PNAS RESEARCH ARTICLE | DEVELOPMENTAL BIOLOGY OPEN ACCESS

Juvenile hormone as a key regulator for asymmetric caste differentiation in ants

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Edited by Lynn Riddiford, University of Washington, Friday Harbor, WA; received April 9, 2024; accepted August 28, 2024

Caste differentiation involves many functional traits that diverge during larval growth and metamorphosis to produce adults irreversibly adapted to reproductive division of labor. Investigating developmental differentiation is important for general biological understanding and has increasingly been explored for social phenotypes that diverge in parallel from similar genotypes. Here, we use *Monomorium pharaonis* ants to investigate the extent to which canalized worker development can be shifted toward gyne (virgin-queen) phenotypes by juvenile hormone (JH) treatment. We show that excess JH can activate gyne-biased development in workers so that wing-buds, ocelli, antennal and genital imaginal discs, flight muscles, and gyne-like fat bodies and brains emerge after pupation. However, ovary development remained unresponsive to JH treatment, indicating that JH-sensitive germline sequestration happens well before somatic differentiation. Our findings reveal important qualitative restrictions in the extent to which JH treatment can redirect larval development and that these constraints are independent of body size. Our findings corroborate that JH is a key hormone for inducing caste differentiation but show that this process can be asymmetric for higher colony-level germline *versus* somatic caste differentiation in superorganisms as defined a century ago by Wheeler. We quantified gene expression changes in response to JH treatment throughout development and identified a set of JH-sensitive genes responsible for the emergence of gyne-like somatic traits. Our study suggests that the gonadotropic role of JH in ovary maturation has shifted from the individual level in solitary insects to the colony level in an evolutionary-derived and highly polygynous superorganism like the pharaoh ant.

juvenile hormone | caste differentiation | canalization | superorganism | modularity

Significance

Morphologically differentiated adult castes in ants require preimaginal differentiation among the colony members of a larval cohort. Adults thus become irreversibly specialized for either reproduction or nonreproductive altruism—analogous to metazoan germline and soma differentiation as established a century ago. However, the phenotypic and transcriptomic details of this process have rarely been studied and whether body size linearly predicts caste has remained controversial. Pharaoh ants are an excellent model for exploring the sensitivity windows by which juvenile hormone induces developmental canalization and for documenting whether this process happens synchronously for gyne and worker development. We show that responses to JH treatment are modular and that caste differentiation for germline and somatic traits is asynchronous and at least partly nonlinear.

Canalization and morphological integration are two key developmental mechanisms regulating phenotypic variation among conspecific individuals (1–3). The ants, corbiculate bees (except orchid bees), and vespine wasps have independently completed irreversible major transitions in evolution (MTEs) to higher-level organismality, characterized by irreversible gyne-worker caste differentiation starting early in larval development. As a result, every colony member has a fixed caste phenotype specialized for either reproduction (i.e. direct fitness) or altruistic helping (indirect fitness) during adult life (4, 5). More than a century ago, William Morton Wheeler noted that particularly the superorganismal colonies of ants are strikingly analogous to metazoan bodies, because queens and workers function as complementary higher (colony)-level germline and soma (4). In recent years this analogy has gained further support because comparative data showed that lifetime parental monogamy (6) and single-zygote foundation (7) are the universal ancestral states of, respectively, colonial superorganismality and obligate multicellularity (8–13). The development of gyne and worker castes involves many morphological and physiological traits associated with Wheeler's colony-level germline and soma concept, even though caste differentiation is simpler than cell-type differentiation in Metazoa.

Adult gynes normally have both higher body mass and wings and ocelli crucial for their mating and dispersal flight, as well as an extended abdomen with large fat bodies producing regulatory molecules and nutrition for egg production in enlarged ovaries, and a spermatheca (sperm storage organ) (14). In contrast, workers are wingless females with smaller body size and degenerated reproductive tracts, usually without a spermatheca (15) but with relatively bigger brain mushroom bodies to coordinate their somatic roles in a.o. foraging, defense and brood care (16). Previous work (17) has extended Waddington's classic metazoan-body canalization paradigm (18, 19) to become applicable to Wheeler's concept of the ant colony as a higher-order developmental system (see *SI Appendix* for detailed explanation). In that view, the immature developmental processes that produce

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2406999121/-/DCSupplemental.

Published November 4, 2024.

adult gynes and workers are thus expected [and shown; (17)] to be strongly canalized to ensure that adult caste phenotypes remain predictable in spite of disturbances in the abiotic, biotic, and social environments. Higher-level canalization in the development of superorganismal colonies thus minimizes individual variation within castes while fixing and stabilizing variation between castes (17), which requires tight coordination of functionally and developmentally related gene-expression modules. However, how caste-specific traits gradually take form during gyne and worker development to produce phenotypes with predictable division of labor remains poorly understood.

Developmental canalization is likely maintained by stabilizing selection in ecological time, but not set in stone over evolutionary time because the emergence of new castes and the disappearance of existing castes can be tracked across the ant tree of life (20). Novel, evolutionarily derived ant castes such as permanently wingless (ergatoid) gynes and soldiers may have evolved from so-called intercaste individuals, i.e. from mosaic phenotypes that recombine gyne- and worker-specific traits (21). This conjecture is consistent with developmental recombination of gyne-specific ovaries and worker-specific wing discs in ergatoid gyne larvae of *Myrmecina nipponica* (22). Larvae of *Pheidole* soldiers display phenotypic recombination of gyne-specific forewing discs and worker-specific hindwing discs (23). Together with other studies (24–28), these findings suggest that caste-related traits are somehow encoded by developmental modules that can be recombined and modified (29), but with the implicit and usually untested assumption that these modules are synchronously expressed. Alternatively, it has been proposed that the growth of caste-related traits is primarily a function of body size, such that large body size is necessary and sufficient for the expression of gyne-like traits (30–32). These interpretational positions have been subject to recent debate, although both parties agree that the juvenile hormone (JH) pathway is always involved.

As a gonadotropin hormone, JH stimulates oogenesis in adult female insects via vitellogenesis (33), while precocious preimaginal maturation is prevented by JH via its interaction with 20-hydroxyecdysone (20E) signaling (34, 35). JH lost its direct association with reproduction in the ants and honeybees (36–40) owing to permanent gyne-worker caste differentiation, but it gained novel functions in mediating brood care behavior (13), longevity (41) of founding queens, and division of foraging-labor among adult workers (42–46). At the same time, developmental functions of JH were elaborated in the larval stages to induce morphological gyne-worker differentiation (47–52), worker-soldier differentiation (53–55), and male polymorphism (56). JH-induced integration of social organization and caste development thus allowed colonies as a whole to operate at optimal efficiency when maximizing survival and lifetime reproductive success (57, 58). In some cases, the JH-sensitive period initiating gyne-worker differentiation may occur as early as embryogenesis, i.e. shortly after egg fertilization, as in the ant *Cataglyphis mauritanica* (59), or even during oogenesis by the mother queen, as in the ants *Pheidole pallidula* (60, 61) and *Pogonomyrmex* (62, 63). However, the extent to which JH treatment can reverse caste trait development after the onset of larval differentiation remains almost completely unexplored (but see ref. 64). This question is particularly opportune in ants with embryonic or oocyte JH-sensitivity, so that larvae already have experienced a period of directional caste biasing before the robustness of canalization can be challenged by manipulative experiments.

Recent studies have confirmed that caste differentiation in the pharaoh ant *Monomorium pharaonis* is blastogenic (65) and characterized by a long process of gradually deepening canalization, during which gynes and workers commit to their typical developmental trajectories regardless of perturbations from the environment (17, 66). Many genes in the JH pathway display caste-specific and body size correlated expression during larval development even though JH treatment of last instar worker larvae can induce larger body size and the emergence of gyne-specific traits (17). This suggested that canalized development can in fact be disrupted, despite its very early initiation during embryogenesis, which prompted the present study to explore how differential JH-related gene expression affects development of all caste-related traits. We used differential feeding of third (last) instar worker larvae of *M. pharaonis* with the JH-mimic methoprene to characterize the phenotypic and transcriptomic details of late-stage disruption of canalized caste development. We show that the JH-sensitivity windows for germline and soma are decoupled, and we identify a set of core genes, regulated by JH, that can remodel the overall expression profile from being worker-like to being gyne-like, but without ever changing the development of ovary function in gyne-like direction. This asymmetry in caste-specific JH-sensitivity may, albeit likely in less extreme form, apply to an unknown number of other ant lineages and thus complement the alternative mosaic and growth explanations for caste hitherto suggested (29–32).

