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Social and molecular regulation of division of labor in ants

présentée et soutenue publiquement par :

Romain LIBBRECHT

le 24 juin 2025

JURY

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Sommaire

Curriculum vitae

Romain LIBBRECHT

Insect Biology Research Institute (UMR 7261)
CNRS / University of Tours
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Academic record

2012	**PhD in life sciences** University of Lausanne, Switzerland
2007	MSc in Ecology, Ethology, Evolution - with Honors University of Rennes, France
2007	Engineering diploma in biology and agronomy Ecole Nationale Supérieure d'Agronomie de Rennes, France

Research experience

2023-present	**CNRS researcher** (*Chargé de Recherche*) Insect Biology Research Institute (UMR 7261) CNRS / University of Tours, France
2016-2022	Assistant professor / Group leader Institute of Organismic and Molecular Evolution Johannes Gutenberg University of Mainz, Germany
2015-2016	Marie Curie postdoctoral fellow - return phase Department of Ecology and Evolution - Supervisor: Laurent Keller University of Lausanne, Switzerland
2013-2015	Marie Curie postdoctoral fellow - outgoing phase Laboratory of Social Evolution and Behavior - Supervisor: Daniel Kronauer The Rockefeller University, New York City, USA
2012	Postdoctoral researcher Department of Ecology and Evolution - Supervisor: Laurent Keller University of Lausanne, Switzerland (3 months)
2007-2012	PhD student Department of Ecology and Evolution - Supervisor: Laurent Keller University of Lausanne, Switzerland
2007	Master student Laboratory of Ecobiology - Supervisor: David Renault University of Rennes, France (6 months)
2005-2006	Internship Laboratory of Applied Ecology - Supervisor: Mark Fellowes University of Reading, UK (6 months)

Research grants

2024	ANR JCJC (*Agence Nationale de la Recherche*) - ANTOGENY (229,135€)
2024	OFB (*Office Français de la Biodiversité*) - FIVALO (138,292€, co-applicant: JL. Mercier)
2024	SSBCV doctoral school - PhD fellowship (ca. 125,000€, co-applicant: M. Goubault)
2020	DFG (German Science Foundation) - Research Grant LI3051/8-1 (351,750€, co-applicants: S. Foitzik and I. Scharf)
2019	DFG (German Science Foundation) - GenEvo Research Training Group (5,000,000€, 1 of 12 PIs in the consortium, ca. 340,000€ per PI)
2018	DFG (German Science Foundation) - Research Grant LI3051/3-1 (245,795€, co-applicants: S. Foitzik and V. Nehring)
2017	DFG (German Science Foundation) - Research Grant LI3051/2-1 (223,125€)
2017	Impulsfonds Grant (15,000€, co-applicant: R. Ketting)
2016	JGU Mainz Intra-University Grant (10,525€)
2013	Marie Skłodowska-Curie International Outgoing Fellowship (264,000€)
2013	Swiss National Science Foundation early.postdoc Fellowship (70,000€ - Declined)

Publications

35 publications, 1963 citations, h-index = 20.
(underlined names indicate students that I have supervised)

1. Lenhart A, Majoe M, Selvi S, Colgan TJ, **Libbrecht R** & Foitzik S (2025) Worker survival and egg production - but not transcriptional activity - respond to queen number in the highly polygynous, invasive ant *Tapinoma magnum*. **Molecular Ecology**, 34(6), e17679. doi.org/10.1111/mec.17679
2. Majidifar V, Psalti M, Coulm M, Fetzer E, Teggers E, Rotering F, Grünewald J, Mannella L, Reuter M, Unte D & **Libbrecht R** (2024) Ontogeny of superorganisms: Social control of queen specialization in ants. **Functional Ecology** 38: 1044-1060. doi.org/10.1111/1365-2435.14536
3. Jaimes-Nino LM, Bar A, Subach A, Stoldt M, **Libbrecht R**, Scharf I & Foitzik S (2024) Transcriptomic signature of spatial navigation in brains of desert ants. **Ecology and Evolution** 14: e70365**.** doi.org/10.1002/ece3.70365
4. Majoe M, Stolarek N, Vizueta J, Xiong Z, Schrader L, Boomsma JJ, Foitzik S, **Libbrecht R** & Nehring V (2024) Queen loss fails to elicit physiological and transcriptional responses in workers of the invasive garden ant *Lasius neglectus*. bioRxiv. doi.org/10.1101/2024.01.26.577224
5. Caminer MA, **Libbrecht R**, Majoe M, Ho DV, Baumann P & Foitzik S (2023) Task-specific patterns of odorant receptor expression in worker antennae indicates a sensory filter regulating division of labor in ants. **Communications Biology** 6: 1004. doi.org/10.1038/s42003-023-05273-4
6. Sistermans T, Hartke J, Stoldt M, **Libbrecht R** & Foitzik S (2023). The influence of parasite load on transcriptional activity and morphology of a cestode and its ant intermediate host. **Molecular Ecology**, 32: 4412-4426. doi.org/10.1111/mec.16995
7. Mier P, Fontaine JF, Stoldt M, **Libbrecht R**, Martelli C, Foitzik S & Andrade-Navarro MA (2022) Annotation and analysis of 3902 odorant receptor protein sequences from 21 insect species provide insights into the evolution of odorant receptor gene families in solitary and social Insects. **Genes** 13: 919. doi.org/10.3390/genes13050919

8. Psalti M, Gohlke D & **Libbrecht R** (2021) Experimental increase of worker diversity benefits brood production in ants. **BMC Ecology and Evolution** 21:163. doi.org/10.1186/s12862-021-01890-x
9. Teggers E, Deegener F & **Libbrecht R** (2021) Fecundity determines the outcome of founding queen associations in ants. **Scientific Reports** 11: 2986. doi.org/10.1038/s41598-021-82559-9
10. Scharf I, Stoldt M, **Libbrecht R**, Höpfner AL, Jongepier E, Kever M & Foitzik S (2021) Social isolation causes downregulation of immune genes and behavioral changes in a social insect. **Molecular Ecology** 30: 2378-2389. doi.org/10.1111/mec.15902
11. Majoe M, **Libbrecht R**, Foitzik S & Nehring V (2021) Queen loss increases worker survival in leaf-cutting ants under paraquat-induced oxidative stress. **Philosophical Transactions of the Royal Society B** 376: 20190735. doi.org/10.1098/rstb.2019.0735
12. Gilad T, Dorfman A, Subach A, **Libbrecht R**, Foitzik S & Scharf I (2021) Evidence for the effect of brief exposure to food, but not learning interference, on maze solving in desert ants. **Integrative Zoology** 17: 704. doi.org/10.1111/1749-4877.12622
13. Korb J, Meusemann K, Aumer D, Bernadou A, Elsner D, Feldmeyer B, Foitzik S, Heinze J, **Libbrecht R**, Lin S, Majoe M, Monroy Kuhn JM, Nehring V, Negroni M, Paxton RJ, Séguret AC, Stoldt M & Flatt T (2021) Comparative transcriptomic analysis of the mechanisms underpinning ageing and fecundity in social insects. **Philosophical Transactions of the Royal Society B** 376: 20190728. doi.org/10.1098/rstb.2019.0728
14. Kramer B, Nehring V, Buttstedt A, Heinze J, Korb J, **Libbrecht R**, Meusemann K, Paxton R, Séguret A & Bernadou A (2021) Oxidative stress and senescence in social insects - a significant but inconsistent link? **Philosophical Transactions of the Royal Society B** 376: 20190732. doi.org/10.1098/rstb.2019.0732
15. Choppin M, Graf S, Feldmeyer B, **Libbrecht R**, Menzel F & Foitzik S (2021) Queen and worker phenotypic traits are associated with colony composition and environment in *Temnothorax rugatulus* (Hymenoptera: Formicidae), an ant with alternative reproductive strategies. **Myrmecological News** 31: 61-69. doi.org/10.25849/myrmecol.news_031:061
16. Körner M, Vogelweith F, **Libbrecht R**, Foitzik S, Feldmeyer B & Meunier J (2020) Offspring reverse transcriptome responses to maternal deprivation when reared with pathogens in an insect with facultative family life. **Proceedings of the Royal Society B** 287: 20200440. doi.org/10.1098/rspb.2020.0440
17. **Libbrecht R**, Nadrau D & Foitzik S (2020) A role of histone acetylation in the regulation of circadian rhythm in ants. **iScience** 23: 100846. doi.org/10.1016/j.isci.2020.100846 (cover of March 2020 issue)
18. Psalti M & **Libbrecht R** (2020) Caste differentiation: Ants. Book chapter in **Encyclopedia of Social Insects**. Springer International Publishing.
19. Kohlmeier P, Alleman AR, **Libbrecht R**, Foitzik S & Feldmeyer B (2019) Gene expression is more strongly associated with behavioural specialisation than with age and fertility in ant workers. **Molecular Ecology** 28: 658-670. doi.org/10.1111/mec.14971
20. **Libbrecht R**, Oxley PR & Kronauer DJC (2018) Clonal raider ant brain transcriptomics identifies candidate molecular mechanisms for reproductive division of labor. **BMC Biology** 16: 89. doi.org/10.1186/s12915-018-0558-8
21. Chandra V, Fetter-Pruneda I, Oxley PR, Ritger A, McKenzie S, **Libbrecht R** & Kronauer DJC (2018) Social regulation of insulin signaling and the evolution of eusociality in ants. **Science** 361: 398-402. doi.org/10.1126/science.aar5723

22. Kronauer DJC & **Libbrecht R** (2018) Back to the roots: the importance of using simple insect societies to understand the molecular basis of complex social life. **Current opinion in insect science** 28: 33-39. doi.org/10.1016/j.cois.2017.12.004
23. Weitekamp CA, **Libbrecht R** & Keller L (2017) Genetics and evolution of social behavior in insects. **Annual Review of Genetics** 51: 219-239. doi.org/10.1146/annurev-genet-120116-024515
24. **Libbrecht R**, Oxley PR, Keller L & Kronauer DJC (2016) Robust DNA methylation in the clonal raider ant brain. **Current Biology** 26: R391-R395. doi.org/10.1016/j.cub.2015.12.040
25. Corona M, **Libbrecht R** & Wheeler D (2016) Molecular mechanisms of phenotypic plasticity in social insects. **Current opinion in insect science** 13: 55-60. doi.org/10.1016/j.cois.2015.12.003
26. Ulrich Y, Burns D, **Libbrecht R** & Kronauer DJC (2016) Ant larvae regulate worker foraging behavior and ovarian activity in a dose-dependent manner. **Behavioral Ecology and Sociobiology** 70: 1011-1018. doi.org/10.1007/s00265-015-2046-2
27. **Libbrecht R** & Keller L (2015) The making of eusociality: insights from two bumblebee genomes. **Genome Biology** 16: 75 – Dispatch. doi.org/10.1186/s13059-015-0635-z
28. **Libbrecht R** & Kronauer D (2014) Convergent evolution: the genetics of queen number in ants. **Current Biology** 24: R1083-R1085 – Dispatch. doi.org/10.1016/j.cub.2014.09.066
29. Schwander T, **Libbrecht R** & Keller L (2014) Supergenes and complex phenotypes. **Current Biology** 24: R288-R294. doi.org/10.1016/j.cub.2014.01.056
30. Corona***** M, **Libbrecht* R**, Wurm Y, Riba-Grognuz O, Studer RS & Keller L (2013) Vitellogenin underwent subfunctionalization to acquire caste and behavioral specific expression in the harvester ant *Pogonomyrmex barbatus*. **Plos Genetics** 9: e1003730 (*contributed equally). doi.org/10.1371/journal.pgen.1003730
31. **Libbrecht R**, Oxley PR, Kronauer DJC & Keller L (2013) Ant genomics sheds light on the molecular regulation of social organization. **Genome Biology** 14:212-219. doi.org/10.1186/gb-2013-14-7-212
32. **Libbrecht R**, Corona M, Wende F, Azevedo D, Serrao J & Keller L (2013) Interplay between insulin signaling, juvenile hormone and vitellogenin regulates maternal effects on caste polyphenism in ants. **PNAS** 110: 11050-11055. doi.org/10.1073/pnas.1221781110
33. **Libbrecht R** & Keller L (2013) Genetic compatibility affects division of labor in the Argentine ant *Linepithema humile*. **Evolution** 67: 517-524. doi.org/10.1111/j.1558-5646.2012.01792.x
34. **Libbrecht R**, Schwander T & Keller L (2011) Genetic components to caste allocation in a multiple-queen ant species. **Evolution** 65: 2907-2915. doi.org/10.1111/j.1558-5646.2011.01348.x
35. **Libbrecht R**, Gwynn DM & Fellowes MDE (2007) *Aphidius ervi* preferentially attacks the green morph of the pea aphid, *Acyrthosiphon pisum*. **Journal of Insect Behavior** 20: 25-32. doi.org/10.1007/s10905-006-9055-y

Academic awards

2017	Young investigator award of the French section of the International Union for the Study of Social Insects (IUSSI)
2014	Jasper Loftus-Hills young investigator award of the American Society of Naturalists
2013	Faculty award for best PhD thesis – University of Lausanne
2011	Best poster award – Doctoriales de l'Unil

Student supervision

Postdocs (2)

09/2023-present Luisa Jaimes – Molecular basis of learning in ants (Johannes Gutenberg University of Mainz, co-supervised with S. Foitzik and I. Scharf)

