArUrCrAGT	This paper	N/A
DsiRNA, *Vg* bottom: rArCrUrGrArUrGrUrUrUrArUrUrGrArAr GrArArArCrUrArUrCrArG	This paper	N/A
DsiRNA, nGFP top: rArGrArGrArArArGrGrUrArGrArUrCrCr ArArArArArArGAA	This paper	N/A
DsiRNA, nGFP bottom: rUrUrCrUrUrUrUrUrUrGrGrArUrCr UrArCrCrUrUrUrCrUrCrUrUrC	This paper	N/A
Recombinant DNA		
pBID-UASC	Wang et al., 2012	Addgene #35200
pBID-UASC-hsal*Crz*	This paper	N/A
Software and Algorithms		
STAR	Dobin et al., 2013	https://github.com/alexdobin/STAR
DEGseq (R package)	Wang et al., 2010	https://www.bioconductor.org/packages/release/bioc/html/DEGseq.html
DESeq2 (R package)	Love et al., 2014	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
OrderedList (R package)	Lottaz et al., 2006	https://bioconductor.org/packages/release/bioc/html/OrderedList.html
Other		
in vivo-jetPEI	Polyplus-transfection	#201
Hank's Balanced Salt Solution	ThermoFisher	#14175095
GlycoBlue Coprecipitant (15 mg/mL)	Invitrogen	# AM9516

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Roberto Bonasio (rbon@mail.med.upenn.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Harpegnathos saltator ants

All *Harpegnathos* ants originated from colonies originally excavated in Karnataka, India in 1999 and propagated as gamergate colonies since, first in the Liebig lab, then in the Reinberg, Berger, and Bonasio lab, in this order. As colonies were split and mixed repeatedly over the years, the genetic background can be considered mixed and homogeneous. However, because ants were housed in separate colonies, colony-of-origin effects were taken into account with appropriate experimental design (treatment and control from the same colony) and statistics (paired tests).

Harpegnathos colonies were housed in plastic boxes with a plaster nest chamber in a temperature (25°C) and humidity (50%) controlled ant facility on a 12 hr light/dark cycle. Ants were fed three times per week with live crickets and plaster was wet with water to prevent desiccation of the ant brood.

Monomorium pharaonis ants

Monomorium ants were maintained in glass nests, between two pieces of 4 cm x 6 cm glass, at 27 ± 1°C and 50% humidity and fed twice weekly with dried mealworms (*Tenebrio molitor*) and an agar-based synthetic diet (Dussutour and Simpson, 2008; Warner et al., 2017).

Drosophila melanogaster

All flies were raised at 25°C and 50% humidity on a 12 hr light/dark cycle using standard Bloomington *Drosophila* Medium (Nutri-Fly). The w^{1118} (5905), OreR (5), *Crz* RNAi (25999), UAS-nGFP (4775) and *elav*-Gal4 driver (8760) lines were purchased from the Bloomington *Drosophila* stock center. The UAS-hsal*Crz* transgenic line was generated by cloning the *Harpegnathos* cDNA for corazonin into pBID-UASC (Wang et al., 2012), followed by PhiC31 integrase-mediated transgenesis into the *attP40* landing site on chromosome 2, performed by BestGene.

METHOD DETAILS

Worker–gamergate transitions

To induce worker–gamergate transitions we transferred 30 newly eclosed female ants (1–3 days old) from mature colonies to a new nest with three males. Every ant was individually painted with a unique two-color combination. Ants were carefully observed for 1–2 hr every morning and evening for the following two weeks to monitor dueling activities. After 120 days, ants were scored for their hunting behavior and sacrificed. Brains (minus optic lobes) were dissected for total RNA extraction and worker versus gamergate status was scored by visual inspection of the ovaries. An individual was classified as gamergate if it had activated ovaries, as judged by the presence of mature oocytes (Figures 1C and S1A).

The transition time-point experiment (Figures 3A and 6A) was performed in the Liebig laboratory at ASU. We set up 5 adoptive colonies lacking dominant reproductives using 70–100 young (4–8 weeks) workers per colony. At the time of transfer, we removed 3 workers for baseline gene expression (“d0”). We monitored colonies each day until more than 8 workers in a colony were observed dueling, usually three days later. On the 3rd, and 10th day after the start of the tournament, we removed the three individuals (“d6” and “d13”) that had been observed to duel most frequently. Finally, three established gamergates were sampled between 104 and 110 days after transfer (“d110”). We identified their status on behavioral grounds (location next to cocoons, submissive response by subordinate workers, standing with high posture) and verified it by dissection and observation of their activated ovaries. Immediately after collection, each individual was dipped in liquid nitrogen and frozen at −80°C until dissection. Whole brains (in this case including the optic lobes) were dissected on dry ice and kept frozen until further processing.