Results

JH Induction of Gyne-Specific Organ Development Except for the Ovaries. Early third instar worker larvae with body length of 0.85 to 1.15 mm (*SI Appendix*, Fig. S1 *A* and *B*) were fed three or six times with controlled doses of the JH-mimic methoprene (5 mg/mL dissolved in 10% ethanol) while control group larvae were fed with the solvent of 10% ethanol (*SI Appendix*, Fig. S1*C*). The exact ingestion of methoprene could not be controlled, so we compensated for some stochasticity by replication to ensure adequate statistical power. Whole-body samples for transcriptomic analysis were then collected for early, mid, and late third instar larvae plus their subsequent prepupal and early pupal stages. Unless otherwise specified, our results were obtained through feeding early third instar worker larvae.

JH-treated workers (JH workers for short) delayed timing of pupation by ca. 1 wk, while most control workers entered the prepupal stage around day 8 of the experiment, comparable to worker larvae fed with a normal diet (*SI Appendix*, Fig. S1*D*). The longer developmental period of JH worker larvae corresponds to gyne larvae which develop more slowly than worker larvae under normal conditions (67). JH treatment significantly and consistently increased larval body length as development proceeded (*SI Appendix*, Fig. S2 *A* and *B*). After pupal eclosion, adult JH workers had different degrees of gyne-like thoraxes, compound eyes, wings, and ocelli. Such abnormalities are also occasionally found in natural *M. pharaonis* colonies (68), suggesting that our JH treatment recapitulated natural variation in the nutritional, hormonal, and/or social factors that regulate caste development. After three successive methoprene doses, 38% of the workers (56/146) only obtained a larger body size without wings or ocelli (JH type-A workers), while 38% (55/146) developed vestigial forewings and parts of the ocelli (JH type-B workers) and 24% (35/146) developed complete forewings, hindwings, and ocelli, resembling normal gyne morphology (JH type-C workers). The obtained phenotypic variation thus covaried with methoprene dose in spite of some unavoidable noise in larval ingestion. Our six-methoprene-dose experiment made all 91 test workers develop the full spectrum of gyne somatic traits (JH type-D workers), indicating this double JH-dose was always adequate to induce gyne soma development (Fig. 1*A* and *SI Appendix*, Fig. S3 and Tables S1 and S2).

The pupal lengths of JH workers were also gyne-like (Fig. 1*B*), suggesting that overall growth is highly correlated with thorax, compound eyes, wings, and ocelli development. We also examined adult antennal-scape-to-head size allometry (75), which differed between normal gynes and control workers in being negatively allometric (log–log slope < 1) and isometric (log–log slope = 1), respectively (Fig. 1*C*, *SI Appendix*, Table S3), indicating that antennal scapes become relatively shorter as head size increases as shown earlier for workers of the ant *Solenopsis invicta* (76). We thus recovered a general pattern of increasing antennal scape length with increasing head width (*SI Appendix*, Fig. S2 *C* and *D*). JH type-A workers followed the same antennal-scape-to-head regression as control workers, while type-B workers (which had partly changed organ development) developed wider heads relative to antennal scape length and shifted allometric slope similar to type-C and type-D workers (with complete somatic responses in gyne-like direction) (Fig. 1*C* and *SI Appendix*, Table S3). The significant change in allometric slope of type-B workers thus appears to represent an essential switch from a worker to gyne developmental trajectory, coinciding with developing all somatic gyne-specific traits, while type-A workers expressed only quantitative changes of increasing body size.

Adult workers of *M. pharaonis* completely lack both individual germline functions and all somatic traits associated with reproduction (70, 71), so we next examined how many of the internal reproductive traits had been restored in JH workers. We found that all somatic traits that normally derive from the genital imaginal discs (oviducts, spermatheca, and bursa copulatrix) had developed in JH workers of types B, C, and D, in proportion to the external markers of gyne development, i.e. thorax size, compound eyes, wings, and ocelli (Fig. 1*D*). In particular, the spermatheca and bursa copulatrix of type-B workers were smaller than those of type-C and -D workers, consistent with, respectively, partial and full development of these somatic gyne traits (Fig. 1*A*). This strongly suggests that the growth of all imaginal discs (wing discs, eye-antennal discs, and genital discs), responded in a tightly coordinated way to the elevated JH titers administered in the early third instar larvae. Previous work has shown that worker genital imaginal discs are maintained throughout larval development (66), consistent with our JH treatment of third instar larvae inducing the organs derived from these discs. However, ovary development was never induced (asterisks in Fig. 1*D*), indicating that worker germline function could not be recovered. This is consistent with that same prior study showing that worker germ cells are completely degenerated from stage 9 in embryonic development (66). We confirmed this embryonic degeneration (*SI Appendix*, Fig. S4) and clarified that somatic gonadal cells, another primary cellular component required for germ cell development (77), were maintained through the first half of the third worker larval instar using *headcase* (78) as marker gene (*SI Appendix*, Figs. S5 and S6).

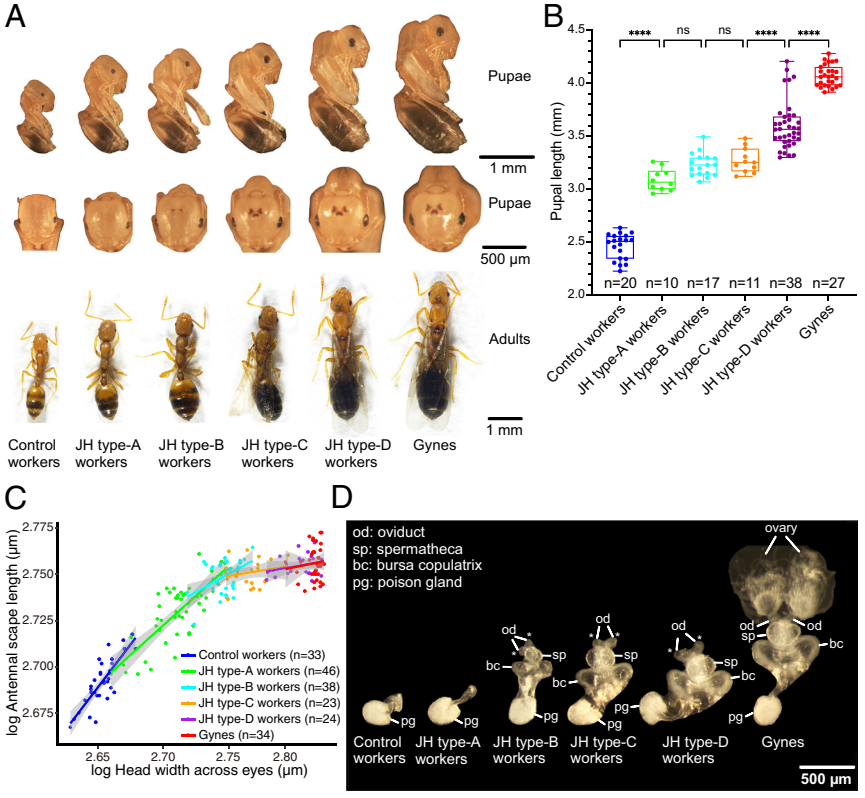

Fig. 1. JH as a key regulator for shifting worker development toward a gyne phenotype except for ovaries. (*A*) Representative images of control workers, JH workers, and gynes on the last pupal day (first two rows) and adult phenotypes (third row), after feeding with three doses of methoprene affecting wing and ocelli development: type-A workers—no wings or ocelli in adults; type-B workers—vestigial forewings and partially developed ocelli; type-C workers—fully developed fore- and hindwings and complete ocelli; type-D workers—fed with six methoprene doses, always had fully developed wings and ocelli. (*B*) Pupal length of control workers, JH workers, and gynes, showing monotonous increase in body length with increasingly gyne-like phenotypic development, but with discontinuities between type-A, type-B, and type-C workers (one-way ANOVAs with Tukey's post hoc tests; ****$P < 0.0001$; ns $P > 0.05$). Plotted are the median (central line), 25% and 75% quartiles (box), outermost values (whiskers), and all data points. (*C*) Adult antennal-scape-to-head-width allometry remained continuous for JH type-A workers, but changed slope in gyne-like direction for type-B, -C, and -D workers, as evaluated by linear regressions and one-way ANCOVAs (69) with Tukey's post hoc tests (see *SI Appendix*, Table S3 for statistical results). Head width across the eyes was assumed to be the predictor variable. (*D*) Comparisons of the reproductive organs of control workers, the four types of JH workers and gynes. Normal *M. pharaonis* workers are completely sterile, lacking both ovaries and associated somatic tissues and organs (70, 71). Images show that JH workers never developed ovaries (asterisks), but that they can develop all somatic components: oviducts (od), spermatheca (sp), and bursa copulatrix (bc), which were identified based on the general reproductive anatomy of *M. pharaonis* (70, 72) and other ants (73, 74). The poison gland (pg), which does not belong to the reproductive organs but is located adjacent to them, developed as well.

