EUSOCIALITY

Social regulation of insulin signaling and the evolution of eusociality in ants

Vikram Chandra[1]*†, Ingrid Fetter-Pruneda[1]*†, Peter R. Oxley[1,2], Amelia L. Ritger[1], Sean K. McKenzie[1,3], Romain Libbrecht[1,4], Daniel J. C. Kronauer[1]*

Queens and workers of eusocial Hymenoptera are considered homologous to the reproductive and brood care phases of an ancestral subsocial life cycle. However, the molecular mechanisms underlying the evolution of reproductive division of labor remain obscure. Using a brain transcriptomics screen, we identified a single gene, *insulin-like peptide 2* (*ilp2*), which is always up-regulated in ant reproductives, likely because they are better nourished than their nonreproductive nestmates. In clonal raider ants (*Ooceraea biroi*), larval signals inhibit adult reproduction by suppressing *ilp2*, thus producing a colony reproductive cycle reminiscent of ancestral subsociality. However, increasing ILP2 peptide levels overrides larval suppression, thereby breaking the colony cycle and inducing a stable division of labor. These findings suggest a simple model for the origin of ant eusociality via nutritionally determined reproductive asymmetries potentially amplified by larval signals.

Eusocial insects exhibit a reproductive division of labor in which queens lay eggs and workers perform other tasks (*1*). Eusociality in ants, and in many other Hymenoptera, likely evolved from a subsocial state in which a female wasp would lay an egg and then care for the resulting larva until pupation (*1*–*3*). Such brood care may have been induced by larval signals, and observations of extant subsocial wasps are consistent with this scenario (*2*–*4*). This temporal reproductive and behavioral plasticity was then modified into a fixed reproductive asymmetry between queens and workers in eusocial colonies (*2*, *5*). This raises three important mechanistic questions: (i) How are subsocial reproductive cycles regulated? (ii) How is the eusocial reproductive division of labor regulated—i.e., what allows queens to lay eggs but prevents workers from doing so? (iii) What is the evolutionary trajectory that gave rise to fixed eusocial division of labor from subsocial cycles? Here we suggest that, in ants, evolutionary innovations in insulin signaling may have played a crucial role in each case.

Eusociality evolved once in a common ancestor of ants and, with the exception of a few derived social parasites, all extant ants are eusocial (*6*) (Fig. 1). To identify conserved potential regulators of division of labor between reproduction and brood care in ants, we conducted an unbiased screen for differentially expressed genes between whole brains or heads of reproductives and nonreproductives across seven ant species, including four previously published datasets (Fig. 1 and tables S1 and S2) (*7*–*11*). We sampled a range of reproductive strategies, from species with morphologically distinct queens and workers to queenless species. Among all 5581 identified single-copy orthologs, we found only one such gene: *insulin-like peptide 2* (*ilp2*). *ilp2* was always significantly up-regulated in reproductives (Fig. 1). Thus, the differential expression of *ilp2* is likely conserved across ants. Consequently, the most recent common ancestor of ants likely had *ilp2* expression that was high in reproductives and low in nonreproductives.

Although our approach is conservative and probably misses genes, it has the advantage of eliminating false positives. When we relaxed the statistical stringency for classifying genes as differentially expressed, our screen still returned *ilp2* as the single candidate gene (fig. S1). Relaxing other inclusion criteria revealed additional genes that might be expected to vary with reproductive state. For example, a total of 24 genes were consistently differentially expressed in subsets of five of the seven studied species (fig. S2 and table S3). This list includes *insulin-like peptide 1* (*ilp1*), as well as other genes implicated in insulin signaling (fig. S3 and table S3). Non–single-copy orthologs were excluded from our screen. One example is *vitellogenin* (*vg*), a gene that has undergone repeated duplications in ants (*12*). The vitellogenin protein is a lipid carrier that provisions developing oocytes with yolk and constitutes a reliable indicator of female reproductive activity (*12*, *13*). Studies of bees and other insects have shown that vitellogenin interacts with insulin signaling (*14*–*16*). *vg* indeed showed consistently higher expression in reproductives in our screen, even though this difference was not statistically significant in two of the ponerines (fig. S3). These findings further bolster the conclusion that insulin signaling played a major role in the evolution of reproductive division of labor in ants.