Corazonin injections

On the 5th day of transitions performed with 30 females and 3 males, ants that had been dueling for three consecutive days in transitioning colonies (i.e., in the absence of a queen or previously established gamergates) were transferred to a new nest in groups of 6 (micro-colony). Three were injected with synthetic corazonin and three with a controlled scrambled peptide. The scrambled peptide control accounted for potential non-specific effects on behavior due to the process of injection. Each ant was injected with a calibrated glass capillary needle directly into the head, right below the antennas, or into the gaster with 100 ng of peptide dissolved in 0.5 μL of 1 × Hank’s balanced salt solution. For the dueling assays, similar transitions (30 females and 3 males) were set up and half of the females were injected in the gaster 4 days after separation. Injected ants were fed with pre-stung crickets every second day. In each micro-colony, the 3 control and 3 injected individuals were obtained from the same 30-individual transition (see above), which was prepared using young individuals all originating from the same large colony. Therefore, in each micro-colony control and treatment individuals originated from the same genetic background and colony-of-origin, and potential confounding effects were taken into account by using paired non-parametric statistical tests (Wilcoxon).

Mass spectrometry

Heads of ants from established colonies and heads of ants injected with corazonin (30 min after injections) were snap frozen in liquid nitrogen and homogenized in 200 μL of protein extraction buffer (8 M urea and 50 mM NH_4HCO_3). The protein extract was spiked with 5 pmol of recombinant corazonin labeled with heavy phenylalanine (^{13}C9, ^{15}N, +10 Da) as an internal standard in 60 μl.

Proteins samples were prepared for MS by subjecting them to solid phase extraction. The bottom of a 200 μl pipette tip was sealed with a 0.4 mm-diameter-disk of C18 material (Millipore) to make a stage-tip. The stage-tip was activated with 100 μl of acetonitrile, equilibrated with 100 μl of 0.1% acetic acid, and loaded with samples, each followed by a brief centrifugation. After washing with 0.1% acetic acid, peptides were eluted into 100 μL of 50% acetonitrile, 5% acetic acid in water. The elution was liophilized in a SpeedVac concentrator and resuspended in 20 μL of 0.1% formic acid.

LC-MS analysis was carried out using an EASY-nLC nano HPLC (Thermo Scientific) coupled to a Q-Exactive mass spectrometer (Thermo Scientific), equipped with a nano-electrospray source. Ionization source parameters were optimized using corazonin and corazonin containing ^{13}C^{15}N-phenylalanine (Thermo Scientific) and set to: positive mode; capillary temperature, 275°C; spray voltage, 2.3kV. Analytes were separated on an in-house analytical column (100 μm ID, 250 mm length) packed with ReproSil-Pur 120 C18-AQ resin 3 μm, (Dr. Maisch GmbH). The mobile phases were 0.1% (v/v) formic acid (A) and acetonitrile with 0.1% (v/v) formic

acid (B). Analytes were eluted using the following 15 min gradient: 4% B at the first 2 min, 4%–30% B in 8 min, 30%–80% B in 2 min and maintained for 3 min at 400 nl/min. The mass spectrometer was set to perform a full MS scan (350 – 1600 m/z) in the Orbitrap with a resolution of 70,000 (at 100 m/z), followed by targeted MS/MS scans of the precursor ion of corazonin (m/z = 685.31 $[M + H]^{2+}$; m/z = 1369.63 $[M + H]^{1+}$) and $^{13}C^{15}N$-corazonin (m/z = 690.33 $[M + H]^{2+}$; m/z = 1379.63 $[M + H]^{1+}$). Fragment ions were scanned from 110–1400 m/z. All MS/MS scans were performed in the Orbitrap mass analyzer (17,500 resolution) using HCD fragmentation (collision energy = 27), and an isolation window of 3.0 m/z. Maximum injection times of 50 and 100 ms were defined for MS and MS/MS scans, respectively. AGC values were set to 10^6 for MS and 5×10^4 for MS/MS. MS data was collected in profile mode.

Behavioral assays

For the hunting assay (a.k.a. ''cricket-in-the-tube test'') ants grouped in micro-colonies (3 treatment + 3 control individuals) were acclimatized to the presence of a test tube for 1–3 days before the actual experiment. On the day of the experiment the transparent tube was replaced with a new tube without crickets (to score baseline interactions with an ''empty tube'') followed by a tube containing four crickets. We scored as interactions any event in which an individual spent more than four seconds in the immediate vicinity of the tube either touching its walls or the cotton plug sealing the tube (see Movies S2 and S3). Attempts to bite the crickets trapped in the tube were also scored as interactions (see Movie S4). We defined a ''cricket test score'' as the number of such independent interactions per individual in a 10 min window and considered each micro-colony of 6 individuals a biological replicate. Micro-colonies where all individuals of one experimental group (corazonin- or control-injected) died before recording were excluded from further analysis. To assay established colonies (Figure 2C), we connected a separate hunting arena to the colony box using clear tubing (see Movie S2).