01/2022-08/2023 Marah Stoldt – Molecular basis of learning in ants (Johannes Gutenberg University of Mainz, co-supervised with S. Foitzik and I. Scharf)

PhD students (9)

10/2024-present Hugo Le Lay – Social and molecular regulation of queen specialization in ants (University of Tours, co-supervised with J. Meunier)

10/2024-present Alice Roux – Impact of thermal stress on colony foundation in ants (University of Tours, co-supervised with M. Goubault and I. Villalta)

07/2022-present Maximilian Bolder – The ontogenic process of colony foundation in ants (Johannes Gutenberg University of Mainz, co-supervised with J. Colgan)

09/2021-present Anna Lenhart – Impact of social environment on aging in ants (Johannes Gutenberg University of Mainz, co-supervised with S. Foitzik)

10/2019-present Martin Coulm – Epigenetic regulation of reproduction in ants (Johannes Gutenberg University of Mainz, co-supervised with R. Ketting)

10/2019-11/2023 Valentine Patterson – Regulation of allele-specific expression in hybrid lizard species (Johannes Gutenberg University of Mainz, co-supervised with P. Baumann)

10/2018-05/2023 Megha Majoe – Reversal of the fecundity / longevity trade-off across social transitions in ants (Johannes Gutenberg University of Mainz, co-supervised with S. Foitzik and V. Nehring)

05/2018-07/2022 Marina Psalti – Consistency and mechanisms of genetic effects on behavior in ants (Johannes Gutenberg University of Mainz)

03/2020-12/2020 Vahideh Majidifar – Social control of worker reproduction in ants (PhD student at the University of Tehran (Iran), 10-month research stay in my research group in Mainz, Germany)

MSc students (M2 or equivalent) (11)

02/2025-07/2025 Léa Droesbeke-Fuente (co-supervised with C. Lucas)

01/2024-06/2024 Tania Tyczynski (co-supervised with I. Villalta)

10/2021-08/2022 Nadine Krämer

10/2021-10/2022 Dogan Barut

08/2020-08/2021 Ebru Fetzer

01/2020-07/2020 Simon de Wever

10/2019-08/2020 Anna Lenhart (co-supervised with S. Foitzik)

09/2018-12/2019 Anna Pfeiffer (co-supervised with C. Grueter)

09/2018-07/2019 Thomas Wagner

08/2018-06/2019 Manuela Beyer (co-supervised with R. Ketting)

04/2017-02/2018 Dennis Nadrau (co-supervised with S. Foitzik)

BSc and M1 students (26)

02/2025-07/2025	Eline Hiron (co-supervised with JL. Mercier)
10/2024-01/2025	Delia Delgado Denis
03/2024-04/2024	Titouan Le Floch
05/2022-09/2022	Anika Zimmermann
09/2021-04/2022	Nathalie Mathee
09/2021-04/2022	Maximilian Simon
09/2021-04/2022	Alexander Buchert
12/2020-08/2021	Hannes Binder
08/2020-02/2021	Maxi Reuter
08/2020-03/2021	Luca Mannella
07/2020-01/2021	Falk Deegener
10/2019-05/2020	Dustin Gohlke
11/2018-06/2019	Johanna Schulz
11/2018-05/2019	Sascha Schlueter
10/2018-04/2019	Lukas Brink
09/2018-05/2019	Frederik Rotering
10/2017-03/2018	Jana Huschka
10/2017-03/2018	Joshua Klein
10/2017-03/2018	Eric Walldorf
06/2017-11/2017	Eva Teggers
05/2017-10/2017	Eva Erb
10/2016-03/2017	Jonas Barkhau
10/2016-03/2017	Sebastian Rauch
10/2016-03/2017	Sophie Steinhausen
06/2014-08/2014	Dominic Burns (co-supervised with Y. Ulrich)
02/2011-04/2011	Elodie Gaide (co-supervised with L. Keller)

Teaching experience

University of Tours (France)

2024	Scientific literature review (practical, 10 hrs/year, BSc level)

Johannes Gutenberg University of Mainz (Germany)

2020-2022	Epigenetic gene regulation in evolution (lecture, 3 hrs/year, PhD level)
2016-2022	Social Behaviors (lecture, 3 hrs/year, MSc level)
2016-2022	Insect Reproductive Behaviors (lecture, 2 hrs/year, BSc level)
2016-2022	Experimental design (lecture, 6 hrs/year, BSc and MSc level)
2016-2022	Introduction to Statistics (lecture, 6 hrs/year, BSc and MSc level)
2016-2022	Sociogenomics (lecture, 2 hrs/year, MSc level)
2016-2022	Statistics with R (practical, 14 hrs/year, BSc and MSc level)
2016-2022	Transcriptomics with R (practical, 6 hrs/year, MSc level)
2016-2022	Insect determination (practical, 8 hrs/year, BSc level)
2016, 18, 22	Excursion in the Alps on plant-pollinator interactions (1 week/year, BSc level)
2019	Experimental design for gene expression studies (lecture, 2 hrs/year, PhD level)
2019	Best practice in gene expression analyses (lecture, 4 hrs/year, PhD level)

University of Lausanne (Switzerland)

2007-2012	Statistics (practical, 40 hrs/year, BSc level)
2009-2012	Marine Ecology master courses in Roscoff, France (2 weeks/year, MSc level)
2009-2011	Introduction to R doctoral course (practical, 20 hrs/year, PhD level)
2009	Mycology (practical, 15 hrs/year, BSc level)
2008-2009	Zoology (practical, 15 hrs/year, BSc level)

Administrative duties

2025-present	International research correspondent of the IRBI
2025-present	Organization of the ESORE team meetings and journal clubs
2024-present	Elected member of the *Conseil de Gestion* of the IRBI
2024-present	Elected member of the *Commission Scientifique Disciplinaire Paritaire (sections 67-68)* of the University of Tours
2023-present	Organization of the IRBI institute seminars

HDR committee member

Jonathan Romiguier (2023) University of Montpellier, France

PhD defense committee member

Marcel Caminer (2024) Johannes Gutenberg University of Mainz, Germany

Thibaut Renard (2023) University of Brussels, Belgium

Nathan Lecocq (2023) University of Brussels, Belgium

Marina Choppin (2022) Johannes Gutenberg University of Mainz, Germany

Anissa Kennedy (2022) Johannes Gutenberg University of Mainz, Germany

Marah Stoldt (2022) Johannes Gutenberg University of Mainz, Germany

Simone Glaser (2021) Johannes Gutenberg University of Mainz, Germany

Juliane Hartke (2021) Senckenberg Institute, Frankfurt, Germany

Philipp Sprenger (2020) Johannes Gutenberg University of Mainz, Germany

Tianfei Peng (2019) Johannes Gutenberg University of Mainz, Germany

Kishor Dhaygude (2019) University of Helsinki, Finland

Maximilian Körner (2019) Johannes Gutenberg University of Mainz, Germany

Matteo Negroni (2019) Johannes Gutenberg University of Mainz, Germany

Austin Alleman (2019) Johannes Gutenberg University of Mainz, Germany

PhD thesis follow-up committee member
(CSI or equivalent)

Simon De Wever (2024) University of Tours, France

Sascha Schlüter (2024) Johannes Gutenberg University of Mainz, Germany

Laura Pasquier (2023, 2024) University of Tours, France

Maïly Kervalla (2021, 2022) University of Strasbourg, France

Jürgen Wierz (2021, 2022) Johannes Gutenberg University of Mainz, Germany

Marah Stoldt (2021) Johannes Gutenberg University of Mainz, Germany

David Ho (2019, 2020) Johannes Gutenberg University of Mainz, Germany

Editorial activity

2023-present Associate Editor for Royal Society Open Science (The Royal Society Publishing)

Peer reviewing activity for scientific journals

Science
Current Biology
PNAS
Plos Biology
Genome Biology
Nature Ecology and Evolution
Evolution
Proceedings of the Royal Society B
Molecular Biology and Evolution
Genome Biology and Evolution
Molecular Ecology
Developmental Cell
Cell Reports
Communications biology
Journal of Experimental Biology
GigaScience
Plos Genetics
Plos One
Epigenetics
Animal Behaviour
Scientific Reports
Behavioral Ecology and Sociobiology
Biological Journal Linnean Society
Evolutionary Ecology
Biology letters
Journal Experimental Zoology Part B
BMC Ecology
Insect Molecular Biology
Molecular Ecology Resources
Insects
Frontiers in Ecology and Evolution
Frontiers in Zoology
Journal of Insect Physiology
EvoDevo
Chemoecology
Journal of Molecular Sciences
PCI Ecology
Physiological Entomology
Myrmecological News
Insectes Sociaux

Peer reviewing activity for funding agencies

Society for the Study of Evolution (SSE)
Human Frontier Science Program (HFSP)
Swiss National Science Foundation (SNF)
German Research Foundation (DFG)
Israel Science Foundation
National Hellenic Research Foundation

Membership of scientific societies

2008-present Member of the IUSSI French section (elected board member since 2023)
2008-present Associate Faculty Member of *Faculty of 1000*
2013-2015 Member of the American Society of Naturalists
2008-2012 Member of the Swiss Zoological Society

Organization of scientific meetings

2021 Co-organizer of the Aging in Social Insects meeting (60 participants), Ingelheim (Germany)
2015 Organization team of the ESEB meeting (1400 participants), Lausanne (Switzerland)
2013 Co-organizer of the SINNERS meeting (50 participants), New York City *(*USA)
2008 Co-organizer of SeeDS (100 participants), Lausanne (Switzerland)

Oral presentations

2024	CNRS Section 26 conference in Marseille, France - Invited
2024	IUSSI European conference in Lausanne, Switzerland
2023	GenEvo Seminar in Mainz, Germany - Invited
2023	LEEC Seminar in Villetaneuse, France - Invited
2023	ECE (European Congress of Entomology) in Heraklion, Crete
2023	IUSSI French Section in Toulouse, France
2023	SFECA in Tours, France
2022	IUSSI International conference in San Diego, USA
2021	IUSSI European conference, Online – Invited
2021	Aging in Social Insects conference in Ingelheim, Germany – Invited
2021	EVOLUTION conference, Online
2021	Biology of Social Insects at Cold Spring Harbor Laboratory, Online
2020	Frontiers in Social Evolution Seminar Series, Online – Invited
2019	IUSSI Central Europe section in Vienna, Austria
2018	CRCA symposium in Toulouse, France – Invited (plenary)
2018	IUSSI International conference in Guaruja, Brazil
2017	IUSSI French section in Paris, France – Invited
2015	IUSSI French section in Tours, France
2015	ESEB conference in Lausanne, Switzerland
2015	Biology of Social Insects at Cold Spring Harbor Laboratory, USA
2014	Ecology and Evolution Seminar at Rutgers University, USA – Invited
2014	IUSSI International conference in Cairns, Australia
2014	EVOLUTION conference in Raleigh, USA – Invited
2012	IUSSI European conference in Florence, Italy
2011	SeeDS conference in Neuchâtel, Switzerland
2011	CIG Symposium “Genetics of behavior” in Lausanne, Switzerland
2011	IUSSI French section in Banyuls, France
2011	Biology11 conference in Zürich, Switzerland
2010	IUSSI International conference in Copenhagen, Denmark
2010	Biology10 conference in Neuchâtel, Switzerland

General-public presentations

2025	Podcast of the association *SFECA*
2024	*Fête de la Science*, presentation of the biology of ants – Tours, France
2024	Multiple interviews (Radio France, Le Figaro, Ouest France, El Pais, etc.) for the publication of our article on the specialization of ant queens
2023	4-day workshop on the biology of ants for elementary school students – Tours, France
2014	Science Saturday, presentation of the biology of ants – Rockefeller University, USA
2011	Conference for the association *Cercle des sciences naturelles* – Vevey, Switzerland
2011	Conference for the association *Paroisse protestante de Vevey* – Vevey, Switzerland
2010-2015	Conferences for the association *Connaissance3* – Switzerland
2008	Interview for the documentary “The ant’s best friend”, RTS - Science Suisse Project
2007-2012	Presentations of the biology of ants to school classes – University of Lausanne
2007-2015	Presentations of the biology of ants for open days – University of Lausanne

Mémoire

A key question in biology is to understand how and why organisms adjust their reproduction, physiology and behavior in response to environmental conditions. This ability to produce several phenotypes from a single genotype, called phenotypic plasticity, is best exemplified by the fascinating diversity in morphology and behavior that can be observed in colonies of social Hymenoptera, such as those of ants, bees and wasps (Wilson, 1971). These insect societies show a division of labor between queens that monopolize egg production and workers that specialize in performing all the other tasks necessary to maintain the colony. These non-reproductive tasks include foraging for food, building and defending the nest, and providing care to eggs and larvae (Wilson, 1971). Such reproductive division of labor between the queen and worker castes is central to the functioning, ecological success and evolution of social insects.