We also performed methoprene treatment of third instar worker larvae in the second half of the third instar when body length is 1.40 to 1.70 mm. This increased body sizes but without developing gyne-specific phenotypic traits (i.e., no wings, ocelli, or reproductive organs and no changes in the antennal-scape to head-size allometry) (*SI Appendix*, Fig. S7 and Table S4). Two conditions thus need to be met simultaneously to induce gyne soma development in worker larvae: 1) JH-exposure during the first half of the third larval instar to initiate the signaling pathways that activate gyne soma development consistent with this stage, with body length of 0.85 to 1.15 mm, and not the second half of that instar with larger body lengths being the critical period for gyne soma determination. 2) Continuation of an adequate amount of JH during the second half of the third worker larval instar to ensure morphogenesis of gyne-specific organs such as wings, ocelli, and spermatheca.

JH Induces Specific Gene Expression Changes that Mediate Phenotypic Caste Traits. To better understand the molecular mechanisms underlying development of the visual and flight systems in response to JH, we probed *Drosophila melanogaster* data to identify transcription factors for orchestrating organ-specific developmental programs and used phylogenetic analysis to identify the *M. pharaonis* orthologs (*SI Appendix*, Fig. S8). Using HCR™ RNA-FISH on prepupal heads, we found that the genes *sine oculis* (*so*) and *eyes absent* (*eya*) were markers for compound eye and ocelli development in *M. pharaonis*, consistent with their roles in *D. melanogaster* (79). Stainings for *so* and *eya* expression in JH workers compared to control workers showed that compound eyes became much larger, and the ocelli fully developed, in fact indistinguishable from these organs in gynes (Fig. 2*A*).

Whole-mount larval staining of nuclei further showed that JH workers develop two pairs of wing discs already in the late third instar just like gynes, while wing discs remained absent in control workers (Fig. 2*B*). We then targeted *vestigial* (*vg*) and *engrailed* (*en*), which in fruit flies are critical transcription factors for patterning and development of wing imaginal discs (80, 81). This revealed that expression patterns of these genes in wing imaginal discs were similar between JH worker larvae and gyne larvae, with *vg* being expressed in the edge region and *en* in the posterior region (Fig. 2*C*). Hematoxylin-eosin staining further showed that flight muscles were more developed in the thorax of adult JH workers and gynes than in control workers (Fig. 2*D*). We thus inferred that JH plays a key regulator role in caste-specific development of eyes and wings.

JH-Induced Gyne-Biasing of Fat Body Function. The fat body is a key organ for nutrient storage, immune response, and energy metabolism in insects, analogous to the liver and adipose tissues in vertebrates (82). Nutrients are eventually stored as glycogen and triglyceride contained in lipid droplets that appear as organelles in the cytoplasm of adipocytes, the basic cell-type of the fat body (83). The size of lipid droplets plus surrounding cytoplasm and the dimensions of cell nuclei are thus likely to reflect their degree of nutrient storage.

We examined how JH titer affects fat body characteristics using the expression of *Krüppel homolog1* (*Kr-h1*) as marker, since *Kr-h1* encodes a core zinc-finger transcription factor that mediates early responses to JH (84). Using HCR™ RNA-FISH of larval adipocytes, we found that mRNAs of *Kr-h1* were more abundant in JH workers than in control workers, while the most intensive signals were detected in gynes (Fig. 3*A*), confirming that JH treatment induces a direct response in the fat body. We then used SYTOX™, phalloidin, and LipidTOX™ stainings, which revealed that JH workers and gynes had larger nuclei, larger cytoplasm volumes, and more abundant lipid droplets in their adipocytes than control workers (Fig. 3 *B*–*F*), indicating enhanced nutrient storage that

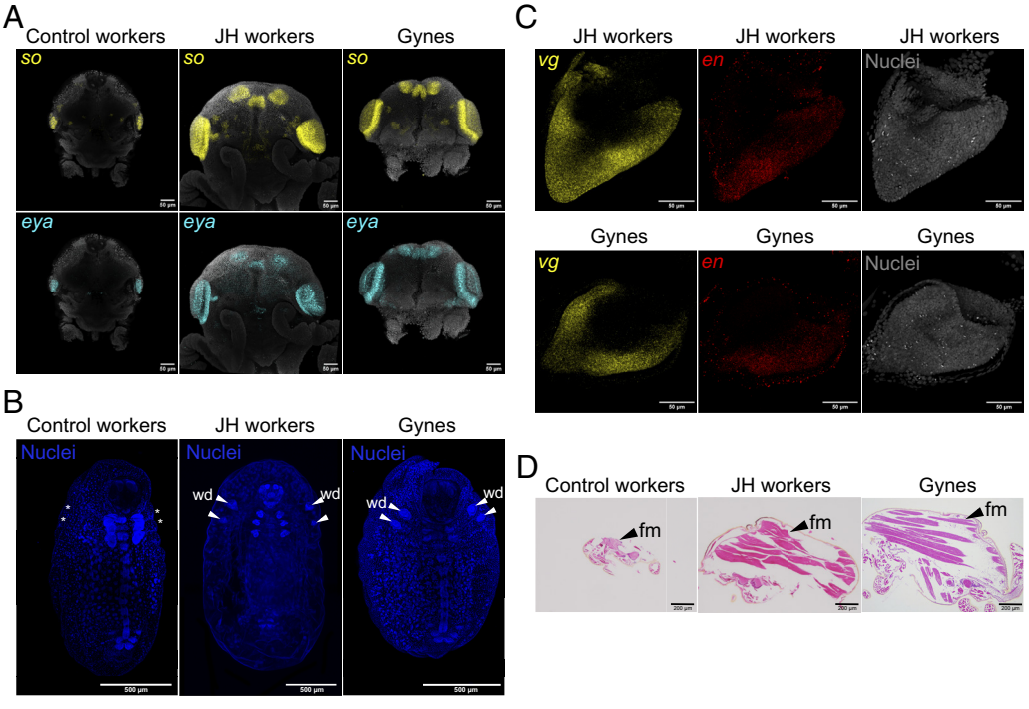

Fig. 2. Larval JH treatment promotes the development of adult visual and flight systems. (*A*) Head capsules with newly-synthesized cuticle were dissected from old wrinkled larvae just after larval-pupal apolysis after which these prepupal heads were stained with *sine oculis* (*so*; yellow), *eyes absent* (*eya*; cyan), and counterstained with nuclei (gray), showing that compound eyes become larger, and ocelli develop in JH workers and normal gynes compared to control workers. (*B*) Whole-mount third instar larvae stained with nuclei (blue) to show that two pairs of wing discs (wd) are absent in control worker (asterisks) while present in JH workers and gynes (arrows). (*C*) Wing discs in late third instar larvae stained with *vestigial* (*vg*; yellow), *engrailed* (*en*; red), and counterstained with nuclei (gray), showing similar expression patterns of *vg* and *en* in wing discs of JH workers and gynes. (*D*) Flight muscles (fm) visualized by hematoxylin-eosin staining in newly eclosed adults, demonstrating that JH workers and gynes developed larger flight muscles than control workers.

may induce larger body size during larval development. Thus, not only adult external morphology but also internal energy storage was affected by JH titer.

JH Regulation of Gyne-Biased Brain Development and Behavior. A previous study of *M. pharaonis* identified *Neuroligin-2* (*Nlg2*) as a gene specifically expressed in a cluster of gyne-specific optic lobe neurons and not at all in workers (16). Our results confirmed this distinction and further showed that JH workers express *Nlg2* in the same way as gynes (Fig. 4*A*), indicating that JH induces development of this gyne-specific cell cluster. We also showed that two neuropeptides mediating adult reproductive division of labor, *Insulin* (*Ins*) and *Neuroparsin-A* (*NPA*; an insulin-like growth factor binding protein) (85–89), have JH-induced gyne-biased expression throughout development (*SI Appendix*, Figs. S9 and S10). However, it remains uncertain whether JH regulates the expression of these brain genes directly or indirectly via altered trajectories of general brain development.