For the dueling assay, ants were observed for dueling behavior twice per day (1–2 hr per session) for the 4 days following peptide injections.

For the open field test, individual injected ants were placed in a glass beaker with Fluon-coated walls to prevent ants from escaping. The ants' movements were recorded for 5 min and quantified using ImageJ. The locomotory activity was expressed as the average distance (cm) traversed in one minute.

For the aggression assay (a.k.a. ''forceps test''), injected ants were challenged with the invasive stimulus (metal forceps) one, three, and five days post-injection. The ants' reactions were classified as follows: ''retreat,'' the ant quickly ran away from the forceps; ''avoid,'' the ant tried to bypass by forceps and resume colony duties; ''threaten,'' the ant stretched her body forward, opened the mandibles and shook the abdomen (more precisely the gaster which is the ant-specific major part of the abdomen); ''attack,'' the ant immediately bit the forceps. ''Threaten'' and ''attack'' were counted as aggressive behaviors.

For all behavioral assays, the investigator performing behavioral scoring was blinded to the experimental groups; specifically, ants were marked with unique color combinations days before analyzing the recordings. The treatment for each color combination was not revealed to the investigator until after scoring was completed.

RNAi for corazonin receptor and vitellogenin

Custom dicer-substrate short interfering RNAs (DsiRNAs) targeting *CrzR* and *Vg* were synthesized by IDT. DsiRNAs were resuspended at 20 μM and complexed with in vivo-jetPEI transfection reagent (Polyplus Transfection) following the manufacturer's instructions. First, equal volumes of 20 μM DsiRNAs and 10% glucose solution were mixed to 20 μl total. Next, 1.6 μl of in vivo-jetPEI reagent was diluted to 20 μl of glucose solution (final concentration of 5%). The two solutions were combined, resulting in 5 μM DsiRNA and incubated for 15 min at room temperature before injections. Each ant was injected with a calibrated glass capillary directly in the head with 0.5 μl of DsiRNA-PEI complexes. For molecular analyses (RT-qPCR, RNA-seq), ant brains were collected 24 hr post injections. Hunting assays were conducted as described above 24 and 48 hr after injections.

RNA isolation, qPCR, and sequencing

Harpegnathos brains without optic lobes (referred to as ''brains'' in the text) were dissected from single individuals and homogenized in TRIzol (Thermo Fisher Scientific, MA). RNA was purified and its quality visualized on agarose-formaldehyde gels. Typical yields amounted to 1.2 μg total RNA per brain. For RT-qPCR, 7 ng were assayed per 10 μl reaction using the RNA-to-Ct single-step kit (Thermo Fisher). The RNA for *Rpl32*, encoding a ribosomal protein, was used as a normalization control.

For library preparation, polyA+ RNA was isolated from 500 ng total RNA using Dynabeads $Oligo(dT)_{25}$ (Thermo Fisher) beads and constructed into strand-specific libraries using the dUTP method (Parkhomchuk et al., 2009). UTP-marked cDNA was end-repaired using end-repair mix (Enzymatics, MA), tailed with deoxyadenine using Klenow exo^- (Enzymatics), and ligated to custom dual-indexed adapters with T4 DNA ligase (Enzymatics). Libraries were size-selected with SPRIselect beads (Beckman Coulter, CA) and quantified by qPCR before and after amplification. Sequencing for Figures 1 (d120 transition) and 5 (peptide and siRNA injections) was performed on a NextSeq 500 (Illumina, CA). Sequencing for Figure 2E (*Monomorium pharaonis*) was performed on a HiSeq 2500 (Illumina). Sequencing for Figures 3A and 6A (transition time points) was performed on a HiSeq2000 (Illumina).

Notes on *Harpegnathos* genome assembly and annotation

All analyses for *Harpegnathos saltator* in this study were performed using our previously reported draft assembly v3 (Bonasio et al., 2010). For protein-coding gene annotation we used official gene set v3.8 (OGS3.8), which only differs from the previously reported

OGS3.5 (Bonasio et al., 2012) by a correction in the boundary between Hsal_09095 and Hsal_09096 (see below). For all genes discussed in this study, gene names and symbols correspond to the closest named *Apis mellifera* homolog or, in its absence, the closest *Drosophila* homolog. The relevant information, including unique OGS3.8 identifier, is reported in Table S5.

By aligning the sequences to those from other species and other databases, we concluded that the OGS3.5 version of Hsal_09096 was a fusion of two genes, the *Harpegnathos* homologs for vitellogenin and alanine-glyoxylate aminotransferase 2-like. We corrected the error and re-annotated the genes as Hsal_09096.2 and Hsal_09095.2, respectively. The new gene boundaries are detailed in Table S5.