Insulin regulates reproduction and food-seeking behavior across a wide range of organisms, making it a prime candidate for the regulation of subsocial cycles and eusocial division of labor (*17*). Most studied hymenopterans have two ILPs: ILP1 and ILP2 (fig. S4). Whereas ILP1 resembles insulin-like growth factor, ILP2 is similar to canonical insulin (fig. S5) (*11*). In other holometabolous insects, these ILPs regulate larval growth, adult metabolism, and reproduction (*17*–*19*). Moreover, caste determination in most ant species relies on nutritional asymmetries during development: Queen-destined larvae eat more than worker-destined larvae, which likely explains how queens acquire higher ILP2 levels (*20*). A study of *Diacamma* sp. found that the asymmetry in reproductive potential between ants was correlated with insulin receptor expression in the ovaries (*21*). This suggests a possible secondary mode of reproductive control downstream of ILPs that may augment the initial reproductive asymmetry reflected by differential *ilp2* expression in the brain.

ILPs have not been studied functionally in eusocial insects in the context of reproductive division of labor between adults. However, insulin signaling has been implicated in other contexts, such as caste development and nonreproductive division of labor (*18*, *22*–*24*). Current data from wasps and bees do not typically indicate that *ilp2* is differentially expressed between adult queens and workers, suggesting that this expression pattern may be ant specific (table S5). This apparent inconsistency may be explained by the fact that eusociality has evolved independently in ants, bees, and wasps (*1*). Therefore, though insulin signaling may have been co-opted repeatedly during social evolution, the details likely differ between independent lineages.

We used the clonal raider ant *Ooceraea biroi* to study ILP2 in ants. *O. biroi* has secondarily lost queens, resulting in a species in which workers reproduce synchronously and asexually (*13*, *25*). Colonies alternate between reproductive and brood care phases. This colony cycle is regulated by the periodical presence of larvae, which suppress reproduction and induce brood care behavior in adults, and is reminiscent of the subsocial cycle presumed to precede eusociality in ants. Despite this unusual biology, *O. biroi* is eusocial. Workers display cooperative brood care, colonies contain overlapping generations of adults, and reproductive asymmetry exists within colonies (*25*).

We found that antibody staining of ILP2 exclusively localized to the brain, primarily in a single medial cluster of ~15 cells in the pars intercerebralis (Fig. 2, A to C, and fig. S6). These insulin-producing cells (IPCs) coincide in location with those of other insects (*26*, *27*). Axons likely project to the corpora cardiaca, the only other brain region staining positive for ILP2 (figs. S6 to S8). We quantified ILP2 in the IPCs and found that its levels are higher in the brood

[1]Laboratory of Social Evolution and Behavior, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA. [2]Samuel J. Wood Library, Weill Cornell Medicine, 1300 York Avenue, New York, NY 10065, USA. [3]Department of Ecology and Evolution, University of Lausanne, Biophore Building, 1015 Lausanne, Switzerland. [4]Institute of Organismic and Molecular Evolution, Johannes Gutenberg University, Johannes-von-Müller-Weg 6, 55128 Mainz, Germany. ***Corresponding author. Email: vchandra@rockefeller.edu (V.C.); ifetter@rockefeller.edu (I.F.-P.); dkronauer@rockefeller.edu (D.J.C.K.)** †These authors contributed equally to this work.

care than in the reproductive phase (Fig. 2D and fig. S6). Peptide levels are thus anticorrelated with transcription. This pattern is known from *Drosophila melanogaster*, in which the rate of ILP secretion correlates with the rate of *ilp* transcription (*27*). This suggests that the mechanisms of *ilp* expression and ILP secretion are conserved in holometabolous insects.