We next performed three-dimensional entire brain reconstructions using confocal image series captured by tissue autofluorescence (*SI Appendix*, Fig. S11*A* and Movies S1–S3). This allowed quantification of brain compartment volumes, including the mushroom bodies [MBs; with their calyces (CAs) and stalks (STs)], the antennal lobes (ALs), the optic lobes [OLs; with their lobulae (LO), medullae (ME) and the laminae (LA)], the three ocelli (OC), the central body (CB), and the supraesophageal zone (SPZ) (Fig. 4*B*). We found that JH workers developed larger absolute volumes for all these brain compartments relative to control workers, with compartments like MBs, ALs, and OLs becoming even larger than those in gynes (Fig. 4*C*). Absolute total brain volumes (the sum of all brain compartments except ocelli), were also significantly higher in JH workers compared to control workers (*SI Appendix*, Fig. S11*B*),

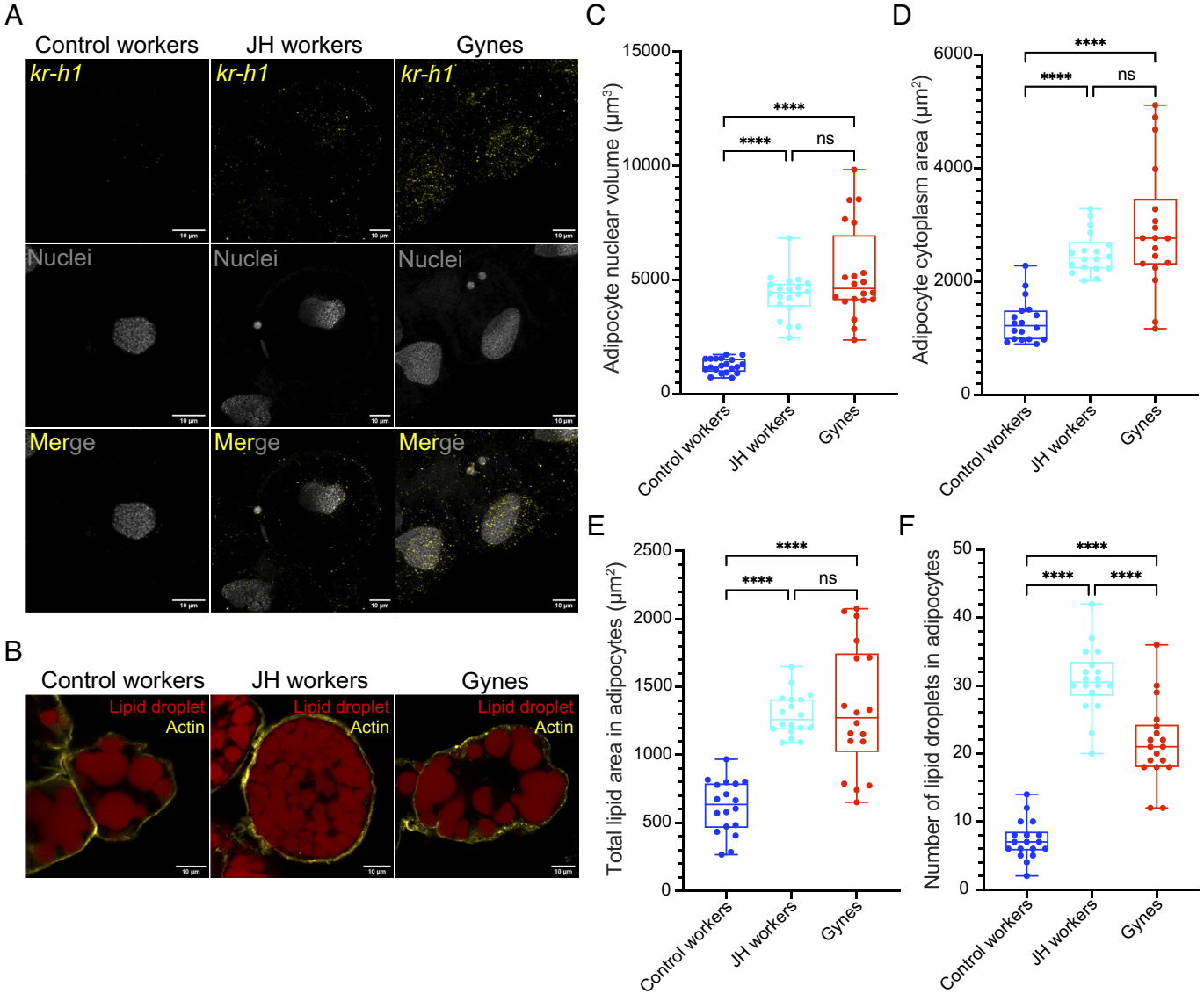

Fig. 3. JH treatment promotes fat body nutrient storage. (*A*) Adipocytes of late third instar larvae stained with *Krüppel homolog1* (*Kr-h1*; yellow) and counterstained with nuclei (gray), showing increased (fluorescence signal) expression across control workers, JH workers, and gynes. (*B*) Adipocytes of late third instar larvae, stained by HCS LipidTOX™ Red neutral lipid stain dye (red) and actin filaments stained with Alexa fluor™ 546-conjugated phalloidin dye (yellow), showing larger adipocyte cytoplasm areas and more lipid droplets in JH workers and gynes compared to control workers. (*C*–*F*) Higher nuclear volume (*C*; n = 20 for each category), cytoplasm area (*D*; n = 18 for each category), total lipid area (*E*; n = 18 for each category), and number of lipid droplets in adipocytes (*F*; n = 18 for each category) in late third instar larvae of JH workers and gynes compared to control workers (overshooting normal gyne development for lipid droplets). Plots have median (central line), 25% and 75% quartiles (box), ranges (whiskers), and all data points, analyzed with one-way ANOVAs and Tukey's post hoc tests: ****$P < 0.0001$; ns $P > 0.05$).