RNA-seq analyses on *Harpegnathos*

RNA-seq reads were mapped to the reference *Harpegnathos saltator* assembly v3 (Bonasio et al., 2010) pre-indexed with transcript models from OGS3.5 (Bonasio et al., 2012) using STAR 2.5.0b (Dobin et al., 2013) with default parameters except –alignIntronMax set to 10,000. Aligned reads were assigned to OGS3.8 gene models using DEGseq (Wang et al., 2010). Reads per kilobase per million (RPKMs) were calculated with a slight modification, whereby only reads assigned to annotated protein-coding genes were used in the denominator, to minimize batch variability due to different amounts of contaminant ribosomal RNA.

Differential expression was analyzed with the DESeq2 package (Love et al., 2014) and visualized by volcano plots using the maximum a posteriori estimated log-fold change (Figure 1D) or by conventional MA plots (Figures S1D, 5A, and 5D) using the $\log_2$(fold-change) calculated from RPKMs.

GO enrichments were calculated using Fisher's exact test with all genes expressed in the brain as background (> 1 RPKM average across samples in at least one experimental group). GO terms for each gene were assigned according to their association with homologous genes in human, fly, and mouse. Only terms without a more specific annotation were considered. Terms scored as enriched but only associated with one gene in the gene set were removed.

Overlap of affected genes (Figures 5B and 5E) was calculated by ranking genes using the product of the absolute log-fold-change multiplied by the log-converted *P*-value and then comparing the lists using the OrderedList package (Lottaz et al., 2006).

RNA-seq on *Monomorium pharaonis* foragers and nurses

For Figure 2E, Replicate colonies containing ~300–400 workers, an equal amount of brood of various stages, and 10 queens, were constructed from 10 mixed large stock colonies. For each replicate, we pooled the heads of 10 workers that were collected in the act of foraging or 10 workers collected nursing brood. RNA was extracted with the RNeasy mini kit (QIAGEN, MD), reverse transcribed, and converted to unstranded libraries using a Nextera kit (Illumina). Analysis was performed as described above (using STAR, DEGseq, and DESeq2), except that the *Monomorium pharaonis* assembly v2.0 and NCBI annotation release 100 were used.

Drosophila fecundity assay

For the fecundity assay, virgin females were collected upon eclosion over a 12 hr period and mated with OreR males for 24 hr. The individual mated females were collected over ice and placed in single wells of 48-well apple juice-agar plates (25% apple juice, 25 mg/ml sucrose and 22.5 mg/ml agar) with a small amount of live yeast paste. Flies were kept in a temperature- (25°C) and humidity- (50%) controlled chamber on a 12 hr light/dark cycle. Eggs were counted 24 hr after isolation.

QUANTIFICATION AND STATISTICAL ANALYSIS

We used DESeq2 for statistical analysis of differential gene expression. All other statistical analyses were performed using GraphPad Prism v7.0a. Sample sizes, type of replicates, and definitions of center and dispersion are reported in each figure legend. Statistical tests employed and significance levels are also reported in the figures and figure legends. We used parametric statistics for normally distributed data (determined by Shapiro-Wilk tests with alpha level of 0.05) and non-parametric tests otherwise.

DATA AVAILABILITY

The SuperSeries accession number for all RNA-seq data reported in this paper is GEO: GSE83807. See details in the Key Resources Table.