Because larvae regulate the *O. biroi* colony cycle, we asked whether larval communication altered *ilp2* expression in adults. When larvae are removed from colonies in the brood care phase, *ilp2* expression levels in adult brains increase markedly within 12 hours (Fig. 2E) (*28*). This increase occurs under identical nutritional conditions. Conversely, when ants in the reproductive phase are given larvae, their *ilp2* levels decrease (Fig. 2E). *vgq*, the vitellogenin gene upregulated in ant queens, responds similarly, albeit more slowly, to these changes (fig. S9A), raising the possibility that ILP2 regulates reproduction at least partly by acting on *vgq*. Although this experiment is highly suggestive, the addition of larvae was always correlated with the removal of pupae, and changes in expression occurring after the 24-hour time point were confounded by nutritional differences. We therefore repeated this experiment without pupae and under nutritionally controlled conditions. We removed larvae from colonies in the brood care phase, waited until the ants in these colonies activated their ovaries, and then compared brain gene expression between these and control colonies. Again, the removal of larvae increased *ilp2* (Fig. 2F) and *vgq* (fig. S9B) expression. This finding suggests that social signals can mediate insulin signaling independently of internal nutritional state and that this is a key regulatory mechanism underlying the *O. biroi* colony cycle. Given the conserved association of caste and *ilp2* expression in all ants, social regulation of *ilp2* may also underlie the life cycle of the subsocial ancestor.

In *D. melanogaster*, insulin signaling is necessary and sufficient to regulate the terminal differentiation of germline stem cells into oocytes. Moreover, it promotes yolk uptake in developing oocytes and is crucial for ovary activation (*29*). It is therefore plausible that the differential expression of *ilp2* in ants has a causal role in regulating ovary activation and reproductive division of labor. We further hypothesized that if the regulation of *ilp2* were freed, at least partially, from larval control, this would yield ants whose physiology is less susceptible to reproductive suppression. Such a mechanism would allow the evolution of distinct reproductive and nonreproductive castes from an ancestral subsocial cycle. To test