In this context, it is important to understand the factors and mechanisms that regulate reproductive division of labor in social insect colonies. Research on this topic can be broadly categorized into two main approaches. The first approach, focusing on caste determination and differentiation, provided key insights into the regulatory processes of the alternative developmental trajectories that lead to the production of queens and workers (Cameron et al., 2013; Collins et al., 2020; Corona et al., 2016; Genzoni et al., 2023; Libbrecht et al., 2011; Libbrecht et al., 2013a; Libbrecht et al., 2013b; Montagna et al., 2015; Mutti et al., 2011; Psalti & Libbrecht, 2020; Schultner et al., 2023; Schwander et al., 2008; Schwander & Keller, 2008; Wheeler et al., 2006). The second approach, based on comparisons of queens and workers at the adult stage, identified a range of caste-specific phenotypic and molecular differences that likely regulate variation in reproductive activity (Bonasio et al., 2012; Chandra et al., 2018; Corona et al., 2007, 2013; Feldmeyer et al., 2014; Grozinger et al., 2007; Kronauer & Libbrecht, 2018; Libbrecht et al., 2013b; Patalano et al., 2015). **Part 1** of this thesis describes how I have implemented both approaches to better understand the genetic and maternal effects on caste determination, and to identify molecular pathways that regulate reproductive variation between queens and workers in ants.

Although the morphological differences between queens and workers are fixed at the adult stage in social Hymenoptera, modifications of the social environment can affect individual physiology and behavior, and thus further refine division of labor among colony members. The most documented example is the profound effects of queen loss on the reproductive activity and aggressive behavior of workers (Choppin et al., 2021; Heinze, 2008; Holman et al., 2010; Holman, 2014; Holman et al., 2016; Negroni et al., 2021; Ronai et al., 2016; Van Oystaeyen et al., 2014). However, other colony members may also influence their social partners in various ways, as

illustrated by larvae and/or pupae affecting worker reproduction and behavior (Ulrich et al., 2016; Villalta et al., 2015), as well as brood survival and development (Santos et al., 2024; Snir et al., 2022). **Part 2** exposes my research on interrogating how the social context shapes phenotypic variation in reproduction, behavior and longevity, as well as its influence on colony efficiency in ants.

While many studies have focused on the effects of the social context on worker behavior and physiology, relatively little is known about its impact on the behavior of queens, whose role is typically assumed to be reduced to reproduction. This bias in the literature has reinforced the widely accepted notion that queens are intrinsically specialized in egg production once they reach the adult stage, and that this robust specialization is independent of environmental conditions. However, the maturation process of social insect queens is not fully completed at the time of their emergence as adults. This is best exemplified by the behavior of queens in the process of establishing their colonies, where founding queens express a broad repertoire of both reproductive and non-reproductive behaviors to produce the first generation of workers (Augustin et al., 2011; Brossette et al., 2019; Cassill, 2002; Majidifar et al., 2024; Norman et al., 2016; Walsh et al., 2018; Wheeler, 1933; Woodard et al., 2013). Only then do queens stop expressing non-reproductive behaviors to become strictly specialized in egg production (Chouvenc, 2022; Majidifar et al., 2024; Woodard et al., 2013). **Part 3** elaborates on my ongoing work on the social and molecular mechanisms that control the specialization of pluripotent founding queens and the maintenance of this specialization in established ant colonies.

Part 1 – The making of queens and workers

The reproductive division of labor in social Hymenoptera is a fundamental aspect of colony organization and a striking example of phenotypic plasticity. Understanding the variation in reproductive activity between queens and workers calls for investigations of both the mechanisms that determine caste fate during larval development and the molecular pathways that regulate reproductive differences between adult queens and workers.

1.1 – Intergenerational effects on caste determination

To understand reproductive division of labor in social Hymenoptera colonies requires elucidating the factors and mechanisms that determine whether an egg develops into a reproductive queen or a functionally sterile worker. Extensively studied in bees and ants, caste determination was long considered to be strictly environmental, with factors such as nutrition and temperature experienced during larval development deciding the caste of the individual (Corona et al., 2016; Psalti & Libbrecht, 2020; Schwander et al., 2010). Thus, the female eggs of social Hymenoptera were thought to be totipotent, with the same likelihood of developing into queens or workers. However, since the early 2000s, several studies have revealed genetic (Hartfelder et al., 2006; Hughes & Boomsma, 2008; Libbrecht et al., 2011; Schwander & Keller, 2008; Smith et al., 2008) and maternal (Libbrecht et al., 2013a; Schultner et al., 2023; Schwander et al., 2008) effects on caste determination, particularly in ants. Some eggs, therefore, appear to have a higher likelihood of developing into queens than others from the moment they are laid. As a PhD student at the University of Lausanne (Switzerland), I investigated the mechanisms underlying genetic and maternal effects on caste determination in ants.

Complex genetic effects influence caste determination

Libbrecht et al. (2011) Genetic components to caste allocation in a multiple-queen ant species. **EVOLUTION** (Appendix 1)

Most studies that revealed genetic effects on caste determination in social insects have focused on species whose colonies contain a single queen, mated with multiple males (Weitekamp et al., 2017). Under these conditions, all female eggs share the same mother but not necessarily the same father. A group of eggs or individuals sharing the same father is referred to as a patriline. Differences among patrilines in the relative proportions of queens and workers have been interpreted as evidence of genetic effects on caste determination (Hughes & Boomsma, 2008; Smith et al., 2008). Such genetic influences have traditionally been considered to be additive genetic effects resulting from simple allelic differences. However, comparisons between patrilines could not distinguish such additive effects from more complex ones, such as those that depend on which parent the allele comes

from. To better understand the nature of genetic effects, it is essential to quantify the relative contributions of both parents to the trait of interest (Schwander & Keller, 2008).

To this end, we used the Argentine ant (*Linepithema humile*), which offers the rare opportunity for controlled crosses in the laboratory, to test the influence of the paternal and maternal lineages on the proportion of queens and workers produced (Libbrecht et al., 2011). We found that the paternal lineage affected the relative production of queens and workers, while no significant effect of the maternal lineage was detected. Finding such parent-of-origin-specific effects reveals that the genetic effects on caste determination in *L. humile* have a complex architecture, as classic additive effects would imply an influence of both parental lineages. Our findings are thus inconsistent with simple allelic differences, and suggest the implication of more complex genetic mechanisms, such as epigenetic factors, on caste determination.

Juvenile hormone regulates maternal effects on caste determination

Libbrecht et al. (2013) Interplay between insulin signaling, juvenile hormone, and vitellogenin regulates maternal effects on polyphenism in ants. **PNAS** (Appendix 2)

Ant colonies start producing sexual offspring (new queens and males) only after several years. Artificial hibernation experiments in the harvester ant (*Pogonomyrmex rugosus*) demonstrated that only colonies that have undergone at least one hibernation produce new queens. Furthermore, this impact of hibernation stems from maternal effects: exposing the queen to cold was both necessary and sufficient to stimulate the production of new queens, regardless of the hibernation status of the workers (Schwander et al., 2008).

To better understand these maternal effects on caste determination, we conducted a series of experiments to study the molecular changes in the queens that made them more likely to produce new queens in response to hibernation (Libbrecht et al., 2013a). By combining hormonal treatments, experimental exposure to cold, and gene expression measurements (via quantitative RT-PCR), we experimentally demonstrated the involvement of juvenile hormone in regulating the maternal effects on caste determination in *P. rugosus*. Hormonal treatments with a synthetic analog of juvenile hormone (methoprene) were able to

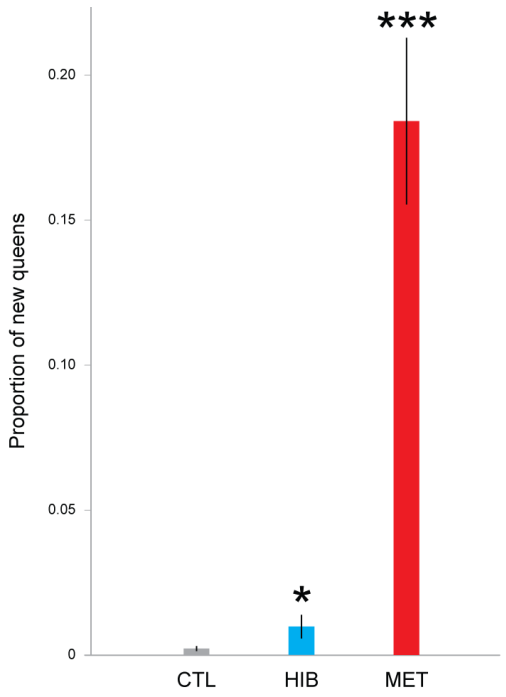

Figure 1. The proportion of queens among the offspring produced (mean±se) was increased in hibernation (HIB, n=25) and methoprene (MET, n=25) treatments compared with control (CTL, n=26) (*P<0.05; ***P<0.001).

successfully replicate the effects of hibernation, both phenotypically (increased production of new queens, Figure 1) and molecularly (similar effects on gene expression and the protein composition of eggs). Based on these results, we proposed a model involving juvenile hormone, insulin signaling and vitellogenin pathways in the regulation of maternal effects on caste determination in *P. rugosus*.

1.2 – Molecular pathways regulating queen reproduction

To understand the reproductive division of labor requires investigating the molecular mechanisms that regulate the variation in reproductive activity between queens and workers. The most common approach to address this question is the comparison of adult queens and workers in mature colonies of social Hymenoptera (Bonasio et al., 2012; Chandra et al., 2018; Corona et al., 2007, 2013; Feldmeyer et al., 2014; Grozinger et al., 2007; Kronauer & Libbrecht, 2018; Libbrecht et al., 2013b; Patalano et al., 2015). These investigations revealed the central roles of the juvenile hormone, vitellogenin and insulin signaling pathways, not only in regulating variation in reproductive activity among colony members, but also in the evolution of reproductive division of labor in social insects (Chandra et al., 2018; Corona et al., 2007, 2013; Grozinger et al., 2007; Kronauer & Libbrecht, 2018; Libbrecht et al., 2013b).

My research work included such comparisons of queens and workers, which contributed to these advances in our mechanistic understanding of division of labor in ants. First, as a PhD student, I investigated the function of vitellogenin in regulating reproduction and behavior (Corona et al., 2013). Second, as a Marie-Curie postdoctoral fellow at the Rockefeller University (New York City, USA), I contributed to a project exploring the central role of the insulin signaling pathway in controlling reproductive variation in ants (Chandra et al., 2018).

Vitellogenin genes perform distinct, caste-specific functions

Vitellogenin is a key protein in insect reproduction, serving primarily as the energy source for the embryo within the developing egg (Hagedorn & Kunkel, 1979). Research on honeybees showed that this protein also has behavioral functions in adult workers (Amdam et al., 2003; Corona et al., 2007). In ants, the gene encoding this protein has undergone duplications, and many ant species possess multiple copies, the functions of which remain largely unknown.

Corona*, **Libbrecht*** et al. (2013) Vitellogenin underwent subfunctionalization to acquire caste and behavioral specific expression in the harvester ant *Pogonomyrmex barbatus*. **PLOS GENETICS** (*contributed equally) (Appendix 3)

To investigate whether *vitellogenin* copies have different functions in ants, we studied the expression of two *vitellogenin* genes (*PbVg1* and *PbVg2*) in queens, nurses, and foragers of the harvester ant (*Pogonomyrmex barbatus*) (Corona et al., 2013). We found that the expression of *PbVg1*, which was higher in queens than in workers, aligns with the ancestral role of vitellogenin in reproduction (Figure 2). However, our results indicate that *PbVg2*, which was expressed at higher levels in foragers than in nurses or queens (Figure 2), has been co-opted to perform non-reproductive behavioral functions. This finding suggests that in *P. barbatus*, the *vitellogenin* gene underwent subfunctionalization after duplication (Lynch & Force, 2000) to acquire caste- and behavior- specific expression associated with reproductive and non-reproductive functions.

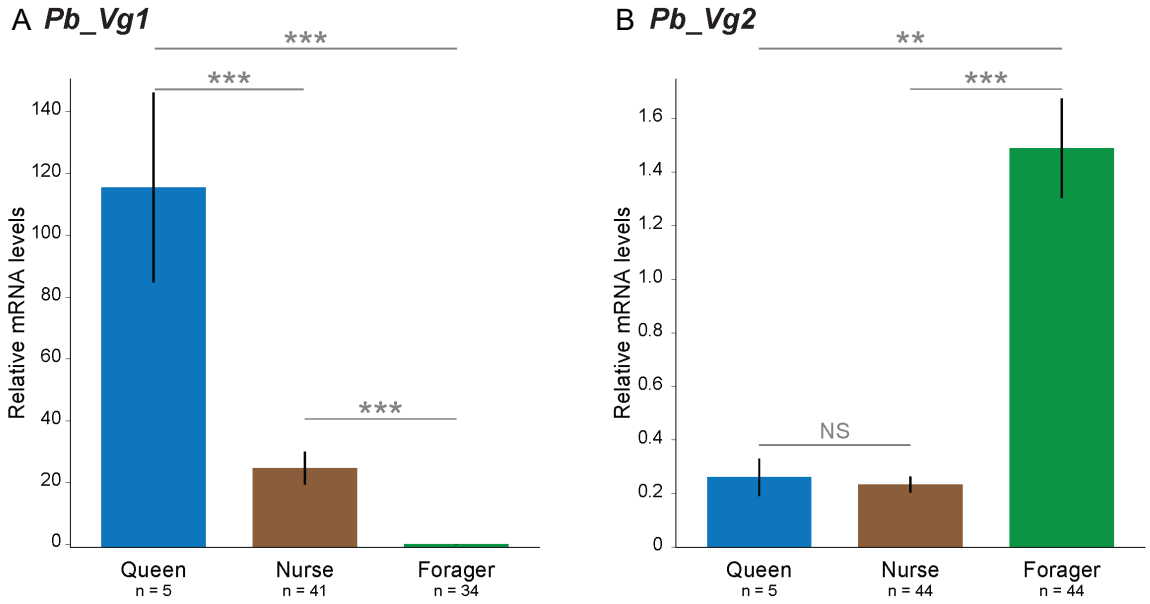

Figure 2. The *vitellogenin* genes in *P. barbatus* showed opposite expression patterns among castes and behavioral groups. (A) *Pb_Vg1* was more expressed in queens than in workers, consistent with the ancestral gonadotropic function of vitellogenin. (B) *Pb_Vg2*, however, was more expressed in foragers than in both nurses and queens, consistent with a role in behavioral regulation. Bar plots represent mean±se (**P<0.01; ***P<0.001).