Cell

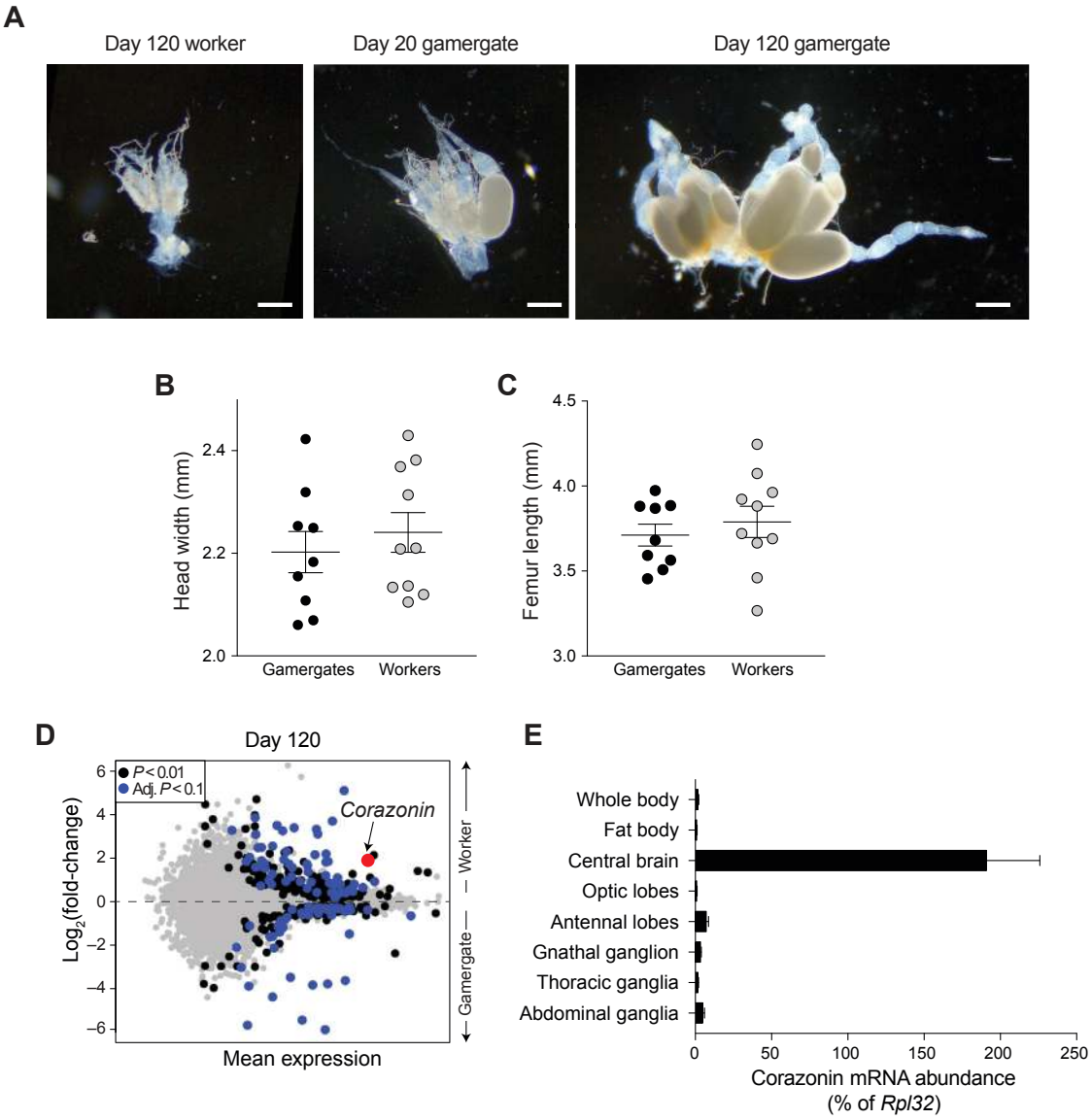

Figure S1. Worker–Gamergate Transitions and Corazonin Expression, Related to Figure 1

(A) Microphotographs of ovaries isolated from individuals scored as workers (left) or gamergates (right) 120 days after transition. A partially activeted ovary from a developing gamergate at an early stage of the transition (day 20) is shown in the middle for comparison. Scale bars correspond to 0.5 mm.

(B) Comparison of head widths in individuals that successfully converted to gamergates or remained workers. Means ± s.e.m. are shown. The difference is not statistically significant.

(C) Same as (B) but femur lengths were measured. The difference is not statistically significant.

(D) MA plot of RNA-seq data from brains of gamergates and workers 120 days after the onset of the transition. Mean expression is plotted on the x axis and the $\log_2$(fold-change) on the y axis. Genes with $p < 0.01$ are in black. Genes with FDR < 0.1 are in blue. Data is from 11 worker and 12 gamergate brain replicates.

(E) Expression levels (RT-qPCR) of corazonin in different ant tissues. The *Rpl32* gene was used as normalization control. Bars represent the mean from ≥ 4 biological replicates (individual ants) + s.e.m.

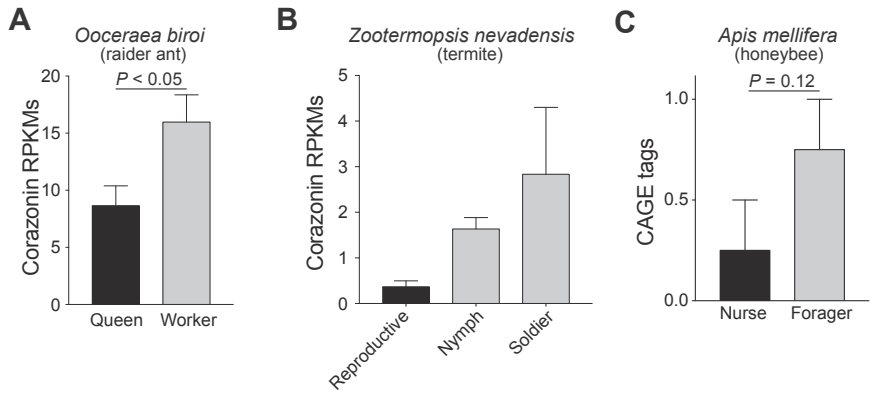

Figure S2. Corazonin Expression in Other Social Insects, Related to Figure 2

(A) Expression levels (RPKMs) of corazonin from brains of *Ooceraea biroi* in their queen and worker phase. Data are from 4 biological replicates per group. *P*-value is from a two-sided t test (Oxley et al., 2014). Bars represent mean + SEM.