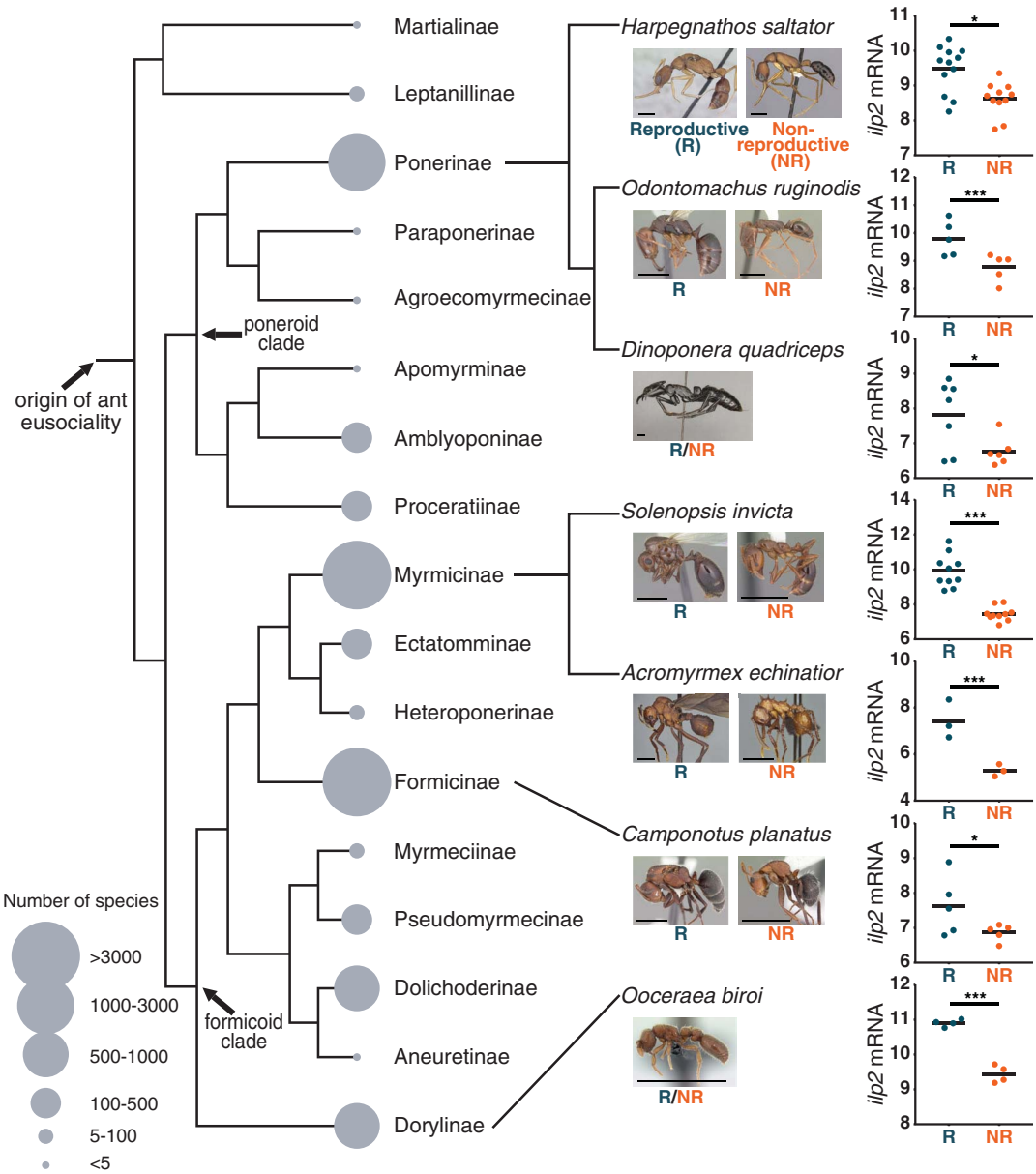

Fig. 1. Brain gene expression in seven ant species identifies one conserved differentially expressed gene. The figure shows the summary cladogram of the seven ant species used in this study in the context of the entire ant phylogeny with all subfamilies labeled. Five of the focal species have queens, whereas two (*Dinoponera quadriceps* and *O. biroi*) are queenless. Although *Harpegnathos saltator* is not queenless, the data compared reproductive and nonreproductive workers (table S1). The dot plots show variance-stabilized transformed read counts for *ilp2*. Blue and orange dots indicate reproductive (R) and nonreproductive (NR) ants, respectively. Horizontal bars indicate means, and asterisks indicate statistically significant differences between groups (Wald test: $*P < 0.05$; $***P < 0.001$). All photos except for those of *Acromyrmex echinatior* are from A. Nobile, S. Hartman, and E. Prado (www.antweb.org). Scale bars, 2 mm. The phylogeny is based on (*30*). Species numbers are from (*6*).