Such subfunctionalization and the acquisition of novel roles in a social context is further supported by the reconstruction of phylogenetic relationships between *vitellogenin* gene copies across different ant species. Indeed, our phylogenetic analyses showed that a first duplication of the ancestral *vitellogenin* gene occurred after the divergence between the poneroid and formicoid clades, and subsequent duplications occurred in distinct ant lineages. This study formed the foundation for several subsequent investigations that confirmed and expanded the diversity of functions fulfilled by *vitellogenin* genes in ants (Kohlmeier et al., 2018, 2019; Morandin et al., 2014; Oxley et al., 2014).

Role of insulin signaling in the evolution of reproductive division of labor

Chandra, …, **Libbrecht** & Kronauer (2018) Social regulation of insulin signaling and the evolution of eusociality in ants. **SCIENCE** (Appendix 4)

In ants, there was a single origin of the reproductive division of labor between the queen and worker castes, which evolved from a subsocial ancestor that would alternate between reproductive and brood care phases (Hunt, 2007; West-Eberhard, 1987). To better understand how these phases were modified into a fixed asymmetry between queens and workers requires investigating the regulation of reproductive division of labor, *i.e.*, the mechanisms that allow queens to lay eggs but prevent workers from doing so.

Using RNA sequencing, we conducted a comparative study to contrast gene expression between the brains of reproductive and non-reproductive individuals in seven species across the phylogeny of ants (Chandra et al., 2018). These comparisons revealed that one candidate gene, *insulin-like peptide 2* (*ilp2*), encoding an insulin-like peptide, was consistently overexpressed in reproductive individuals in all seven species (Figure 3), thus indicating an ancestral role of *ilp2* in the regulation of reproduction in ants. The most parsimonious explanation for this finding is that *ilp2* was already associated with reproduction in the common ancestor of ants, thus we hypothesized that this gene, and more broadly the insulin signaling pathway, played a role in the emergence of reproductive division of labor.

We tested this hypothesis using *Ooceraea biroi*, a species of clonal ants where larval signals inhibit adult reproduction by suppressing *ilp2*, thus producing an alternation of reproductive and brood care phases in a colony cycle reminiscent of ancestral subsociality. We found that injecting the peptide produced by *ilp2* generated ants whose reproduction was no longer inhibited by the presence of larvae. This result not only provides experimental confirmation of the role of *ilp2* in regulating reproduction in response to the social environment but also suggests a potential role in the evolution of the queen and worker castes. By experimentally increasing *ilp2*, we produced ants that reproduced continuously, regardless of larval presence. These ants thus resembled queens. Complementary experiments showed that such an increase in *ilp2* could naturally occur via an increase in food intake during larval development.

Overall, these results suggest that inter-individual variation in larval food intake, and thus in *ilp2* expression could be at the evolutionary origin of reproductive division of labor in ants because it would have resulted in variation across individuals in their physiological response to the presence of larvae (Chandra et al., 2018). Such variation in the effect of larvae on reproduction could have paved the way toward the evolution of the queen and worker castes. We synthesized these findings into a model implicating *ilp2*, and more broadly the insulin signaling pathway, in the evolution of reproductive division of labor in ants (Chandra et al., 2018).

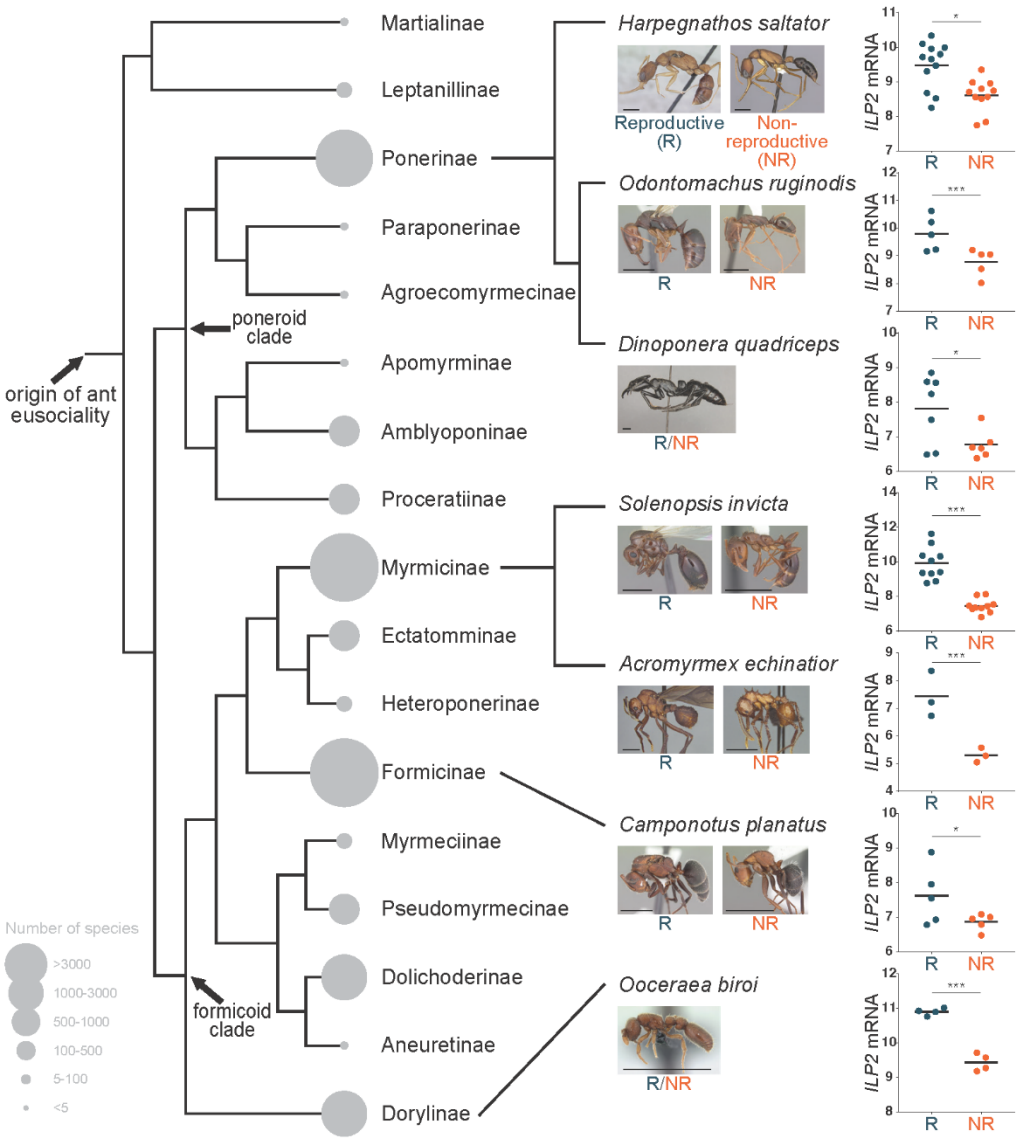

Figure 3. Brain gene expression in seven ant species identifies one conserved differentially expressed gene. The figure shows the summary cladogram of the seven ant species used in this study in the context of the entire ant phylogeny with all subfamilies labeled. The dot plots show variance-stabilized transformed read counts for *ilp2*. Blue and orange dots indicate reproductive and non-reproductive individuals, respectively. Horizontal bars indicate means, and asterisks indicate statistically significant differences between groups (*P<0.05; ***P<0.001).

1.3 – Conclusion of Part 1

Reproductive division of labor is central to the functioning and evolution of insect societies. To better understand its regulation requires studying what makes a queen, *i.e.*, the mechanisms that underlie the process of caste determination and that control adult reproductive activity. Part 1 describes how my research work in ants contributed to our understanding of genetic and maternal effects on caste determination, and of the implication of the juvenile hormone, vitellogenin and insulin signaling pathways in the regulation and evolution of reproductive division of labor. These studies were conducted relatively early in my

academic career, as all but one were part of my PhD thesis, and the results opened thematic and technical perspectives that have impacted my research in multiple ways.

The first impact was produced by our findings that genetic effects on caste determination (Libbrecht et al., 2011) and behavioral specialization (Libbrecht & Keller, 2012) were not consistent with mere allelic differences, which suggested more complex genetic influences such as parent-of-origin and/or epistatic effects. More specifically, the results identified epigenetic mechanisms as candidates for the regulation of phenotypic plasticity, which initiated my interest in the potential role of epigenetic processes in underlying reproductive and behavioral division of labor in social insects (Libbrecht et al., 2016, 2020).

Second, I became aware of the limitations of comparing morphologically distinct queen and worker castes to investigate the molecular mechanisms of division of labor (Kronauer & Libbrecht, 2018). The first limitation is that castes differ in many interrelated traits (*e.g.*, reproduction, behavior, morphology, nutrition, genetics), and it is challenging to associate molecular differences between castes with a specific phenotype. The second limitation is that castes in Hymenoptera are typically fixed at the adult stage. Thus, studies comparing them are necessarily correlative, and do not allow experimental manipulations for functional validation of candidate mechanisms.

Finally, I discovered that an alternative approach to study reproductive division of labor is to use more flexible study systems where reproduction is more plastic and can be manipulated (Kronauer & Libbrecht, 2018). *O. biroi* offers such flexibility, as it allows fine manipulations of the presence of larvae, and thus of reproductive activity (Libbrecht et al., 2016, 2018; Oxley et al., 2014; Ravary et al., 2006; Ulrich et al., 2016). I further realized that conducting such experimental manipulations of the social environment does not need to be restricted to *O. biroi* and can be broadly applied to investigate the impact of the social environment on behavior and reproduction in social insects.

Part 2 – The importance of the social environment

While the division of labor between queens and workers is often viewed as a fixed outcome of caste determination, the social context can dynamically influence individual phenotypes, leading to remarkable plasticity in behavior and reproduction. This part explores how social interactions regulate phenotypic variation in ants, from the molecular mechanisms underlying reproductive plasticity to the broader consequences for individual longevity and colony efficiency.

2.1 – Molecular regulation of the social control of reproduction

Figure 4. Clonal raider ants tending their larvae.

In the last decade, the clonal raider ant (*Ooceraea biroi*) (Figure 4) has emerged as a powerful study system to investigate the factors and mechanisms that control variation in reproductive activity in ant colonies (Libbrecht et al., 2016, 2018; Oxley et al., 2014; Ulrich et al., 2016). This species has lost the queen caste, and all workers reproduce via parthenogenesis. Their reproduction is regulated by the presence of larvae, which inhibit egg-laying (Ravary et al., 2006). This effect of the social environment is interesting for multiple reasons. First, it results in a phasic life cycle in which each colony alternates between a reproductive phase (in the absence of larvae) and a brood care phase (in the presence of larvae). Since this socially regulated cycle is analogous to the subsocial cycle of the ancestor of ants (Kelstrup et al., 2018; Kronauer & Libbrecht, 2018; West-Eberhard, 1987), *O. biroi* provides a unique model for the study of the evolution of reproductive division of labor (Chandra et al., 2018). Second, we found that *O. biroi* larvae inhibit the reproductive activity of adult workers in a dose-dependent manner (Ulrich et al., 2016), enabling precise experimental manipulations of the reproductive activity. Finally, *O. biroi* allows for the control of confounding factors typically associated with caste comparisons (*e.g.*, age, individual experience, genetic background) (Kronauer & Libbrecht, 2018). For all these reasons, as a Marie-Curie postdoctoral fellow at the Rockefeller University (New York City, USA), I have used this study system to investigate the transcriptomic changes and epigenetic processes that regulate reproductive activity in ants.

Transcriptomic regulation of the social control of reproduction

Libbrecht et al. (2018) Clonal raider ant brain transcriptomics identifies candidate molecular mechanisms for reproductive division of labor. **BMC BIOLOGY** (Appendix 5)

To investigate the gene expression changes that underlie the activation or inhibition of reproduction, we developed a protocol in *O. biroi* to collect genetically identical, same-age ants at different time points when they modify their reproductive activity in response to the presence of larvae (Libbrecht et al., 2018). First, we produced clones of the same age that either reproduced (in the absence of larvae) or did not reproduce (in the presence of larvae). We then manipulated the presence of larvae to initiate either the stimulation (via removal of all larvae) or inhibition (via addition of larvae) of reproduction. For each of these two transitions, we collected individuals at various time points and analyzed changes in gene expression in the brain (via RNA sequencing) over time following the manipulations of the social environment (Figure 5).