(B) Expression levels (RPKMs) of corazonin in the whole bodies of different social castes from the termite *Zootermopsis nevadensis* (Terrapon et al., 2014). Differences are not statistically significant but show a trend consistent with our observations in other species. Bars represent mean + SEM.

(C) Expression levels of corazonin inferred from the number of CAGE tags from the brains of *A. mellifera* nurses and foragers (Khamis et al., 2015). Data are from 8 biological replicates per group. *P*-value is from a two-sided t test. Bars represent mean + SEM.

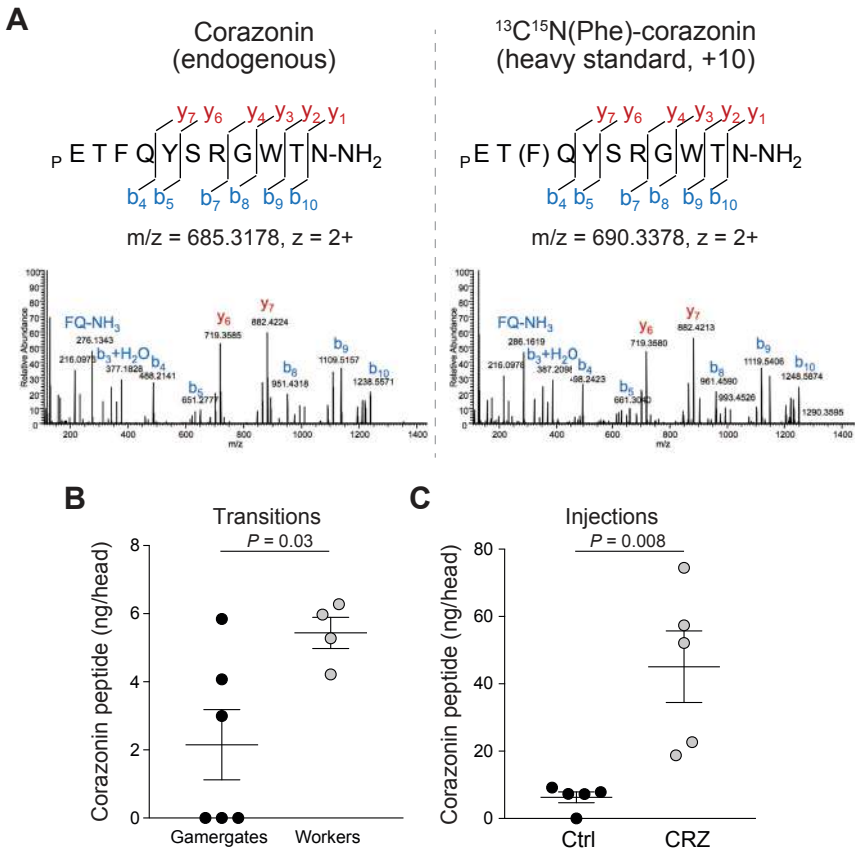

Figure S3. Quantification of Corazonin Peptide in *Harpegnathos* Heads, Related to Figure 3

(A) Peptide ion map (top) and MS2 fragmentation pattern (bottom) used for relative and absolute quantification of the endogenous corazonin peptide (left) compared with a heavy, isotope-labeled spike-in control (right). Only b ions were used for quantification.

(B) Absolute quantification of corazonin peptide amounts in gamergates and workers. Each circle represents mass spectrometry data from a single head. Means ± s.e.m. are shown. *P*-value is from a Mann-Whitney test.

(C) Absolute quantification of corazonin peptide amounts in transitioning workers 30 min after injection of 100 ng synthetic corazonin (CRZ) or mock injection control (Ctrl). Each circle represents mass spectrometry data from a single head. Means ± SEM. are shown. *P*-value is from a Mann-Whitney test.