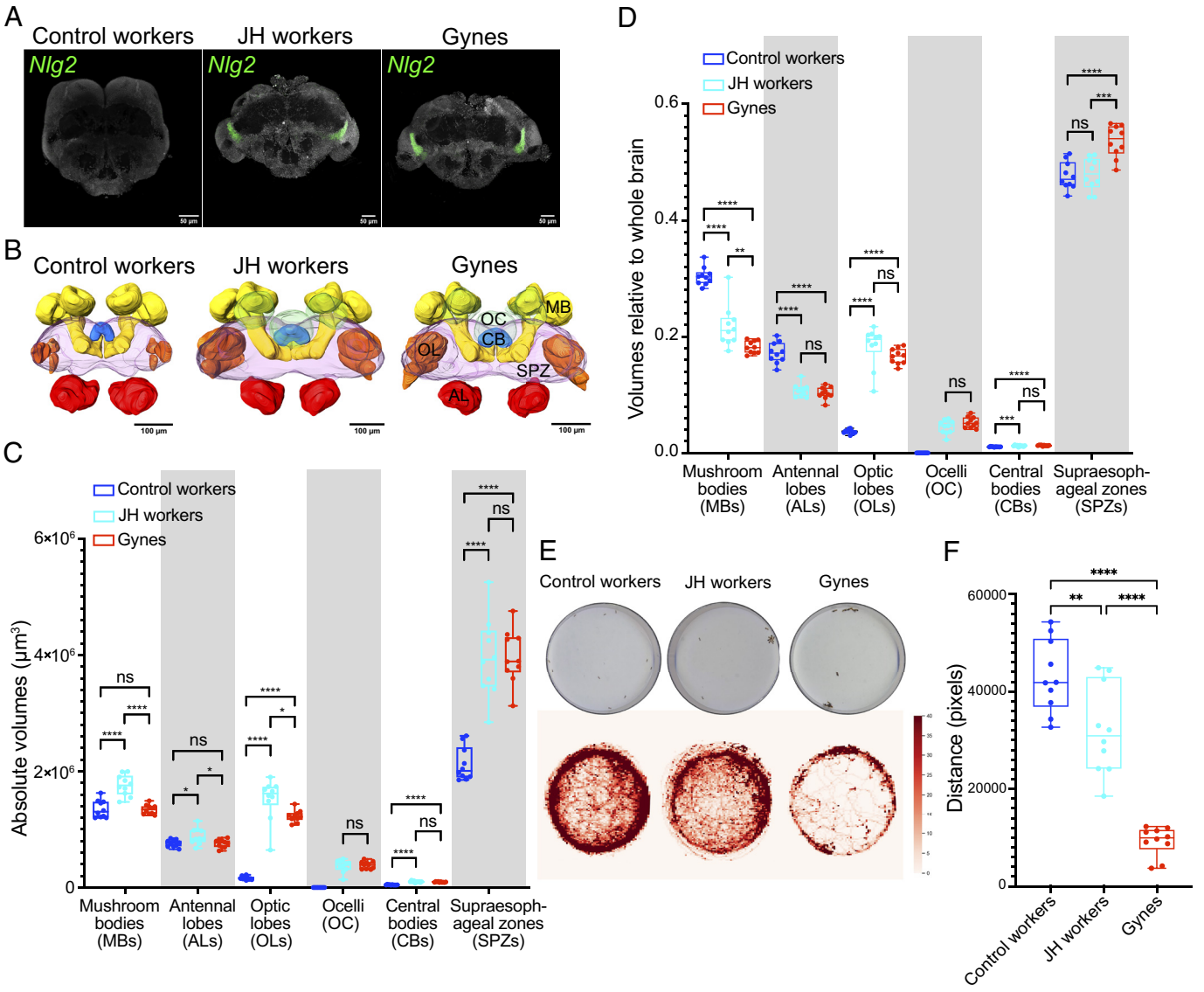

Fig. 4. JH treatment generates gyne-biased brain anatomy and locomotion. (*A*) Brains of 12-d-old pupae stained with *Neuroligin-2* (*Nlg2*; green) and counterstained with nuclei (gray), showing that *Nlg2* is expressed in the optic lobes of JH workers and gynes but not in control workers. (*B*) Representative reconstructions of brain anatomy of 12-d-old pupae of control workers, JH workers, and gynes, obtained from image stacking of heads; 30 brains (n = 10 for each category) were reconstructed in parallel series, after which volumes were quantified as the sums of paired mushroom bodies (MB), antennal lobes (AL), olfactory lobes (OL), and triple ocelli (OC). (*C* and *D*) Absolute (*C*) and relative (*D*) volumes of brain compartments, assessed by one-way ANOVAs with Tukey's post hoc tests, across control workers, JH workers, and gynes; OC are absent in control workers so two-sided *t* test were used to evaluate OC differences between JH workers and gynes: **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001; ns *P* > 0.05; n = 10. (*E*) Locomotory activities of adults, showing that control workers always keep moving, while JH workers and gynes are more likely to aggregate (*Upper* panel) with locomotion of JH workers being intermediate (*Lower* panel heatmaps; n = 10 for each category). (*F*) Individual ant distances moved per unit of time confirming that locomotion of gynes is lower than control worker locomotion, while JH workers were intermediate (n = 10 for each category; one-way ANOVAs with Tukey's post hoc tests; ***P* < 0.01; *****P* < 0.0001). Plots have the same information as in Fig. 3.

indicating that JH enlarged the size of all brain compartments, potentially by increasing overall cell number.

For relative volumes, we found that both JH workers and gynes developed smaller MBs and ALs but larger visual systems (OLs and OC) and CBs than control workers. While the absolute SPZ volumes in JH workers became as large as in gynes (Fig. 4*C*), the corresponding relative volumes did not differ between control and JH workers (Fig. 4*D*) because mushroom bodies, antennal lobes, and optic lobes are overdeveloped in JH workers. Feeding JH mimic to third instar worker larvae thus resulted in a consistent shift in developmental brain trajectory toward gyne phenotypes for neuropil volume, the size of cell clusters, and the expression of specific genes, extending into the prepupal and pupal stages where individuals did not receive JH treatment. This is presumably because transcription of five out of the six copies of the JH biosynthesis enzyme *Jhamt* was enhanced, while transcription of 2 of the 3 JH degradation enzyme *Jheh* was reduced in JH workers throughout development, relative to control workers (*SI Appendix*, Fig. S12). Feeding with JH mimic thus appears to increase endogenous JH titers with positive downstream effects on brain remodeling during subsequent developmental stages.

Division of labor between gynes and workers is obvious in *M. pharaonis* because the completely sterile workers (70, 71) are highly mobile nurses, foragers, sanitation laborers, and nest-defenders, while gynes and inseminated queens are "inward-looking" aggregative, particularly when laying eggs. Having established that these caste differences are governed by differentiated brain functions mediated by expression differences of multiple genes (16 and present study), we next investigated whether the JH-induced gyne-like brain anatomy

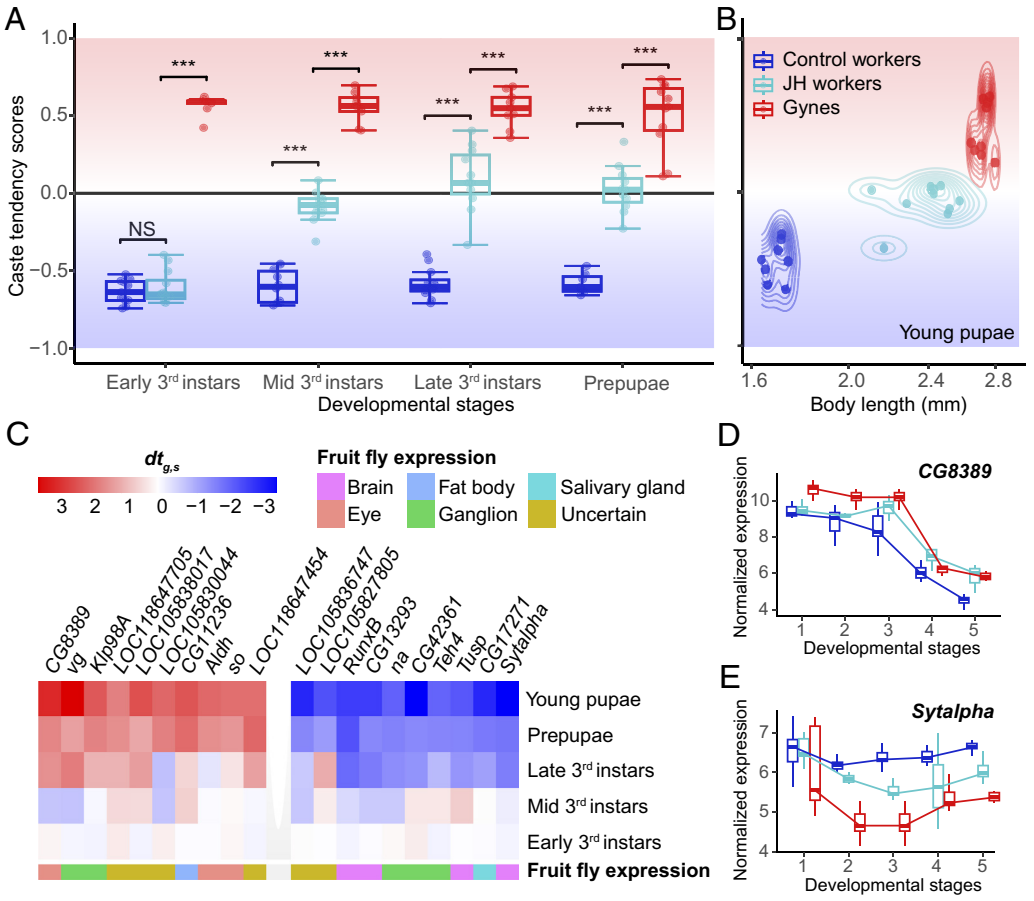

Fig. 5. Overall transcriptomic changes underlie phenotypic shifts in JH workers. (*A* and *B*) Caste tendency scores (CTS) of total transcriptomes (*A*), reflecting the normalized similarities between an individual and the average same-age gyne and worker, with values closer to 1 and −1 implying similarity to these naturally diverging developmental endpoints (Student's *t* tests; ***$P < 0.01$; NS $P > 0.1$); scores of young pupae scaled by body length (*B*) indicate that JH workers were intercastes in terms of their final developmental phenotypes. (*C*) The top 10 gyne-biased genes (*Left* panel) and worker-biased genes (*Right* panel) based on canalization-disruption (*cd*) scores, with heat-map colors representing $dt_{g,s}$ values (Dataset S1) that quantify JH treatment effects across developmental stages (*Materials and Methods*). Each gene is color-coded according to the organ where its *D. melanogaster* homologue is primarily expressed, except for *vestigial* (*vg*) labeled as highly expressed in the ganglion because no wing disc-related dataset is available for fruit flies. (*D* and *E*) Expression patterns of *CG8389* (*D*) and *Sytalpha* (*E*), the top gyne-biased and top worker-biased JH-regulated genes throughout development, with stages 1 to 5 standing for early third instar, mid third instar, late third instar larvae, prepupae, and young pupae. Normalized expression abundances on the *y* axis were obtained by variance stabilizing transformation.