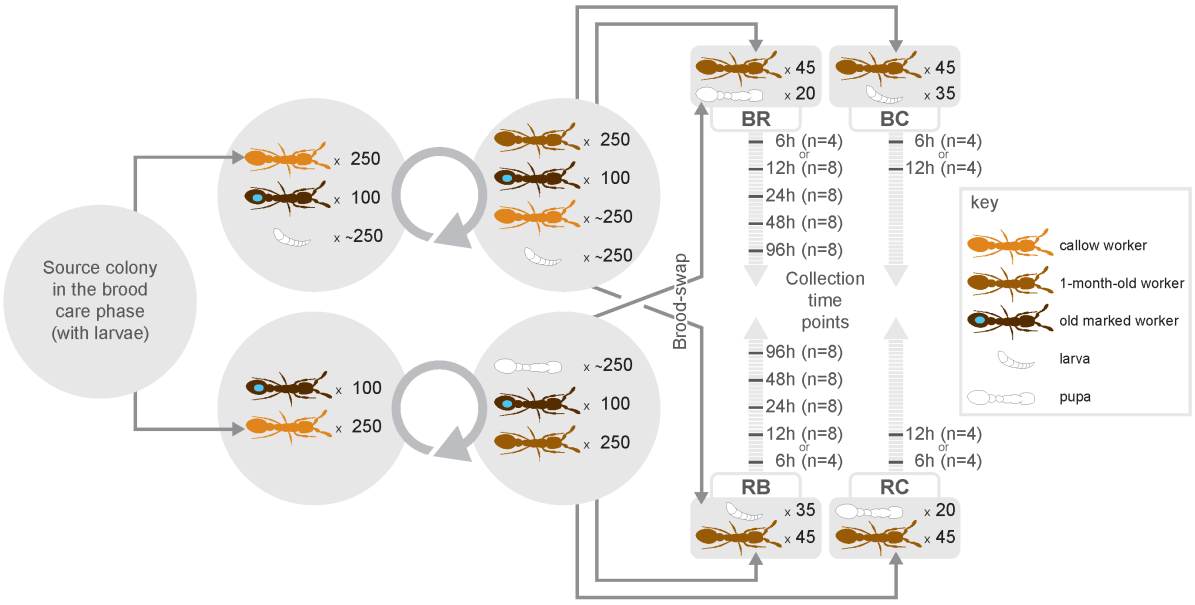

Figure 5. Experimental protocol to investigate brain transcriptomic changes over time during the activation and inhibition of reproduction in same-age, genetically identical ants. For each biological replicate, a large source colony in the brood care phase was used to establish two colonies of 250 1-month-old workers and 100 marked ≥ 3-month-old workers. One of these colonies received approximately 250 larvae. After a full colony cycle, each colony contained a complete cohort of brood and workers and was in either peak brood care phase (with larvae) or early reproductive phase (with eggs and pupae). On the day the first eggs were laid, the 1-month-old workers were subdivided in colonies of 45 workers each. One colony from each phase served as the control colony and was given brood from the mother colony. The remaining colonies received brood from the mother colony in the opposite phase of the cycle, triggering the transition toward the alternative phase. Colonies were subsequently collected 6, 12, 24, 48, or 96 h post treatment. BR: workers transitioning from the brood care phase to the reproductive phase (after larvae were removed and pupae added); RB: workers transitioning from the reproductive phase to the brood care phase (after pupae and eggs were removed and larvae added), BC: workers from the brood care phase with larvae (brood care phase control); RC: workers from the reproductive phase with pupae (reproductive phase control).

Our analyses revealed key differences between the two transitions in the nature of the genes involved and in the temporal dynamics of gene expression changes (Libbrecht et al., 2018). We found that introducing larvae that inhibited reproduction caused much faster changes in brain gene expression than removing larvae (Figure 6A). This finding that the removal of the brood signal is accompanied by a delay in gene expression and physiological adjustments is consistent with larval cues acting as a reinforcement signal for the inhibition of reproduction. Such a delay is necessary in *O. biroi* to prevent premature transitioning to reproduction, such as during foraging, when some individuals frequently exit the nest during the brood care phase and are thus only sporadically exposed to larval cues. Our analyses of the gene expression changes over time also identified candidate genes for the neural control of the effects of the social environment on reproduction, including genes encoding neurotransmitters, neurohormones, and neuropeptides (Figure 6B-C).

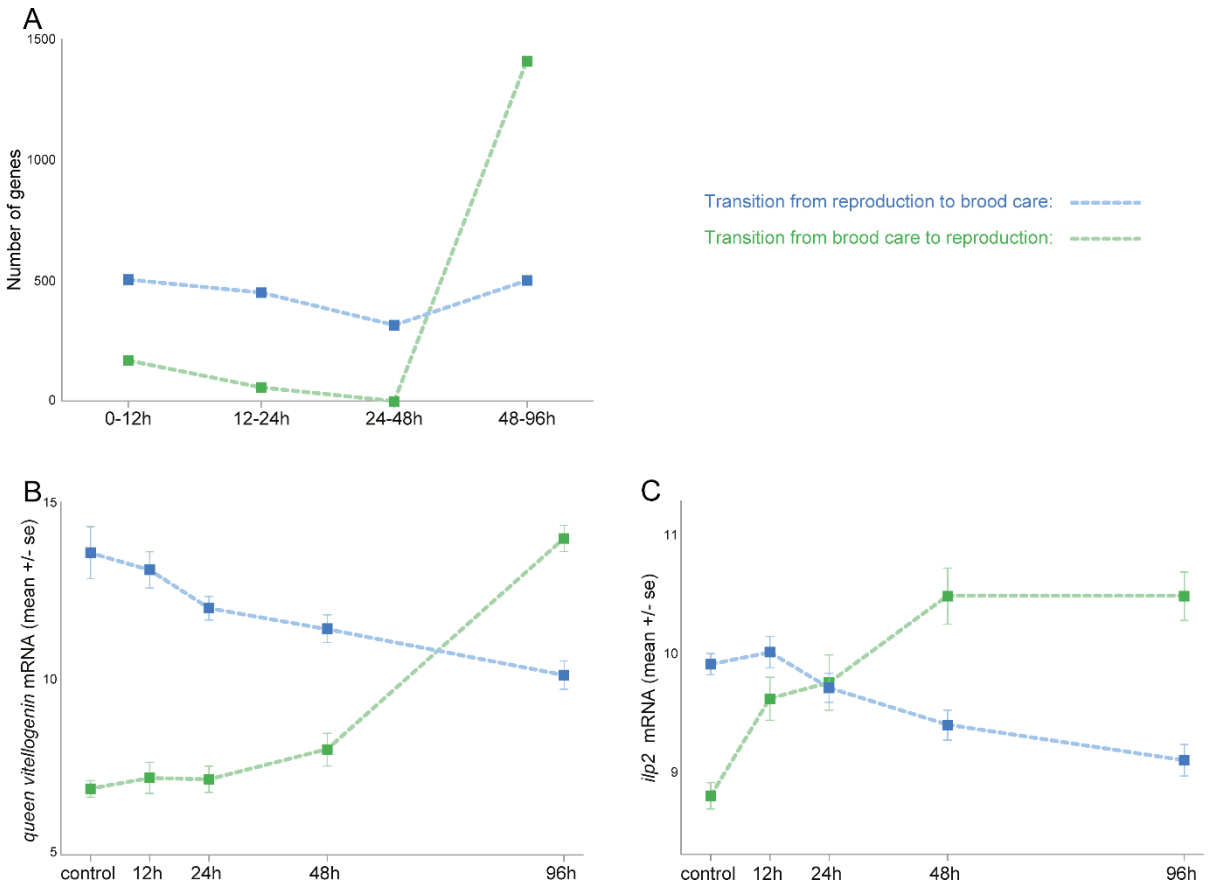

Figure 6. The experimental activation (green) and inhibition (blue) of reproduction induced distinct time dynamics of gene expression changes. A) Number of genes in clusters (enriched for DEGs) with maximal change in expression for each time interval. The distribution of such numbers across time intervals differed significantly between transitions (P<0.001). B) *queen vitellogenin* and C) *ilp2* expression changes over time after adding or removing larvae. Gene expression is shown as variance-stabilized transformed read counts.

Recently, we have used a modified version of the experimental protocol detailed in Figure 5 to investigate the transcriptomic changes associated with the activation of reproduction in two additional tissues susceptible of translating larval cues into reproductive variation: the antennae and the ovaries (Coulm et al., in preparation). In addition to providing general insights into the regulation of the reproductive cycle in response to the presence of larvae, these studies identified specific candidate genes involved in the control of reproduction, thereby contributing to our molecular understanding of the regulation of reproductive division of labor in ants.

Epigenetic regulation of the social control of reproduction

Libbrecht et al. (2016) Robust DNA methylation in the clonal raider ant brain. **CURRENT BIOLOGY** (Appendix 6)

To better understand the molecular mechanisms regulating gene expression changes associated with reproductive variation in response to modifications of the social context in *O. biroi*, we investigated the role of epigenetic processes. One of the most common epigenetic modifications is DNA methylation, which involves the addition of a methyl group to specific nucleotide bases, particularly cytosines (Allis et al., 2007). The discovery of genes encoding the enzymes responsible for this epigenetic process in the genome of the honeybee (Weinstock et al., 2006) elevated DNA methylation as a candidate mechanism for the evolution and regulation of social life in insects. Subsequent studies described differences in DNA methylation between queens and workers in the honeybee and several ant species (Bonasio et al., 2012; Foret et al., 2012; Glastad et al., 2014; Lyko et al., 2010). However, the challenges of comparing morphologically distinct castes (Kronauer & Libbrecht, 2018), coupled with the absence of biological replication in most of these studies, prompted us to investigate the role of DNA methylation in regulating reproductive variation in *O. biroi* (Libbrecht et al., 2016). Using this study system allowed us to control for confounding factors such as genetic, age and morphological variation, thus enabling a better controlled analysis of the role of DNA methylation in regulating reproductive variation.

We performed whole-genome bisulfite sequencing to identify genome-wide methylation differences between the brains of same-age, genetically identical reproductive and non-reproductive individuals. We did not find any evidence that DNA methylation patterns were associated with variation in reproduction or gene expression, despite using several statistical approaches (including random permutation tests). Furthermore, we found that DNA methylation was either very robust (many cytosines were consistently either methylated in all samples or unmethylated in all samples) or highly variable (many cytosines were methylated in a single sample, *i.e.*, sample-specific methylation) (Libbrecht et al., 2016).

As these findings stood in contrast with previous reports of caste-specific DNA methylation (Bonasio et al., 2012; Foret et al., 2012; Glastad et al., 2014; Lyko et al., 2010), we hypothesized that studies lacking biological replication may have conflated sample-specific methylation with caste-specific methylation. To test this hypothesis, we simulated the results that our data would have produced under the experimental design and statistical analyses used in earlier studies (in absence of biological replication). We found that we would have indeed detected differential methylation in all four comparisons of one reproductive sample and one non-reproductive samples (one comparison per source colony) but revealed that these differences in DNA methylation would not have been consistent across source colonies (Figure 7). This result suggests that, in prior studies lacking biological replication, sample-specific methylation may have been falsely interpreted as caste-specific methylation. Our study had a strong and positive impact on the emerging field of social insect epigenetics by stressing the need for proper controls, replication levels and statistical analyses. Since then, other investigations have confirmed the concerns raised in our publication regarding the role of DNA methylation in the regulation of reproductive division of labor (Herb et al., 2012; Patalano et al., 2015; Standage et al., 2016).

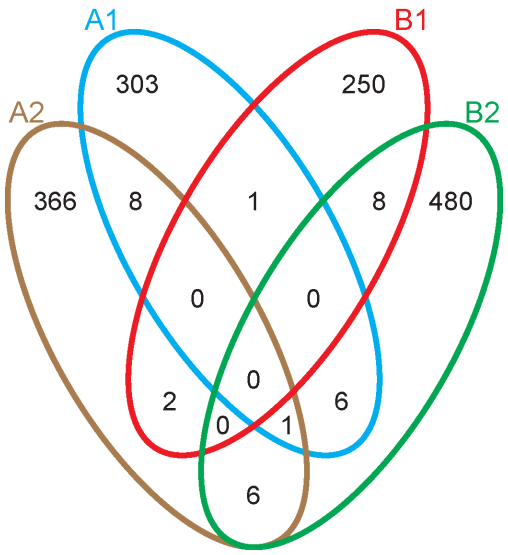

Figure 7. The lists of differentially methylated exons returned by the statistical method used in previous studies without biological replicates are random or colony-specific lists of exons. Number of differentially methylated exons between the reproductive phase and the brood care phase for each source colony: 319 in colony A1, 383 in colony A2, 261 in colony B1, and 501 in colony B2. There was no exon that was consistently differentially methylated between phases in all four source colonies. This shows that the statistical method used in previous studies lacking biological replication is prone to return random or colony-specific lists of differentially methylated exons.

2.2 – Role of social interactions in other species and contexts

Investigating the impact of social partners on reproduction in clonal raider ants raised my interest in whether and how social interactions regulate phenotypic variation in other contexts and/or other species of ants. As an assistant professor at the University of Mainz (Germany), I have designed and supervised the thesis projects of multiple BSc, MSc and PhD students that harness the potential of experimental manipulations of the social environment in ant colonies to investigate the role of social interactions in shaping phenotypic variation in terms of reproduction, lifespan, aggression and brood production.

Larvae stimulate worker reproduction upon queen loss

Majidifar, Fetzer, Wagner & **Libbrecht** (in prep.) Larvae stimulate worker egg production upon queen loss in *Temnothorax* ants.