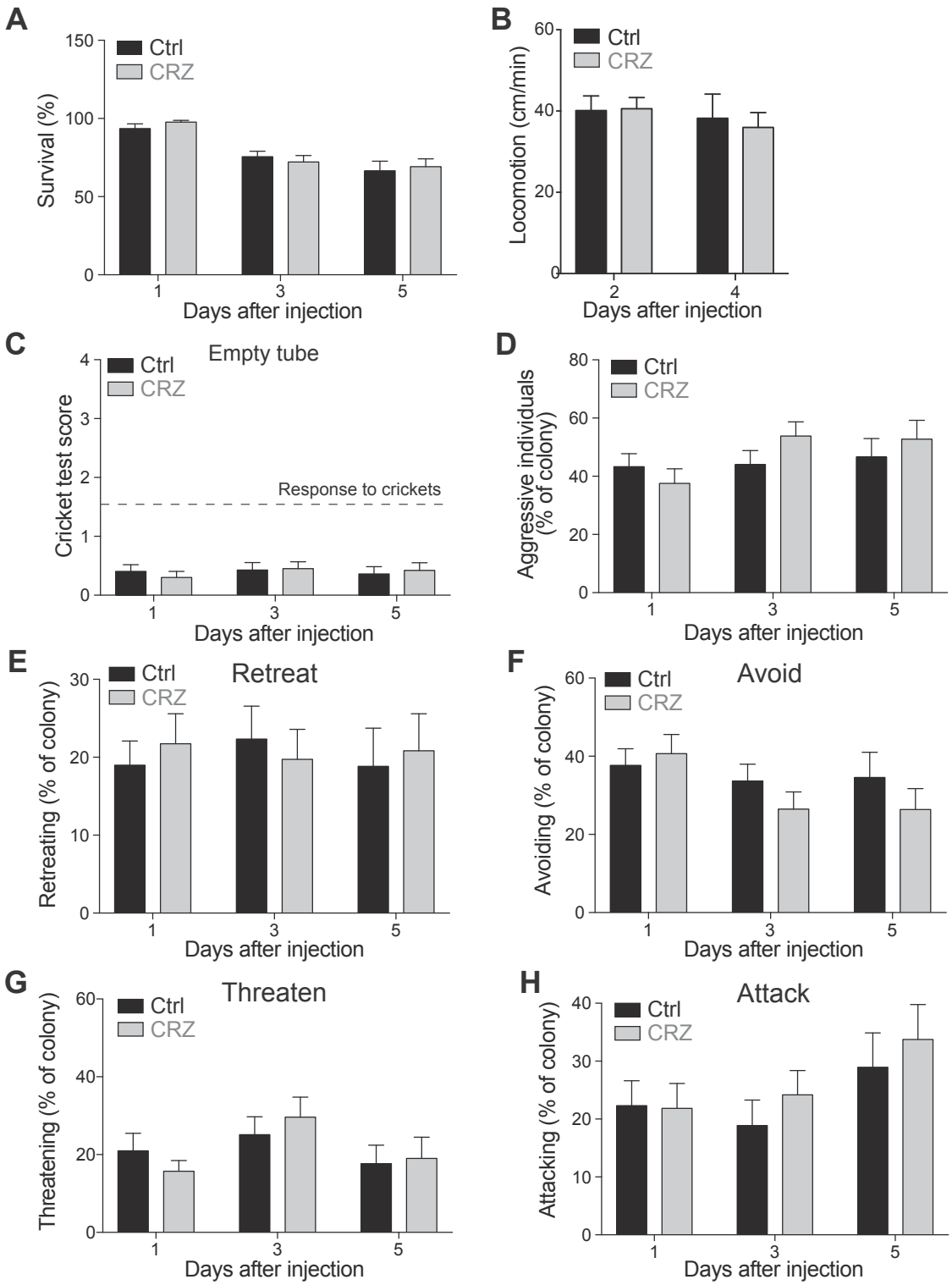

Figure S4. Mortality and Behavioral Controls for Corazonin Injections, Related to Figure 3

(A) Mortality rates for experiment shown in Figure 3C. Differences between ctrl and CRZ are not statistically significant. Bars represent mean + SEM.

(B) Locomotory activity plotted as cm per minute as measured in an adapted open-field test. Bars show the mean of ≥ 8 biological replicates (individual ants) + SEM. Differences are not statistically significant.

(legend continued on next page)

(C) Interaction scores for corazonin- and control-injected individuals quantified as in Figure 3C but toward empty tubes with no crickets. Bars show the mean scores of $\geq$ 20 biological replicates (micro-colonies of 3 + 3 ants housed in the same box) + SEM. Differences are not statistically significant according to two-sided Wilcoxon tests.

(D) Percentage of corazonin- and control-injected ants reacting in an aggressive manner (threat or attack) to a challenge with metal forceps at different times after injection. Bars show the mean of $\geq$ 29 biological replicates (colonies) + SEM. The differences are not statistically significant.

(E–H) Reactions from (D) segregated in four categories: retreat (E), avoid (F), threat (G), and attack (H).

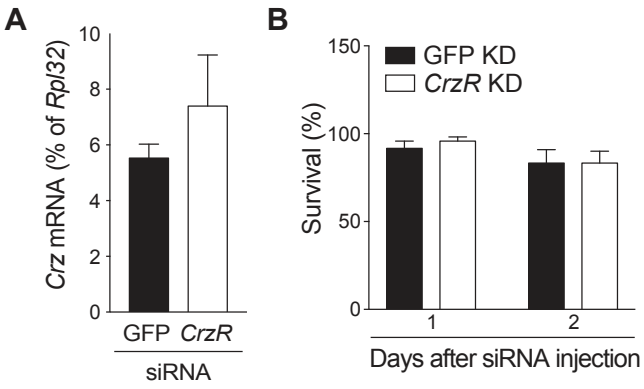

Figure S5. Controls for Corazonin Receptor Knockdown, Related to Figure 4

(A) Expression levels (RT-qPCR) for corazonin in the brains of ants injected with siRNAs against the corazonin receptor or control siRNAs against GFP. Bars represent the mean from $\geq$ 7 biological replicates + s.e.m. The difference is not statistically significant.