and associated gene expressions were sufficient to shift worker-like behavior to gyne-like behavior. To this end, we tracked the locomotion of JH workers, control workers, and gynes. We isolated female ants from males in the pupal stage to avoid any effect of mating after eclosion, since both JH workers and gynes develop a spermatheca and could thus potentially be inseminated. We then selected test adults after 30 d, i.e. at a sufficiently advanced adult age for workers to predictably forage outside the nest and for gynes to lay unfertilized eggs inside the nest (*SI Appendix*, Fig. S11*C*). Our results showed that the locomotory activities of JH workers were dramatically reduced and that about one-third of the JH workers displayed the same aggregation behavior as gynes (Fig. 4*E*). Total locomotion distances of JH workers were significantly shorter than those of control worker, but still longer on average than those of gynes (Fig. 4*F*).

Overall, these results led us to conclude that JH treatment during the early third larval stage modulates brain development and significantly affects many phenotypic traits, in spite of possible constraints that may have been due to ovary development not being inducible. Ovaries would normally comodulate behavior by signaling via the hemolymph, comparable to the release of ecdysone and imaginal morphogenesis protein-Late 2 (Imp-L2) as in adult *Aedes aegypti* mosquitoes (90, 91) and adult *Harpegnathos saltator* gamergates, which have workers that can be inseminated (85).

Transcriptomic Evidence for a Universal Gyne-Biased Somatic Developmental Trajectory Induced by JH. Transcriptomic data across developmental stages can elucidate developmental processes with unprecedented molecular detail (92). We therefore collected whole-body RNA-seq data of control workers, gynes, and JH workers, spanning five time points across the third larval instar, prepupae, and early pupae, involving 142 individuals with 6 to 12 biological replicates (*SI Appendix*, Fig. S1*C* and Table S5). The overall similarities calculated by Spearman's correlation coefficients (ρ) showed, as expected, that individuals clustered according to developmental stages, with the larval–prepupal transition exhibiting the most profound changes (*SI Appendix*, Figs. S13 and S15*A*) consistent with rapid morphological remodeling during metamorphosis. Gene expression profiles of JH workers gradually diverged from those of control workers after the first time point and became more gyne-like in caste tendency scores (CTS) as development proceeded (Fig. 5 *A* and *B*; also see *SI Appendix*, Figs. S15 *B* and *C* and S16).

Next, we identified a set of robust differentially expressed genes between control workers and gynes (caste-DEGs) and between control workers and JH workers (JH treatment DEGs) for each developmental time point (*Materials and Methods*). This revealed significant overlap between JH treatment DEGs and caste-DEGs confirming that expression levels in JH workers generally correspond to those of

gynes (*SI Appendix*, Fig. S17). Focusing on these overlapping DEGs (oDEGs) as prime candidates for driving the developmental transition of JH workers toward gynes, we first performed functional analyses with KEGG and GO databases. The results suggested that oDEGs were involved in processes mediating cuticle and muscle development, as well as fatty acid metabolism (*SI Appendix*, Fig. S18*A*), which might account for the larger body size and higher number of adipocyte lipid droplets of JH workers. The insulin signaling pathway also stood out among the oDEGs (*SI Appendix*, Fig. S18*B*), consistent with our FISH results (*SI Appendix*, Figs. S9 and S10).

Second, we examined the expression levels of oDEGs across tissues using their orthologues in *D. melanogaster* as reference (*Materials and Methods*). This showed that genes that were both JH-down-regulated and more highly expressed in control workers, relative to gynes, were enriched in the central nervous system (CNS) of both third instar larvae and early pupae (brains, ganglia, and heads; *SI Appendix*, Fig. S19 *A* and *B*), suggesting that downregulation by JH contributes to the formation of the less elaborate brain structures and behaviors of gynes (Fig. 4). In addition to these CNS-enriched genes, the JH treatment DEGs also revealed upregulation of genes enriched in fat bodies and larval hindguts, and downregulation of genes enriched in larval tubules and pupal eyes (*SI Appendix*, Fig. S19 *C* and *D*). This is consistent with our JH treatment having remodeled multiple tissues in parallel.

Finally, based on the canalization scores obtained previously (17) via quantification of between-caste and within-caste gene expression differences across development, we calculated a canalization-disruption score (*cd* score) to quantify the effect of JH treatment on canalized gene expression (*Materials and Methods*). An arbitrary threshold ($|cd|$ *scores* > 3, $P < 0.05$) was applied to narrow down the list of candidate genes which produced 95 genes (*cd* genes for short) whose expression was clearly influenced by our JH treatment that resulted in gyne-like development (Dataset S1). Fourteen of these *cd* genes had positive *cd* scores consistent with JH-upregulation, while 81 *cd* genes had negative *cd* scores likely reflecting JH-downregulation. The genes *CG8389* (Fig. 5*D*) and *Sytalpha* (Fig. 5*E*) were at the top of these respective lists. Among the 14 JH-up-regulated *cd* genes, *vestigial* (*vg*) and *sine oculis* (*so*) stood out again (Figs. 2 *A* and *C* and 5*C*). In addition, the up-regulated *singles bar* (*sing*) gene (Dataset S1) encodes a MARVEL domain-containing transmembrane protein required for myoblast fusion in *D. melanogaster* (93), and may thus contribute to the more pronounced flight muscle developed in JH workers (Fig. 2*D*). Among the 81 JH-down-regulated *cd* genes, many were enriched in the brain, eye, ganglion, or the entire head (*SI Appendix*, Fig. S19*E*). *Sytalpha* is expressed in the neuroendocrine cells (94) and encodes a critically important developmental protein of the synaptotagmin family which regulates both synapsis activity and exocytosis of steroid hormones (95, 96). Also the *Tusp* gene expressed in nervous systems is important for synaptic chemistry because it participates in the assembly of the trimeric SNARE complex (97, 98). Finally, the genes *Teh4*, *na*, and *Obp83a* (Dataset S1) are also expressed in neurons or sensory organs, which may mediate social aggregation and pheromone detection (99–101).

Overall, our analysis of total transcriptome profiles confirmed that JH treatment of worker larvae induces a generally gyne-biased developmental trajectory, characterized by a set of core genes with tissue-specific expression, often in good correspondence with our cellular (Figs. 2 *A* and *C*, 3*A*, and 4*A* and *SI Appendix*, Figs. S9 and S10), morphological (Figs. 1 *A*–*C* and 2 *B* and *D*), anatomical (Figs. 1*D*, 3 *B*–*F*, and 4 *B*–*D*), and behavioral (Fig. 4 *E* and *F*) data.

Discussion

In this study, we have shown that recurrent JH-analogue treatment through the third larval instar of the ant *M. pharaonis* can redirect canalized development of workers to a somatically functional gyne phenotype. This included the genital imaginal discs that give rise to the bursa copulatrix, spermatheca and oviducts, the eye-antennal imaginal discs that give rise to the antennae, ocelli, and compound eyes, the wing imaginal discs that produce the forewings, hindwings, and the dorsal half of the mesothorax, the flight muscles, the fat body, and the optic lobe, central body, and supraesophageal zone in the brain, but never the most fundamental ovary component (see *SI Appendix*, Table S6 for details). The likely explanation is that the *M. pharaonis* germline is irreversibly sequestered during the embryonic (egg) stage (66). This has led to strongly canalized gyne development and only slightly less canalized worker development, facilitated by nonoverlapping JH-sensitivity windows for gyne and worker development that could not be reversed for the ovaries. We used RNA-seq to identify a substantial set of the JH-affected genes, which appear to have crucial functions in specifying caste-specific traits related to morphology, anatomy, and behavior.