(Underlined names indicate students that I have supervised)

In most species of social Hymenoptera, workers in queenless colonies develop their ovaries and lay haploid, male-destined eggs (Holman et al., 2010; Van Oystaeyen et al., 2014). While this effect of the queen presence on worker reproduction is well documented, it remains unclear whether additional changes in the social environment (*e.g.*, the presence of brood) affect the egg production of workers. One hypothesis is that the presence of larvae would inhibit egg production because caring for the brood bears energetic costs and queenless workers would prioritize resource allocation toward caring for existing larvae over investing in egg production. Previous studies supported this hypothesis by reporting inhibitory effects of larvae on worker egg production in bumblebees and ants (Ebie et al., 2015; Starkey et al., 2019; Ulrich et al., 2016; Villalta et al., 2015). Another hypothesis is that the presence of brood would stimulate egg production, as larvae provide various, passive and active nutritional benefits to adult colony members (Schultner et al., 2017). While such positive impact of brood on reproduction was documented in mature colonies that contain queens (Schultner et al., 2017), there is no evidence that larvae stimulate worker reproduction upon queen loss.

To better understand the effect of larvae on worker reproduction in ants, we investigated this issue in the ant *Temnothorax nylanderi* (Majidifar et al., in preparation). We found that the experimental removal of the queen stimulated egg production in *T. nylanderi* workers, but that the increase over time in the number of eggs was higher for workers that were provided with larvae compared to workers that were kept without larvae (Figure 8). We found this effect to be dose-dependent, as higher larva-to-worker ratios provided weaker benefits in terms of egg production compared to

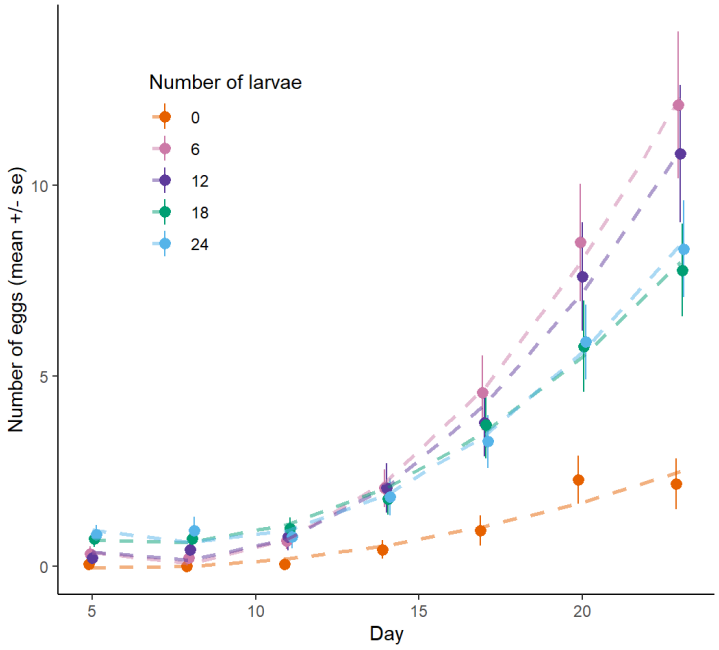

Figure 8. Larvae had a positive, dose-dependent effect on egg production in *T. nylanderi* queenless workers. The nonlinear increase over time in the number of eggs differed among treatments (n=18 in each treatment, P<0.0001). All treatments with larvae showed a stronger increase than the treatment without larvae (all P<0.0001). We found no evidence that the experimental colonies with 6 and 12 larvae differed (P=0.59), but both showed a higher increase over time than experimental colonies with 18 and 24 larvae (all P<0.015). Experimental colonies with 18 and 24 larvae did not differ from each other (P=0.99). All experimental colonies contained 12 workers. The dotted lines depict the output of the model for each treatment.

lower larva-to-worker ratios (Figure 8). Our analyses also indicated that larvae did not stimulate egg production when they had been starved beforehand (P=0.57), suggesting that the stimulatory effect of larvae on worker egg production may stem from nutritional benefits. However, we found that workers kept with larvae had a lower glycogen content than those kept without larvae (P<0.0001), possibly reflecting the mobilization of energy reserves to care for the larvae and/or invest in egg production.

Our results thus support a positive, dose-dependent effect of larvae on worker egg production in absence of the queen in *T. nylanderi*. This finding illustrates how the presence and number of distinct social partners (queen and larvae) interact to influence worker reproduction and physiology. More generally, because it stands in contrast with previous reports of an inhibitory effect of larvae on worker egg production in other ant species (Ebie et al., 2015; Ulrich et al., 2016; Villalta et al., 2015), this study highlights that the impact of social interactions on division of labor depends on species-specific ecology and characteristics.

Manipulations of the social context affect worker lifespan

Lenhart, Majoe, Nehring, Foitzik & **Libbrecht** (in prep.) Experimental inhibition of reproduction decreases the lifespan of clonal ants.

(Underlined names indicate students that I have supervised)

Social insect colonies display fascinating variation not only in reproductive activity but also in lifespan. The typical trade-off between longevity and fecundity that can be observed in most living organisms (Gems & Partridge, 2013; Partridge et al., 1987; Westendorp & Kirkwood, 1998) appears to be absent in social insects: fertile queens live longer than non-reproducing workers (Heinze et al., 2013; Keller & Genoud, 1997; Kramer et al., 2015), and workers that become reproductively active extend their lifespan (Kohlmeier et al., 2017; Kuszewska et al., 2017; Lopes et al., 2020; Majoe et al., 2021; Negroni et al., 2021). However, comparing queens and workers or experimentally manipulating worker reproduction via queen removal makes it challenging to disentangle reproductive activity from confounding factors such as age, morphology, individual experience and/or genetic background (Kronauer & Libbrecht, 2018).

In this study, we used the clonal raider ant (*O. biroi*) to investigate the impact of reproduction on lifespan, as well as its transcriptomic basis, while controlling for all these confounding factors (Lenhart et al., in preparation). We experimentally manipulated randomly selected, same-age, monomorphic and genetically identical workers to either inhibit or stimulate their reproduction shortly after their emergence and for a period of five months. We did so by forcing them to constantly reproduce via regular removal of eggs (forced reproductive phase) or to never reproduce via regular addition of larvae (forced brood care phase). We also included a treatment where experimental colonies followed the natural phasic cycle (control). We

monitored worker survival over the entire experimental period and investigated age-related transcriptomic changes in the brain and the fat body.

Our main finding is that the experimental inhibition of worker reproduction resulted in a reduced lifespan: workers forced to remain in the non-reproductive brood care phase exhibited a decreased survival compared to workers that were forced to continuously reproduce or to control workers that alternated between reproduction and brood care phases (Figure 9). In addition, we identified age-associated variation in gene expression that differed between workers in the forced reproduction and forced brood care treatments. We found 13 genes in the brain and 54 genes in the fat body that exhibited this differential expression pattern (interaction between age and treatment, $P<0.05$), and thus represent candidate molecular mechanisms that could underlie the decreased survival in response to our experimental inhibition of reproduction.

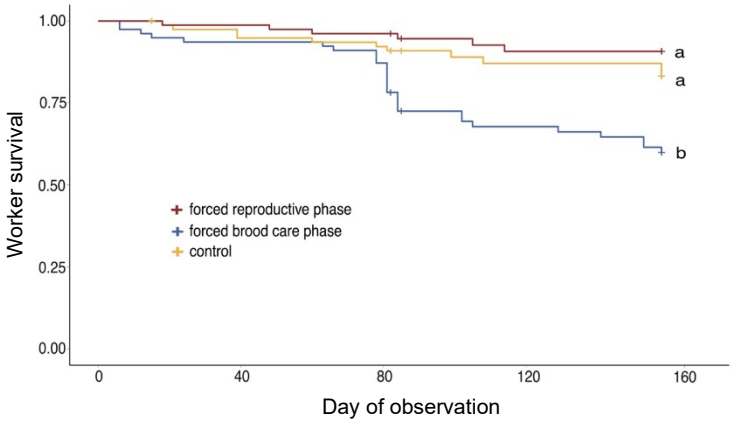

Figure 9. Experimental manipulations of the presence of larvae affected worker survival in *O. biroi*. Workers in the forced brood care phase (blue line, constant reproductive inhibition via regular addition of larvae) lived shorter than control workers (yellow line) (P=0.008) and workers in the forced reproductive phase (red line, constant reproductive activation via regular removal of eggs) (P=0.0004). Each treatment consisted in 6 groups of 13 workers.

In this study, we used experimental manipulations of the social context to demonstrate that the typical trade-off between longevity and fecundity is absent in clonal raider ants. This work and our recent studies of the impact of the queen presence on worker survival in other ant species (Lenhart et al., 2025; Majoe et al., in preparation) illustrate the importance of conducting experimental manipulations of the social context for robust investigations of the unusual association between longevity and fecundity found in social insects.

Ant colonies with a more diverse worker force show increased brood production

A central question to understand social evolution in insects is why ants, bees and wasps repeatedly evolved reproductive strategies that lower genetic relatedness within their colonies, such as queens mated with multiple males and/or colonies headed by multiple queens (Hughes et al., 2008). Selection for such strategies is unexpected, as it

Psalti, Gohlke & **Libbrecht** (2021) Experimental increase of worker diversity benefits brood production in ants. **BMC ECOLOGY EVOLUTION** (Appendix 7)

(Underlined names indicate students that I have supervised)

apparently impedes the evolution of altruism toward kin. One hypothesis is that the increased genetic diversity associated with these reproductive strategies facilitates division of labor among workers and enhances colony fitness. Previous studies tested this hypothesis, but the evidence supporting a beneficial effect of genetic diversity remains weak. Many of these studies reported correlations between levels of genetic diversity and colony performance (Cole & Wiernasz, 1999; Fjerdingstad et al., 2002; Fjerdingstad & Keller, 2004; Goodisman et al., 2007; Modlmeier et al., 2012) but failed to show a causal link between the two. The few experimental studies published so far mostly relied on artificial inseminations of queens with the sperm of one or multiple males (Fuchs & Schade, 1994; Jones et al., 2004; Mattila & Seeley, 2007). Such manipulations not only affect the diversity in the offspring produced, but also the queens that are inseminated artificially. Therefore, any effects on colony performance could also be explained by maternal effects (*e.g.*, on egg production, brood survival and development).

In this study, we manipulated the social composition of experimental colonies to investigate whether increased diversity in the worker force enhances colony performance in the black garden ant (*Lasius niger*) (Psalti et al., 2021). Colonies of this species are headed by a single queen that usually mates with one male (Boomsma & Van der Have, 1998). We produced experimental colonies of either low (by mixing workers from a single colony) or high (by mixing workers from multiple colonies) diversity. We controlled for any maternal effects by randomly assigning one, unrelated queen to each experimental colony.

Our main finding was that more diverse experimental colonies produced more larvae over time (Figure 10). In addition, we found that the increased diversity was apparent in term of worker size variation. We argue that the benefits of diversity likely stemmed from an improved division of labor that facilitated brood development and enhanced its survival. Our study provides experimental evidence for a positive influence of increased worker diversity on colony performance in a species with naturally low levels of genetic diversity. Such benefits could explain the evolution of reproductive strategies decreasing genetic relatedness in colonies of social insects.

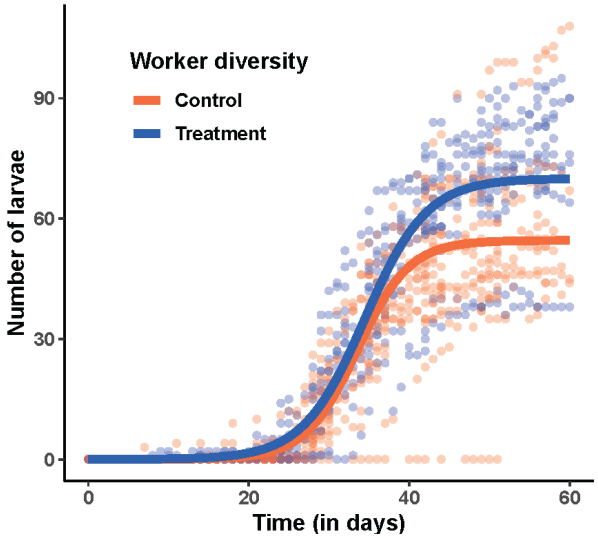

Figure 10. Colonies experimentally manipulated to have a more diverse worker force produced more larvae. Number of larvae in control (n = 23) and treatment (n = 18) colonies over time. The dots show the raw data for all colonies and time points. The curves depict the output of the models for control (orange) and treatment (blue) colonies. Two parameters of the logistic growth function differed significantly between control and treatment colonies (asym, P=0.011; scal, P=0.046).

Central role of fecundity in pleometrotic associations of ant queens

Teggers, Deegener & **Libbrecht** (2021) Fecundity determines the outcome of founding queen associations in ants. **SCIENTIFIC REPORTS** (Appendix 8)

(Underlined names indicate students that I have supervised)

One classic case of animal cooperation among unrelated individuals is pleometrosis in ants (Bernasconi & Strassmann, 1999). Pleometrosis is the association of founding queens after the nuptial flight to establish a new colony together. While this cooperative behavior favors colony growth (Madsen & Offenberg, 2017; Rissing & Pollock, 1987; Sommer & Hölldobler, 1995), the mechanisms behind its benefits remain unclear. We experimentally induced pleometrosis to dissect out its benefits, and proposed that they stemmed from a nutritional boost to the larvae (Teggers et al., 2021).