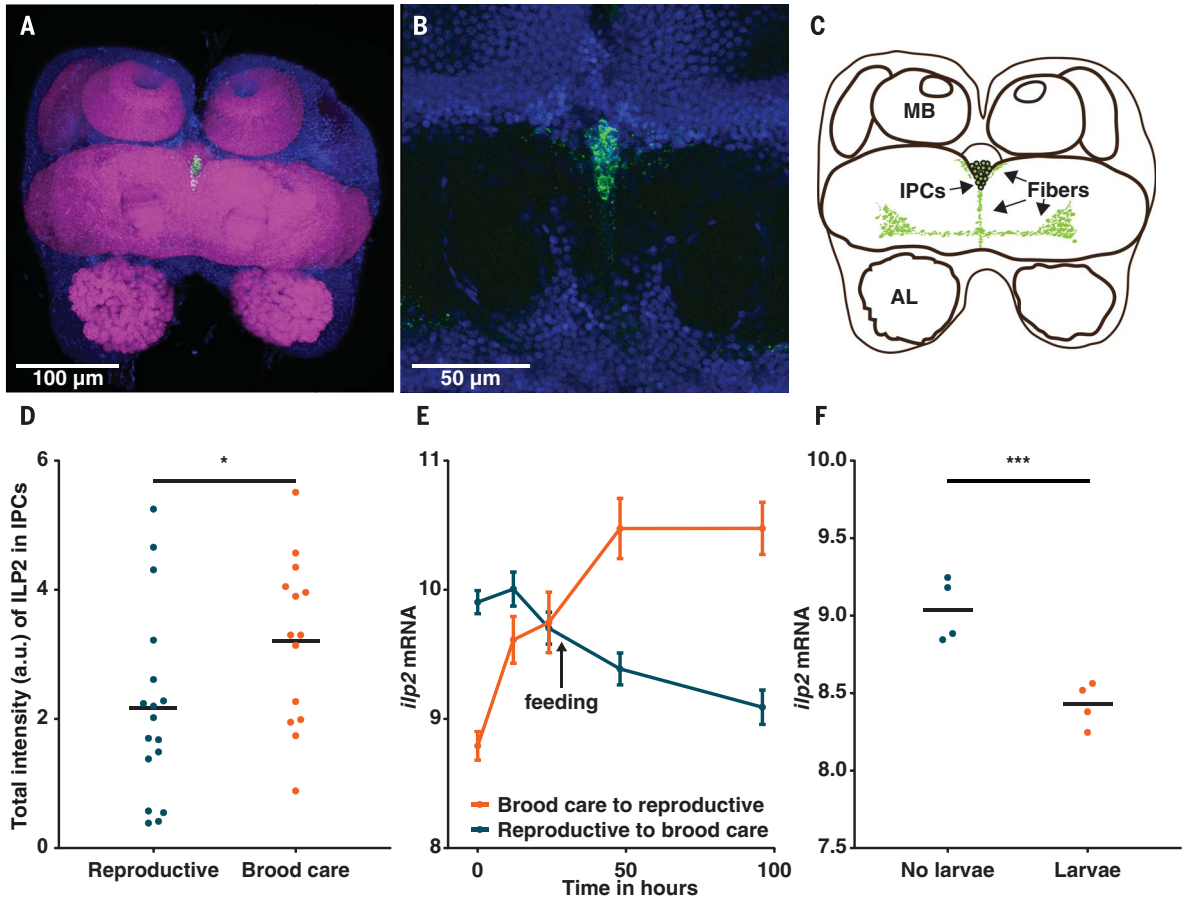

Fig. 2. Larvae regulate *ilp2* in adults. (**A** to **C**) Immunohistochemistry with antibody against ILP2 (anti-ILP2) on an *O. biroi* brain localizes ILP2 peptide to a single cluster of insulin-producing cells (IPCs) in the pars intercerebralis (body-axis dorsal view). Green, anti-ILP2; blue, DAPI (4′,6-diamidino-2-phenylindole); magenta, phalloidin. MB, mushroom body; AL, antennal lobe. (**D**) Total intensity of ILP2 in the IPCs is higher in the brood care phase than in the reproductive phase ($n \geq 14$ ants, *t* test; $^{*}P = 0.046$). a.u., arbitrary units. (**E**) RNA sequencing (RNA-seq) time course shows that the addition of larvae down-regulates *ilp2*, whereas the removal of larvae up-regulates *ilp2* [$n \geq 4$ biological replicates, time-transition interaction, likelihood ratio test with 5% false discovery rate (FDR) correction; $P < 10^{-15}$]. The arrow indicates when ants with larvae were fed (i.e., changes in expression beyond that time point are confounded by differences in nutrition). Error bars depict SEM. Data are from (*28*). (**F**) RNA-seq on ant brains shows that under nutritionally controlled conditions, *ilp2* is up-regulated 8 days after larvae are removed from *O. biroi* workers in the brood care phase ($n = 4$ biological replicates, Wald test with 5% FDR correction; $^{***}P < 10^{-6}$). Data are variance-stabilized transformed read counts. Horizontal bars indicate means.

this hypothesis, we injected synthetic *O. biroi* ILP2 mature peptide into workers in colonies with larvae. As a control, we injected the inactive B chain of this peptide (fig. S11A) (*19*). Injecting ILP2 mature peptide caused strong ovary activation despite the presence of larvae (Fig. 3, A to C, and fig. S10A). Higher doses of ILP2 caused ants to develop more eggs simultaneously (fig. S10, B and C), suggesting that quantitative differences in ILP2 levels vary the ants' positions along a spectrum of reproductive potential. To ensure that ILP2 does not have inhibitory effects during the opposite phase of the colony cycle, we injected ants in the reproductive phase with ILP2 and found no detectable effect on ovary state (fig. S11, B and C).

Finally, we hypothesized that as developmental nutritional asymmetries determine caste in most ants, this might be a general and natural mechanism that produces asymmetries in baseline adult ILP2 levels and consequently in reproductive potential. Whereas most *O. biroi* workers have two ovarioles, some individuals ("intercastes") have four or more (*25*) (fig. S12, A and B). We found that these differences can be determined by the amount of food a larva receives (fig. S13). Intercastes have longer and more active ovaries compared with those of regular workers in the brood care phase, suggesting intercastes are less sensitive to larval signals that suppress ovarian activity (Fig. 4A and fig. S12C). This finding is consistent with previous work showing that some intercastes fail to regress their ovaries during the brood care phase (*25*). Additionally, we found that the IPCs of intercastes contained more ILP2 than those of regular workers (Fig. 4, B and C). As we have shown above, ILP2 peptide levels are negatively correlated with *ilp2* expression, ovary state, and, by extension, circulating ILP2 levels in workers between the different phases of the cycle, probably owing to higher rates of peptide release during the reproductive phase (Fig. 2D). The phase-matched comparisons between different types of workers, on the other hand, show that intercastes consistently have higher ILP2 levels in their IPCs, and, given their more active ovaries and decreased sensitivity to larval signals (*25*), it is likely that they also have consistently higher levels of ILP2 in circulation.