Caste-Specific Canalization Constraints May Explain Tissue-Specific JH-Responses. We showed that JH treatment in the last larval stage extended the larval growth period, consistent with earlier work on *M. pharaonis* (17) and with similar results in other ants (50, 51, 54, 102). This implies that the terminal growth period (TGP), defined as the time between reaching the critical body size above which a larva is committed to undergo pupation and the actual cessation of growth (103), are both important for determining final larval body size. Reprogramming the critical size for cessation of growth and the TGP itself have both been suggested previously to be important for further differentiation of the somatic ant caste into workers and soldiers (53, 104). Our findings that JH treatment of *M. pharaonis* workers extended the final larval stage of workers to include the longer gyne-like TGP, required to produce a proper reproductive phenotype, is consistent with these earlier results and suggests that the same mechanisms regulate gyne-worker differentiation.

Compared to solitary insects, JH appears to have gained novel functionality in ants by up-regulating caste-specific imaginal disc growth in preparation for metamorphosis. Interruptions of normal spatial-temporal gene expression in the disc-specific gene-regulatory network of workers may thus be sufficient to account for caste-specific JH regulation of growth, consistent with earlier studies of wing polyphenism in ants (23, 105). In contrast to the suppressive effect of JH on imaginal disc growth in the last instar of solitary Lepidoptera such as *Manduca sexta* (106, 107) and *Precis coenia* (108), and the absence of any effect in *D. melanogaster* (109), JH is also known to elicit the appearance of hindwing- and forewing discs when applied to larvae of soldiers (55) and minor workers (69) of *Pheidole* ants. Likewise, gyne-like imaginal structures emerged when JH was applied to late larval instars of *Apis mellifera* honeybee workers (47, 110).

Although it is a reproductive organ, the spermatheca is part of the queen soma. It evolved to store male spermatozoa until their later use for egg fertilization, which is necessary in Hymenoptera to produce diploid female offspring while haploid males develop from unfertilized eggs. The spermatheca is degenerated and often completely lost in worker ants, in contrast to the ovaries which are normally retained in workers except for a few genera such as *Monomorium* (15, 111, 112). It is interesting, therefore, that JH treatment of final instar larvae recovered somatic spermatheca development but not germline ovary development, consistent with the deep canalization

of ovaries discussed above (17). If degree of canalization merely determined how much experimentally applied JH would be needed to also induce germline functionality in the somatic caste, one could argue that our experimental protocol failed because we should have administered more than six JH treatments. However, if the JH sensitivity windows for germline and soma induction occur asynchronously over time and without overlap our results would reflect that germline induction in third instar larvae was developmentally impossible. We will return to this in the next section.

When castes are determined well before pupation—the hallmark of all superorganismal hymenopteran lineages whose origins represent irreversible MTEs (4, 5), the immature brain obtains specific functions to coordinate regulation of organ-specific gene expression to implement the required differential canalization of caste development. The brains of individual caste members thus become pivotal for homeostatically producing an integrated syndrome of phenotypic traits in superorganismal lineages rather than affecting primarily single traits such as ovary development, as is typical for open family-based societies of social Hymenoptera characterized by mere adult reproductive role differentiation. Our JH treatment of larvae enhanced the absolute volume of all brain compartments in JH workers so they were anatomically indistinguishable from gyne brains on the day before pupal eclosion. The antennal lobes, mushroom bodies, and optic lobes of JH workers actually became larger than those of gynes even though mushroom bodies and antennal lobes did not differ between control workers and gynes. We also found that the number of lipid droplets in fat body adipocytes of JH worker larvae surpassed the typical numbers of these droplets in fat bodies of gyne larvae. JH supplementation thus had disproportional effects on these brain and fat body traits even though the larger body size and reduced locomotion of JH workers did not entirely bridge the developmental gap with naturally developing gynes, as also the transcriptomic profiles indicated. This suggests that there may be selection against overexpression of JH under natural conditions in order to maintain homeostatic growth patterns in each caste phenotype. Some of these inconsistencies between JH workers and normal gynes may also have arisen from disturbed brain-body communication via signaling pathways because the ovaries were absent in JH workers.

Modular Developmental Responses and (A)Synchrony in JH-Sensitivity. As introduced above, the hypotheses that caste development in ants is merely a function of body size (the hourglass model) or requires a more complex JH-driven interaction-network explanation (the standard model) have been subject to debate (29–32). Our present results in *M. pharaonis* support the standard model but

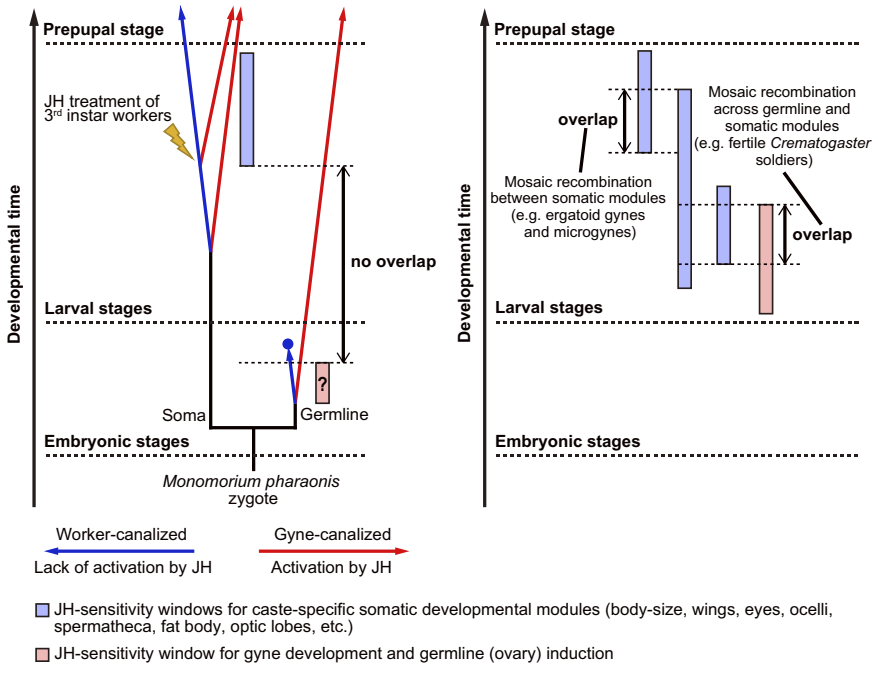

Fig. 6. A proposition to explain caste differentiation in ants assuming that JH is a key regulator and emphasizing the importance of (a)synchronous caste-specific JH-sensitivity windows. *Left* panel. In *Monomorium pharaonis*, gynes (colony germline individuals) start their specific canalized development early in embryonic development (17, 66) well before the onset of somatic trait canalization in worker-individuals (the colony soma) during larval development. That process starts in the first larval instar and becomes controlled by JH in the third larval instar (17). The trigger mechanism of irreversible germline differentiation in gynes remains unknown but might also be JH-induction (labeled with a question mark) because JH affects migration of primordial germ cells in *D. melanogaster* embryos (116) and induces ovary differentiation between gyne and worker caste individuals in the early fifth larval instar of *A. mellifera* honeybees (114, 115). In contrast, germline degeneration in *M. pharaonis* workers is complete at embryo stage 9 well before egg hatching (66) (*SI Appendix*, Figs. S4–S6) (dot at the end of the bottom blue line). The red arrows illustrate the results obtained in the present study, which imply that functional ovaries should be inducible in *M. pharaonis* workers provided recurrent JH treatment could be administered in the embryonic stage. The diagram also suggests that recombination of developmental modules failed because the JH-sensitivity windows did not overlap (black vertical arrow). *Right* panel. A schematic plot of the more common situation where caste differentiation is completed before pupation (the hallmark of all superorganismal hymenopteran lineages) but developmental divergence starts later, i.e. somewhere in the larval phase so that overlap of gyne and worker JH-sensitivity windows is more likely. Then it is reasonable to assume that JH titers affect gene expression in gyne- and worker-destined larvae synchronously, so that natural variation in JH exposure across individuals may decouple and recombine developmental modules for body size, and reproductive, sensory, flight, and fat body organs, as well as for some of the brain modules (see *SI Appendix*, Table S6 for a list of such traits). Mosaic individuals should thus become possible, initially by chance and later as developmental routines when natural selection occasionally promotes such recombined phenotypes as adaptations to environmental changes that colonies face. For mosaic recombination across germline and somatic modules, producing for example *Crematogaster* soldiers with gyne-like ovaries, body size, ocelli, and thorax while wings and spermatheca remain worker-like (24), we thus expect a zone of overlap across JH-sensitivity windows for germline (pink bar) and soma but not for all somatic modules (blue bars). For mosaic recombination between somatic modules such as ergatoid gynes or microgynes, only traits like thorax, wings, and body size become worker-like, while ovaries and other reproduction-related somatic modules such as the spermatheca remain gyne-like. In such cases, we expect a zone of overlap for JH-sensitivity windows for at least part of the somatic modules.

cannot decisively refute the simpler alternative in other ant lineages (see *SI Appendix* for a more detailed discussion). To resolve this debate at a more general level, we suggest that (a)synchrony in caste-specific JH-sensitivity windows deserves to be explicitly considered to enable a more general understanding of the developmental dynamics of caste differentiation across the (superorganismal) social insects with preimaginal caste differentiation.