Importantly, those benefits are conditional, as pleometrotic associations are only transitory: the queens stay together to produce eggs and raise the first larvae, but upon worker emergence, they engage in fights (sometimes initiated and/or joined by the workers) until a single queen survives (Bernasconi & Strassmann, 1999). It is thus critical for founding queens to decide whether to engage in an association, and to select pleometrotic partners depending on the associated likelihood of surviving the association. For example, if a phenotypic trait is positively correlated with the likelihood to survive the association, there may be selective pressure on founding queens to choose pleometrotic partners with low values for this trait. While several traits are indeed associated with queen survival (*e.g.*, size, mass, fecundity) (Aron et al., 2009; Nonacs, 1990, 1992), they tend to be confounded, and it is unclear which factor specifically determines the outcome of pleometrosis. Similarly, whether founding queens choose their partners according to those traits remains unknown.

In this study, we conducted experimental manipulations of the social context to address these questions. We experimentally paired queens that differed in fecundity but not in size, and vice versa, to disentangle the effect of these factors on queen survival. We also provided pleometrotic pairs with unrelated workers (via brood replacement) to prevent queen fecundity from being confounded with worker parentage (the proportion of daughters in the pool of

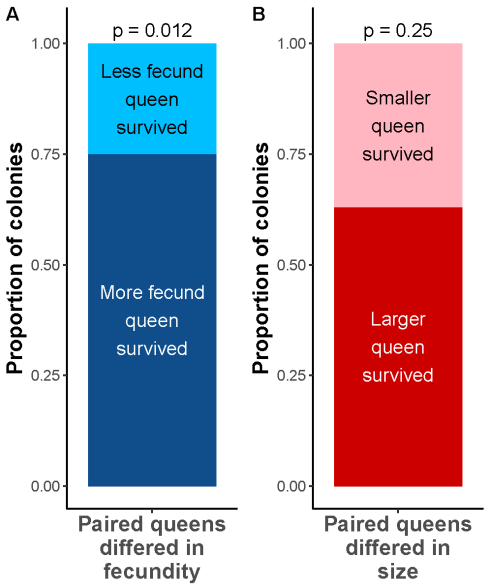

Figure 11. More fecund queens were more likely to survive the pleometrotic associations. The likelihood of surviving pleometrosis depended on queen (A) fecundity (n=28), but not (B) size (n=27). P values come from binomial tests.

workers) when cooperation ended. Our results showed that the most fecund queens more frequently survived the associations, even when controlling for size and worker parentage (Figure 11). Then, to study the traits associated with partner selection, we provided founding queens with the choice between two randomly selected queens. We found that the queens that were chosen as pleometrotic partners were less fecund, but of similar size, compared to the queens that were not chosen (Teggers et al., 2021). Thus, when selecting cooperation partners, *L. niger* founding queens show a preference that increases the likelihood that they survive the association. This study revealed that the fecundity of social partners is central to both the onset and the outcome of pleometrotic associations in ants.

2.3 – Conclusion of Part 2

Social insect colonies are complex, integrated biological systems that function based on the cooperation of specialized colony members (Wheeler, 1911). In addition to intrinsic differences between morphologically distinct castes, the constant interactions among social partners produce phenotypic variation at multiple levels, from physiology to behavior. While the influence of queen presence on worker reproduction and behavior has been well studied (Heinze, 2008; Holman et al., 2010; Lenhart et al., 2025; Van Oystaeyen et al., 2014), the role of other types of social interactions in shaping division of labor and/or ensuring colony efficiency remains relatively less understood.

Part 2 describes how my research work improved our mechanistic understanding of the impact of larvae on worker reproduction (Libbrecht et al., 2016, 2018; Majidifar et al., in preparation; Ulrich et al., 2016) and longevity (Lenhart et al., in preparation), and the colony-level benefits of having a more diverse worker force (Psalti et al., 2021). These studies confirmed the importance of experimentally manipulating the social environment to uncover how social interactions ensure proper colony functioning and raised my interest for understudied influences of the social context, such as the effect of workers and larvae on the queen behavioral specialization in egg production.

Part 3 – Research perspectives on the behavioral specialization of queens

The functioning of biological systems relies on the cooperation of specialized components. Typical examples of such biological systems include multicellular organisms that are composed of specialized cells, and insect societies that are composed of specialized individuals (Szathmary & Maynard Smith, 1995). Indeed, social insect colonies (also called superorganisms) are analogous to multicellular organisms in that they have queens that monopolize reproduction (similar to germ cells), and functionally sterile workers that perform all non-reproductive tasks and thus act as somatic cells (Boomsma & Gawne, 2018; Wheeler, 1911). Both types of biological systems evolved from solitary and non-specialized ancestors in major evolutionary transitions (Szathmary & Maynard Smith, 1995): multicellular organisms from unicellular organisms, and superorganisms from solitary insects.

Interestingly, in both cases, the specialization also needs to be established in every generation during the ontogeny of these biological systems (*i.e.*, the developmental process that produces the self-assembly and specialization of their components from a single unit). Extensive research efforts have focused on the ontogeny of multicellular organisms, building up the entire field of developmental biology. This demonstrated that studying the ontogeny is a powerful approach to understand the evolution and emergence of specialization (Brunet & King, 2017; Sogabe et al., 2019). However, this approach has not been applied to social insects, and there are few experimental investigations of the ontogeny of insect societies (Chouvenc, 2022; Majidifar et al., 2024; Woodard et al., 2013). In recent years, I have developed a new research agenda that aims to address this knowledge gap and provide a comprehensive understanding of the ontogeny of superorganisms. My objective is to identify the mechanisms that induce and maintain the individual specialization that emerges in the ontogenic process of insect societies and provide a better understanding of its evolutionary history.

In most social Hymenoptera species, mated queens establish their colony independently (Peeters, 2020) and are similar to zygotes in that they are the earliest developmental stage of superorganisms (Figure 12). The development of colonies from founding queens thus constitutes the ontogeny of superorganisms. These pluripotent founding queens express a broad repertoire of behaviors and fulfill multiple functions to produce the first workers. It is only once the colonies are established that the queens become strictly specialized in egg production (Wilson, 1971). The queen specialization is a central process in the ontogeny of superorganisms, as is cell differentiation in the ontogeny of multicellular organisms. However, studies of colony foundation have been limited to natural history descriptions, ecological

observations, and investigations of specific exceptions (Bernasconi & Strassmann, 1999; Hölldobler & Wilson, 1990; Peeters, 2020; Sommer & Hölldobler, 1992; Wilson, 1971). There is a need for experimental investigations of the factors and mechanisms that control the specialization of pluripotent founding queens.

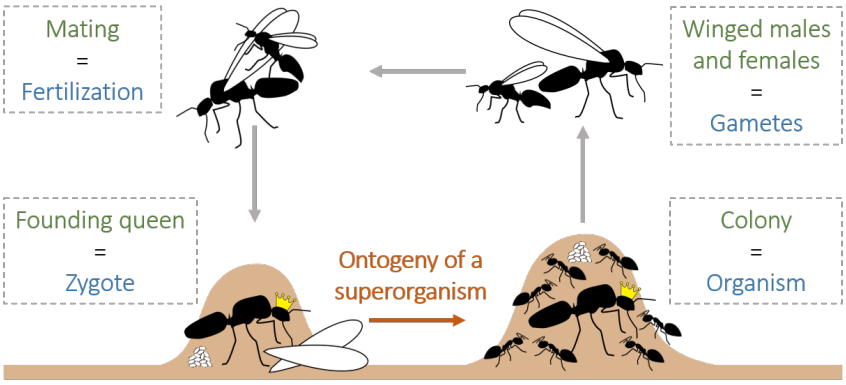

Figure 12. Ontogeny of a superorganism. Insect societies with reproductive division of labor are superorganisms: queens resemble germ cells, and workers somatic cells. Mature colonies produce winged males and females that are analogous to gametes, and their mating is analogous to fertilization. Then, newly mated queens, which are analogous to zygotes, found new colonies. I am interested in the transition from founding queens to established colonies, which corresponds to the ontogeny of superorganisms.

The pluripotency of founding queens is best illustrated by their behavior toward the brood. Brood care behavior has evolved repeatedly in insects, and its emergence represented critical early steps in the multiple evolutionary origins of insect sociality (Kronauer & Libbrecht, 2018). Furthermore, variation between individuals in their response to brood likely played a central role in the evolution of reproductive division of labor in ants (Chandra et al., 2018). Providing care to the developing brood is a critical task that is typically performed by workers, but that queens must fulfill in the foundation stage. This is necessary because in social Hymenoptera, the successful development from eggs to adults requires care by other individuals (Schultner et al., 2017).

To investigate the ontogeny of superorganisms, I have established the black garden ant (*Lasius niger*) as a model for the study of queen behavioral pluripotency and specialization. Several features of *L. niger* make it an optimal species for these investigations. First, *L. niger* queens show independent colony founding, meaning that founding queens (Figure 13A) express behavioral pluripotency before becoming exclusively specialized in egg production once the first workers have been produced (Figure 13B). This specialization typically lasts several decades, as the lifespan of queens in this species may approach 30 years (Hölldobler & Wilson, 1990). Second, *L. niger* is very common in Europe, with very large nuptial flights. This allows the collection of up to 1,500

Figure 13 - *Lasius niger* queens.
(A) without workers; (B) with workers.

founding queens per year, which easily accommodates most experimental requirements. Third, *L. niger* offers the genomic resources that are required to implement this project, such as a chromosome-level genome assembly (Masson et al., 2024) and multiple published (Lucas et al., 2016, 2017; Lucas & Keller, 2018) and unpublished transcriptomes (section 3.2). Finally, we have established a behavioral setup that allows the parallel analysis of many queens, which can be provided with brood and/or workers depending on the experiment (Figure 14).

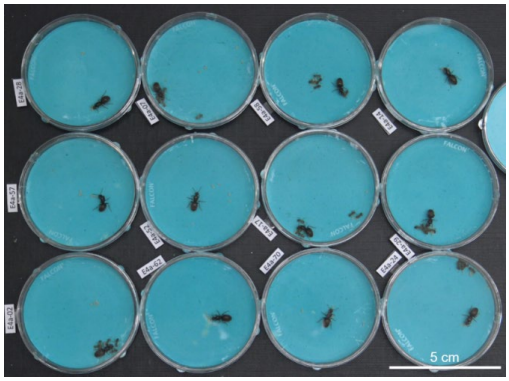

Figure 14. The setup for parallel behavioral analyses. This setup allows the filming of up to 12 queens in parallel with one camera.

In this part, I present our recent, ongoing and future work on the study of brood care behavior in ant queens. I have initiated this research line as an assistant professor at the University of Mainz (Germany) and have been expanding it further since my recruitment in 2023 as a CNRS researcher (*Chargé de Recherche*) at the Insect Biology Research Institute (IRBI, UMR CNRS 7261) of the University of Tours (France). The work presented below is part of the projects of BSc, MSc and PhD students under my supervision, funded by grants from the *Deutsche Forschungsgemeinschaft* (DFG) and the *Agence Nationale de la recherche* (ANR), in particular the ANR JCJC grant "ANTOGENY" (2024-2028).

3.1 – Social control of queen specialization

To better understand the behavioral transition of queens in the process of colony foundation, we study whether and how the presence of workers - and more generally the social interactions among colony members – regulates the queen specialization in egg production.

The presence of workers both initiates and maintains queen specialization

The behavioral specialization of *L. niger* queens is associated with the presence of workers, as demonstrated by our finding that founding queens observed before worker emergence provided more care to larvae than the same – then established - queens observed after worker emergence (Majidifar et al., 2024). While it is tempting to implicate a causal effect of the presence of workers on queen behavior, other confounding factors could explain the behavioral changes, such as queen age (founding queens are younger than established queens) or nutritional status (founding queens are

Majidifar, Psalti, Coulm, Fetzer, Teggers, Rotering, Grünewald, Mannella, Reuter, Unte & **Libbrecht** (2024) Ontogeny of superorganisms: Social control of queen specialization in ants. **FUNCTIONAL ECOLOGY** (Appendix 9)

(Underlined names indicate students that I have supervised)

starved, while established queens are fed by workers). To demonstrate the effect of the presence of workers requires experimentally providing workers to founding queens than did not produce any workers yet. To do so, we sampled brood from *L. niger* field colonies and kept it in laboratory colonies that we monitored regularly to collect workers that recently emerged from the pupae. These very young workers did not elicit aggression from foreign individuals and were readily accepted by founding queens, possibly because they did not possess the signature chemical profile of their own colony yet (Dahbi et al., 1998).

This protocol allowed us to experimentally manipulate the presence of workers in same-age founding queens, while controlling for the nutritional status. We confirmed that the experimental addition of workers did cause a reduction in queen brood care behavior (Figure 15A). This experiment demonstrates that the presence of workers is necessary and sufficient to initiate the behavioral specialization of *L. niger* queens. Then, we investigated whether the presence of workers is necessary to maintain the queen specialization in established colonies. To do so, we used *L. niger* colonies that were founded in the laboratory several years before starting the experiment. We first recorded the queen brood care behavior after removing all but five workers. Then, we removed the last five workers, and quantified queen brood care behavior on the next two days. We found that queens expressed elevated brood care levels after the experimental removal of the workers (Figure 15B). This shows that the presence of workers not only initiates the specialization of *L. niger* queens during colony foundation, but also constantly maintains it in established colonies.