How the ancestral subsocial cycle was regulated remains unknown. However, assuming that similar mechanisms underlie the *O. biroi* colony cycle, our findings suggest a plausible scenario for the evolution of ant sociality. First, during the transition from solitary to subsocial life, some signaling systems (probably including insulin signaling) in adults must have become responsive to larval signals. This shift allowed behavioral and physiological responses in adults to be appropriately modified for the nutritional requirements of the larvae. During the transition from subsocial to eusocial life, increased developmental variation may have caused some

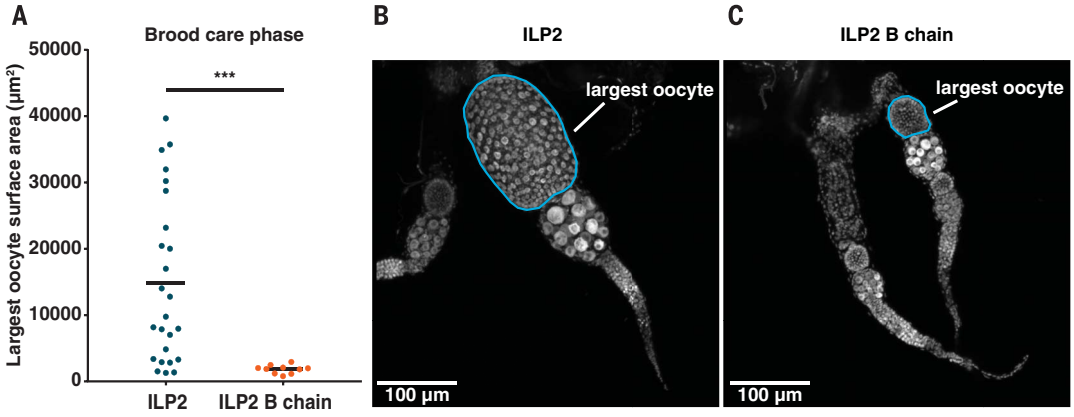

Fig. 3. ILP2 supplementation overrides larval suppression of adult reproduction. (**A**) Workers injected with 100 µM ILP2 in the brood care phase activate their ovaries relative to controls injected with 100 µM ILP2 B chain, despite being in contact with larvae [$n \geq 10$, Welch's t test with Bonferroni correction (related data in fig. S8); ***$P = 0.0005$]. (**B** and **C**) Confocal images of ovaries from ants injected with either 100 µM ILP2 (B) or 100 µM ILP2 B chain (C). Shown are the pairs of ovaries closest to the mean value from each treatment; the largest oocyte in each pair is circled in blue.