(B) Mortality rates for experiment shown in Figure 4C. Differences are not statistically significant. Bars represent mean + SEM.

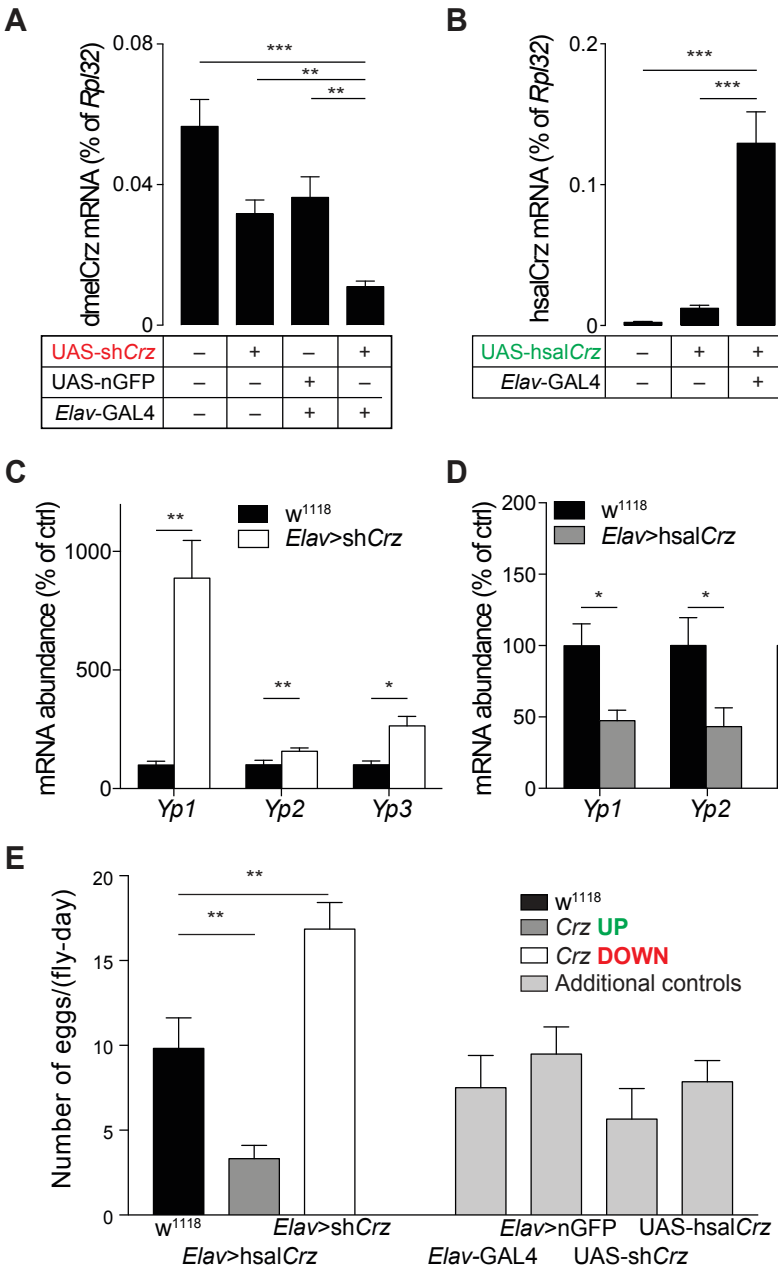

Figure S6. Corazonin Regulates Vitellogenin Levels and Egg-Laying in *Drosophila*, Related to Figure 6

(A) Expression levels (RT-qPCR) for *Drosophila* corazonin in the indicated transgenic lines. Bars represent the mean from ≥ 5 biological replicates + s.e.m per line. *P*-values are from ANOVA and Holm-Sidak test.

(B) Expression levels (RT-qPCR) for transgenic *Harpegnathos* corazonin in the indicated transgenic lines. Bars represent the mean from ≥ 5 biological replicates + s.e.m per line. *P*-values are from ANOVA and Holm-Sidak test.

(C and D) Expression levels (RT-qPCR) for the three vitellogenin homologs in *Drosophila* in the indicated transgenic lines. Bars represent the mean from ≥ 5 biological replicates + s.e.m per line. *P*-values are from ANOVA and Holm-Sidak test.

(E) Egg-laying rates in the indicated *Drosophila* lines expressed as number of eggs laid per fly per day. Bars represent the mean from ≥ 18 biological replicates + s.e.m per line. *P*-values are from ANOVA and Holm-Sidak test.