In all our JH treatments, the *M. pharaonis* worker caste always remained monomorphic because growth of the various caste-specific somatic traits (e.g., wing-buds, ocelli, antennal and genital imaginal discs) was never decoupled when larvae responded to JH treatment. Similar results were obtained for JH-treated larvae in *S. invicta* fire ants (52, 113) and *A. mellifera* honeybees (47, 110), two other species with continuous variation in worker body size. A notable difference was that JH treatment of honeybee worker larvae could also shift ovary development to a gyne phenotype (114, 115). The explanation may be that the germline sequestration in *Solenopsis* and *Apis* happens later (during larval development) rather than in the embryonic (egg) stage. As shown in Fig. 6, *Left* panel, the distinct lack of overlap in gyne and worker JH-sensitivity windows (black vertical arrow) may well explain that induction of germline functionality in JH-treated workers remained out of reach in *M. pharaonis* while all somatic traits responded in a mutually coordinated way.

The fact that JH-sensitivity windows of germline (ovary) and caste-specific somatic traits do not overlap in developmental time apparently also precludes complete mosaic recombination across the germline and soma modules in *M. pharaonis*. In Fig. 6, *Right* panel, we illustrated the more common situation where preimaginal caste differentiation starts somewhere in the early larval stages and is completed in the last or pre-last larval stage (47–52). While natural variation in JH exposure (more modest than the contrasts in our experimental setup) would be unlikely to have effects in *M. pharaonis* (Fig. 6, *Left* panel), it would allow recombination between developmental modules when variable JH-exposure affects caste-specific developmental trajectories simultaneously. Intercaste phenotypes consistent with recombination of caste-developmental modules have been generated by in vitro rearing of honeybee larvae and shown to be often characterized by intermediate ovariole numbers combined with somatic worker traits (117–119). Intercaste phenotypes have been particularly well documented in the ants *Myrmica rubra* (120), *Mystrium rogeri* (25, 121), *Aphaenogaster rugulosa* (122), and several *Temnothorax* species (26, 123–125). When such modular recombination is feasible because caste-specific JH-sensitivity windows overlap (Fig. 6, *Right* panel), colony-level selection may occasionally elaborate such recombined caste-phenotypes so they can become established as ergatoid (permanently wingless) gynes, microgynes (distinctly smaller queen morphs), soldiers, or other female anomalies (summarized in ref. 27).

Ergatoid gynes evolved in 55 ant genera (126) and microgynes, which may in fact be intraspecific inquiline social parasites of normal queens (macrogynes), might be even more common (127). Mosaic recombination across germline and somatic modules of caste differentiation may also give rise to unexpected diversification, for example in *Crematogaster* ants, where soldiers developed larger and clearly gyne-biased ovaries (24). Selection for novel response thresholds or slight shifts in the JH-sensitivity windows might then explain that JH could induce supersoldiers rather than normal soldiers in some *Pheidole* ants (55). This type of modularity in tissue growth is also prevalent among solitary insects (128) and has been well documented in male rhinoceros beetles (*Trypoxylus dichotomus*) where nutrition affects wing size in proportion to body size while genital discs remain unaffected and horn discs respond in a hypersensitive manner. Tissues within the same beetle bodies all had distinct response thresholds to insulin/IGF signals (129) reminiscent of what variation in JH titers can achieve in ants.

Based on these considerations, our study invites complementary work to untangle the molecular mechanisms underlying development of morphological differentiation in other ants where caste is determined later during larval development. We would expect phenotypically analogous, but not homologous molecular underpinning of JH-driven caste differentiation in the superorganismal lineages of corbiculate bees and vespine yellowjacket wasps, because they represent convergent irreversible MTE's to higher-level colonial organization. We also note that additional caste phenotypes are unknown in honeybees, stingless bees, and bumblebees, with the exception of a soldier caste in some species of Neotropical stingless bees (130, 131). This may be related to gyne-worker size dimorphism in superorganismal bees and wasps being modest (21) in comparison to many ants where worker caste individuals lack the constraint of having to forage on the wing (112). However, the results of our study are not directly relevant for the society-forming lineages of polistine and stenogastrine wasps, halictid bees, and allodapine bees where there is no preimaginal caste differentiation. Here, individual development should have been selected to preserve reproductive totipotency until pupal eclosion because the much shallower breeder-helper division of labor in these lineages is based on condition-dependent and potentially reversible forms of adult reproductive role differentiation driven by JH (132–135).

Materials and Methods

Methoprene Feeding. Details pertaining to the maintenance of *M. pharaonis* colonies and the experimental design of methoprene feeding of first-half and second-half third instar worker larvae are described in *SI Appendix*.

Morphometric Measurements. Larval and pupal body length, adult antennal scape length, and adult head width across the eyes were measured based on the images of live individuals captured by a stereo microscope. See *SI Appendix* for the methods of measurements.

Staining, Microscopy, and Image Analysis. The reagents and methods used for performing HCR™ RNA fluorescence in situ hybridization to visualize gene expression, hematoxylin-eosin staining to visualize flight muscles, and adipocyte staining to visualize lipid droplets and cytoplasm volume are described in *SI Appendix*. Image analysis methods for the quantification of gene expression levels based on fluorescence intensity and cell number, as well as the quantification of lipid droplets, cytoplasm volume, and nuclei size in adipocytes, are also included in *SI Appendix*.

Volume Quantifications of Brain Neuropils. Pupae of control workers, JH workers, and gynes were collected on the last (twelfth) day before eclosion for volume quantification of brain neuropils. See *SI Appendix* for details about sample preparation, imaging, and three-dimensional reconstructions.

Analysis of Locomotory Behavior. 30-d-old adults of control workers, JH workers, and gynes were collected for analysis of locomotory behavior. See *SI Appendix* for details.

Sampling, RNA Sequencing, and Transcriptome Profiling. A total of 142 individual transcriptomes of control workers, JH workers, and gynes across the developmental stages were acquired for downstream analysis. See *SI Appendix* for details.

Quantification of Individual-Level Caste Tendency Scores (CTS), Gene-Level Canalization-Disruption Scores (*cd* score), and Tissue-Specific Gene Expression. Methods were elaborated based on a previous study (17); further details can be found in *SI Appendix*.

Orthology Assessment and Phylogenetic Analysis. Orthologs of the core-set of genes that induced a gyne-biased developmental trajectory after JH treatment of worker larvae (listed in Dataset S1) were assessed across the Formicidae, the Apoidea, and the fruit fly *D. melanogaster*. Orthologs of the genes *so*, *eya*, *vg*, *en*,

and *Kr-h1* were identified in *M. pharaonis* using phylogenetic analysis. Detailed methods are explained in *SI Appendix*.

Data, Materials, and Software Availability. All transcriptomic data in this paper have been deposited at NCBI repository (BioProject Accession Number: PRJNA1143187) and are now publicly available (136).

ACKNOWLEDGMENTS. We thank the Center for Advanced Bioimaging Denmark at the University of Copenhagen for providing the confocal microscope and matching software, which were necessary for collecting our imaging data, and Dr. Takashi Koyama in the Department of Biology, University of Copenhagen, for comments on a previous version of the manuscript. We thank Fuqiang Lin in the Centre for Evolutionary and Organismal Biology, Zhejiang University, for depositing the transcriptomic data into NCBI, and Alivia Lee Price for helping to establish the RNA-FISH protocol. This work was supported by the National Natural Science Foundation of China (Grant No. 32388102 to G.Z.), the National Natural Science Foundation of China (Grant No. 32170631 to W.L.), the Yunnan Provincial Science and Technology Department (Grant No. 202301AT070291 to W.L.), and the Villum Foundation (Villum Investigator Grant, Grant No. 25900 to G.Z.).

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