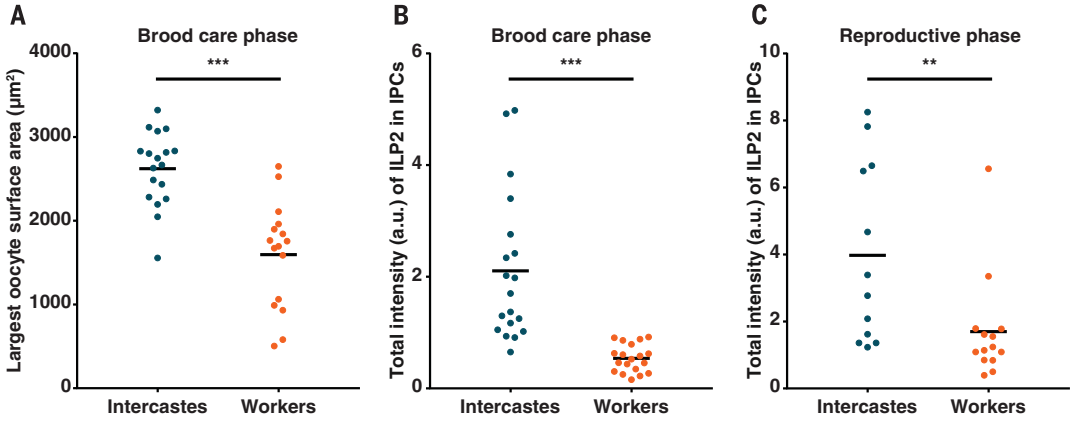

Fig. 4. Intercastes respond less to larvae and have more ILP2 than regular workers. (**A**) Intercastes have more active ovaries than age-matched regular workers in the brood care phase, despite both groups being in contact with larvae ($n \geq 16$, Welch's t test; ***$P < 0.0001$). (**B**) In the brood care phase ($n = 19$, Mann-Whitney U test; ***$P < 0.0001$) and (**C**) in the reproductive phase ($n \geq 12$, Mann-Whitney U test; **$P = 0.0043$), intercastes have more ILP2 in their IPCs than age-matched regular workers. Horizontal bars indicate means on all dot plots.

adults to emerge from the pupa with low nutritional stores and low ILP2 levels. These subfertile individuals would have been more sensitive to larval signals that suppress reproduction and would consequently have foregone nest founding and ovary activation and instead assumed brood care roles. Other adults, meanwhile, would have emerged with high nutritional stores and high ILP2 levels. These adults would have had reduced sensitivity to larval signals and would have been more likely to reproduce despite the presence of larvae. This reproductive asymmetry could then have been enhanced or modified by natural selection to ultimately produce the obligately reproductive queens and sterile workers of advanced eusocial species (fig. S14). This scenario constitutes an explicit molecular version of Mary Jane West-Eberhard's model for the evolution of hymenopteran eusociality (*5*).

REFERENCES AND NOTES

1. E. O. Wilson, *The Insect Societies* (Belknap Press, 1971).
2. W. M. Wheeler, *Ants: Their Structure, Development and Behavior* (Columbia Univ. Press, 1910).
3. J. Hunt, *The Evolution of Social Wasps* (Oxford Univ. Press, 2007).
4. J. Field, *Behav. Ecol.* **16**, 770–778 (2005).
5. M. J. West-Eberhard, *in Animal Societies: Theories and Facts*, Y. Itô, J. L. Brown, J. Kikkawa, Eds. (Japan Scientific Societies Press, 1987), pp. 35–51.
6. P. S. Ward, *Annu. Rev. Ecol. Evol. Syst.* **45**, 23–43 (2014).
7. R. Libbrecht, P. R. Oxley, L. Keller, D. J. C. Kronauer, *Curr. Biol.* **26**, 391–395 (2016).
8. S. Patalano *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **112**, 13970–13975 (2015).
9. Q. Li *et al.*, *Nat. Commun.* **5**, 4943 (2014).
10. J. Gospocic *et al.*, *Cell* **170**, 748–759.e12 (2017).
11. Materials and methods are available as supplementary materials.
12. M. Corona *et al.*, *PLOS Genet.* **9**, e1003730 (2013).
13. P. R. Oxley *et al.*, *Curr. Biol.* **24**, 451–458 (2014).
14. L. Badisco, P. Van Wielendaele, J. Vanden Broeck, *Front. Physiol.* **4**, 202 (2013).
15. M. Corona *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 7128–7133 (2007).
16. K.-A. Nilsen *et al.*, *J. Exp. Biol.* **214**, 1488–1497 (2011).
17. A. L. Toth, G. E. Robinson, *Trends Genet.* **23**, 334–341 (2007).
18. Y. Wang, S. V. Azevedo, K. Hartfelder, G. V. Amdam, *J. Exp. Biol.* **216**, 4347–4357 (2013).
19. M. R. Brown *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **105**, 5716–5721 (2008).
20. W. Trible, D. J. C. Kronauer, *J. Exp. Biol.* **220**, 53–62 (2017).
21. Y. Okada *et al.*, *J. Insect Physiol.* **56**, 288–295 (2010).
22. S. V. de Azevedo, K. Hartfelder, *J. Insect Physiol.* **54**, 1064–1071 (2008).
23. D. E. Wheeler, N. Buck, J. D. Evans, *Insect Mol. Biol.* **15**, 597–602 (2006).
24. S. A. Ament, M. Corona, H. S. Pollock, G. E. Robinson, *Proc. Natl. Acad. Sci. U.S.A.* **105**, 4226–4231 (2008).
25. S. Teseo, D. J. C. Kronauer, P. Jaisson, N. Châline, *Curr. Biol.* **23**, 328–332 (2013).
26. M. A. Riehle, Y. Fan, C. Cao, M. R. Brown, *Peptides* **27**, 2547–2560 (2006).
27. C. Géminard, E. J. Rulifson, P. Léopold, *Cell Metab.* **10**, 199–207 (2009).
28. R. Libbrecht, P. R. Oxley, D. J. C. Kronauer, bioRxiv 223255 [Preprint]. 22 November 2017. https://doi.org/10.1101/223255.
29. L. LaFever, D. Drummond-Barbosa, *Science* **309**, 1071–1073 (2005).
30. M. L. Borowiec *et al.*, bioRxiv 173393 [Preprint]. 8 August 2017. https://doi.org/10.1101/173393.
31. P. Oxley, V. Chandra, Social-evolution-and-behavior/insulin_signaling: Data and code: Social regulation of insulin signaling and the evolution of eusociality in ants, Version 1.1, Zenodo (2018); https://doi.org/10.5281/zenodo.1311222.

ACKNOWLEDGMENTS

We thank C. Zhao, H. Zebroski III, T. Tong, and the Rockefeller University Resource Centers for sequencing, peptide synthesis, and help with image analysis; M. Deyrup for assistance with ant collection; L. Olivos-Cisneros for dissecting brains; and

L. Vosshall, C. Bargmann, B. Chait, H. Yan, C. Desplan, and the entire Kronauer laboratory for helpful discussion. This is Clonal Raider Ant Project paper no. 9. **Funding:** This work was supported by grant 1DP2GM105454-01 from the NIH, a Searle Scholar Award, a Sinsheimer Scholar Award, a Hirschl/Weill-Caulier Trusts Award, a Klingenstein-Simons Award, a Pew Scholar Award, and an HHMI Faculty Scholar Award (D.J.C.K.); a Leon Levy Neuroscience Fellowship (P.R.O.); a Rockefeller University Women & Science Fellowship (I.F.-P.); and a Marie Curie International Outgoing Fellowship (PIOF-GA-2012-327992; R.L.). **Author contributions:** D.J.C.K., V.C., I.F.-P., and P.R.O. designed the study; S.K.M., I.F.-P., and V.C. performed fieldwork; P.R.O., V.C., R.L., S.K.M., I.F.-P., and A.L.R. performed genomic analyses; I.F.-P., V.C., A.L.R., R.L., and S.K.M. performed immunostains; V.C., A.L.R., I.F.-P., and P.R.O. performed pharmacological experiments; V.C., I.F.-P., and D.J.C.K. wrote the manuscript with feedback from all authors; and D.J.C.K. supervised the project. **Competing interests:** The authors declare no competing interests. **Data and materials availability:** Raw sequence data are available through NCBI (BioProject PRJNA472392); scripts are available on GitHub (*31*).

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/361/6400/398/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S14
Tables S1 to S6
References (*32*–*76*)

22 November 2017; resubmitted 1 April 2018
Accepted 30 May 2018
10.1126/science.aar5723

Science

Social regulation of insulin signaling and the evolution of eusociality in ants

Vikram Chandra, Ingrid Fetter-Pruneda, Peter R. Oxley, Amelia L. Ritger, Sean K. McKenzie, Romain Libbrecht and Daniel J. C. Kronauer

Science **361** (6400), 398-402.
DOI: 10.1126/science.aar5723

The benefits of being well fed

In eusocial insects, the vast majority of individuals sacrifice their reproductive potential to support the reproductive queen. Although this system has evolved repeatedly, there is still much debate surrounding its origin. Working with seven different species of ants, Chandra *et al.* used a transcriptomic approach to show that a single gene is consistently up-regulated in queens. This gene seems to confer reproductive status through integration with increased nutrition. In a clonal ant, larval signals disrupt this gene up-regulation, destabilizing the division of reproductive labor. Increasing levels of the associated peptide override these larval signals and establish eusociality.

Science, this issue p. 398