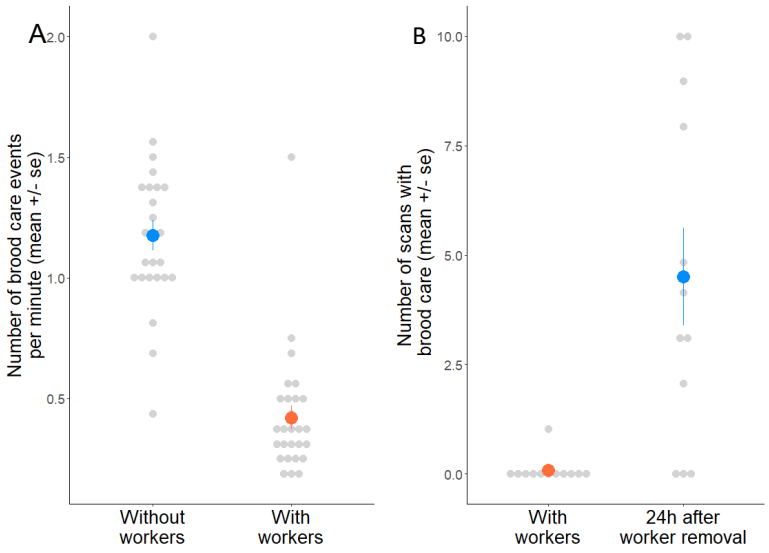

Figure 15. The presence of workers initiates and maintains the behavioral specialization of *L. niger* queens. (A) The experimental addition of workers to same-age founding queens that had not produced workers yet inhibited their expression of brood care behavior (without workers: n=25, with workers: n=29; P<0.00001). (B) The experimental removal of workers from 2.5-year-old established queens caused them to revert to expressing brood care behavior (n=12, P=0.0007).

After demonstrating that the presence of workers controls the queen specialization, we set out to determine whether cues of the presence of workers were sufficient to inhibit brood care behavior in queens, or whether the actual presence of workers was required. Because social insects detect social partners via the blend of hydrocarbons on their cuticle (Sprenger & Menzel, 2020), we extracted cuticular hydrocarbons (CHC) from pools of *L. niger* workers, and applied the CHC to glass beads that we provided to same-age founding queens. We did not detect any effect of the CHC treatment on the expression of brood care by queens (Figure 16A), indicating that queens do not modify their behavior in response to the mere detection of worker CHC. This result was confirmed by three additional experiments (Majidifar et al., 2024), including an investigation of whether the queen behavior was affected by workers separated from the queen and brood by a wire mesh. This setup enabled workers to make antennal contacts with the queen and brood, but prevented closer interactions such as fluid exchange via trophallaxis (LeBoeuf, 2021). We found that queens kept with workers separated by a wire mesh expressed as much brood care as queens kept without workers (Figure 16B). These experiments show that worker cues are not sufficient to drive the queen specialization and suggest that workers require close interactions with queens and/or larvae to inhibit the brood care behavior of queens.

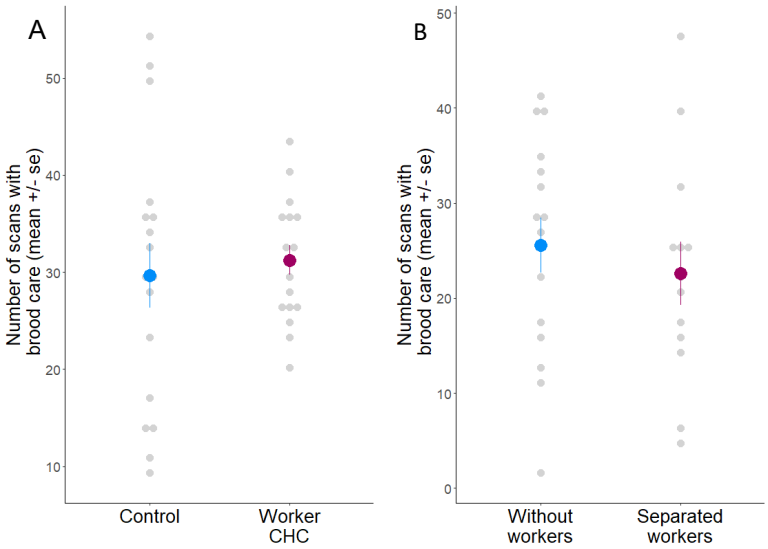

Figure 16. Cues of worker presence do not induce the queen behavioral specialization. (A) Founding queens exposed to glass beads coated with the cuticular hydrocarbons (CHC) of workers (n=18) show similar levels of brood care behavior as control founding queens exposed to the solvent (hexane, n=18) (P=0.45). (B) Founding queens provided with workers separated from the queen and the brood by a wire mesh (n=13) show similar levels of brood care behavior as founding queens kept without workers (n=16) (P=0.58).

Mechanistic understanding of the effect of workers on queen specialization

One of our current objectives is to test hypotheses that could explain how the actual presence of workers - but not mere signals of worker presence – inhibits brood care behavior in queens (Majidifar et al., 2024).

Ongoing PhD thesis
Hugo Le Lay (2024 – present)

One possible explanation is that the queen and the workers should interact closely for the effect of workers on queen behavior to occur, for example through an exchange of fluids via trophallaxis. Indeed, trophallactic fluids not only contain food, but also molecules that influence

behavior (LeBoeuf et al., 2016). We will determine whether fluid exchange between the queen and the workers triggers the queen behavioral specialization. We will use continuous video recordings of founding queens to investigate whether trophallaxis is required to initiate the queen behavioral specialization. We will also collect the trophallactic fluid of workers and experimentally provide it to founding queens to test its effect on brood care behavior. Should our experiments show that the exchange of fluids via trophallaxis drives the effect of workers on the queen behavior, we would investigate the content of the trophallactic fluid via proteomic characterization (Hakala et al., 2021; LeBoeuf et al., 2016).

Another possible explanation for mere worker cues not triggering behavioral changes in queens is that workers inhibit the queen brood care behavior via their effect on larvae. By providing care to larvae, workers would reduce the larval need for care, up to the point when larvae receive all the care they need from workers, and queens would stop providing brood care. This hypothesis implies that founding queens adjust their brood care to the needs of the larvae. We will thus observe the behavior of founding queens toward larvae that require more or less care, obtained via manipulations of their nutritional or health status.

Social insect larvae communicate their presence and needs to workers via chemical cues (Schultner et al., 2017). We hypothesize that founding queens also respond to such signals. These signals may be fixed and merely communicate the presence of brood, or they may be dynamic and indicate larval needs. We will determine whether and how larvae signal their presence and/or their needs for care. To test whether larvae do so via their cuticular hydrocarbons (CHC), we will analyze the larval CHC to identify chemical compounds that are specific to larvae and/or correlate with larval needs, as well as study the behavioral response of founding queens to CHC extracted from larvae. We will also consider the importance of other chemical cues (e.g., volatile compounds) produced by the larvae, as well as physical signals, such as begging behavior and body posture (Schultner et al., 2017).

3.2 – Molecular regulation of queen specialization

While we have accumulated robust evidence that the social environment affects brood care behavior in *L. niger* queens (Majidifar et al., 2024), the molecular mechanisms that translate the presence of workers into behavioral modifications remain to be understood. To address this question, we combine transcriptomic analyses with functional manipulations of candidate mechanisms to build an integrated mechanistic model of the queen behavioral specialization.

Transcriptomic basis of the queen behavioral specialization

Ongoing PhD theses
Maximilian Bolder (2022 – present)
Hugo Le Lay (2024 – present)

Transcriptomic analyses by RNA sequencing (RNA-seq) provide a powerful approach to describe gene expression changes that control behavioral plasticity. In my research, I have used this tool to reveal the implication of molecular pathways (Kohlmeier et al., 2019; Libbrecht et al., 2018), and/or identify candidate genes for further functional studies (Chandra et al., 2018). In the past years, we have used RNA-seq to generate 277 *L. niger* queen transcriptomes from tissues that regulate insect behavior (brain and fat body) or detect environmental cues (antennae). The tissues were dissected from queens at many different stages of colony development, and from founding queens that were experimentally manipulated to show variation in brood care behavior (Table 1) (Majidifar et al., 2024).

Table 1. RNA-seq samples used to investigate the transcriptomic basis of queen behavioral specialization.

	Species	Queen phenotypes	Behavioral data	Tissue	Number of RNA-seq samples
Dataset #1	*Lasius niger*	Founding queens before and after worker emergence	Yes	Brain	10
Dataset #2	*Lasius niger*	Same-age founding queens experimentally provided with workers or without workers, and with food or without food	Yes	Brain	23
Dataset #3	*Lasius niger*	Founding queens collected after the nuptial flight, then with eggs, with larvae, with first workers, and established queens of different ages: 6 months, 1 year, 2 years and 3 years	No	Brain Fat body	112
Dataset #4	*Lasius niger*	Same-age founding queens experimentally provided with brood or without brood	Yes	Brain	24
Dataset #5	*Lasius niger*	Same-age founding queens experimentally provided with workers, without workers or with workers separated from the queen and the brood by a wire mesh	Yes	Brain Antennae	72
Dataset #6	*Lasius niger*	Same-age founding queens experimentally provided with workers, without workers or with workers that were removed after 3 days	Yes	Brain	36

We are currently using these existing resources to perform large-scale transcriptomic analyses to identify candidate genes and/or pathways that correlate with the queen behavioral specialization. To ensure a robust identification of candidate genes and/or pathways, we apply the following analytical strategy. First, we perform differential gene expression analyses to compare pluripotent queens (usually without workers) and established queens (usually with workers). We explore doing so independently for each RNA-seq dataset and/or with a large analysis over all datasets. The differential expression analyses are performed by comparing models with and without the variable of interest with the likelihood ratio test (LRT) function of the R package DESeq2 (Love et al., 2014). This approach generates several lists of potential candidate genes, allowing us to rank genes according to the consensus across lists. Second, we explore the candidates to extract genes with putative behavior-related functions in the brain.

Third, because the queen behavioral specialization is a complex phenotypic response, we favor candidates that have regulatory functions and/or sit relatively upstream of broader physiological cascades. To do so, we use the available RNA-seq database (Table 1) to build gene expression networks using weighted gene co-expression network analyses (WGCNA) (Langfelder & Horvath, 2008). We use these networks to assess the centrality of candidates and characterize their position and influence in their respective modules of co-expression. More generally, these networks may provide a more integrated, finer approach to identify relevant transcriptomic signals and/or a complementary approach to search for entire pathways that correlate with the queen behavioral variation.

Development of a protocol for functional validation of candidate mechanisms

Ongoing PhD thesis
Maximilian Bolder (2022 – present)

Although powerful, transcriptomic analyses remain correlative in nature and cannot demonstrate a causal link between candidate genes and/or pathways and phenotypic variation. For this reason, we are currently developing a protocol to functionally validate the role of candidate mechanisms. We have already established a micro-injection protocol that allows injecting volumes up to 0.5µl in the head capsule of *L. niger* queens, with very limited mortality (<5%). This opens the possibility to deliver small molecules to manipulate specific pathways in the brain or associated glands via injection of activators or inhibitors of these pathways (*e.g.*, using methoprene or precocene to manipulate the JH pathway) and/or double-stranded RNA to knockdown the expression of specific genes via RNA interference (RNAi). We are currently troubleshooting our RNAi protocol to produce a robust, highly repeatable downregulation of the target gene expression.

Our next objective will be to use the RNAi protocol to investigate whether downregulating the expression of candidate genes affects the queen behavioral specialization in response to the presence of workers. For example, if a candidate that is overexpressed when workers are present actually induces the queen behavioral specialization in response to the presence of workers, we predict that its downregulation will produce queens that always care for the brood, irrespective of the presence of workers. Once candidates will be validated, we will characterize the temporal dynamics of candidate gene expression by performing time course expression analyses after manipulating the presence of workers (Libbrecht et al., 2018) and use RNA-seq to investigate the transcriptome-wide impact of the downregulation. It may be that the behavioral responses to such experimental manipulations of molecular mechanisms require finer assessments of the queen behavior than what can be provided by our setup for parallel behavioral analyses (Figure 14). To address this potential issue, we are in the process of developing another setup that consists in close-up filming of the head and antennae of

individual queens harnessed to a fixed support (Figure 17). This method may be combined with the use of an open-access software, such as SLEAP (Pereira et al., 2022), for the automated detection and tracking of specific body parts (*e.g.*, head, antennal segments). With this protocol, we will be able to expose queens to experimental manipulations via micro-injection while already being filmed, which will allow detecting early behavioral responses, as well as quantifying how behavioral modifications change over time after treatment.

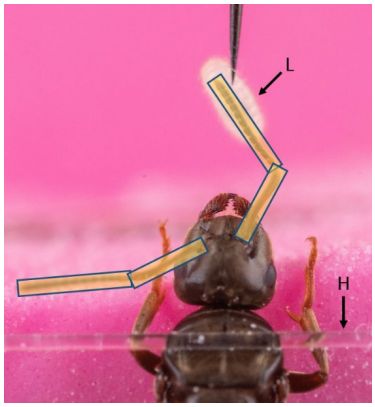

Figure 17. The setup for close-up behavioral analyses. Queens are harnessed (H) to prevent them from moving. We film the queens and use automated detection of multiple body parts to quantify the behavioral response to brood or other cues (L). As an example, the detection of the antennae is illustrated here by colored rectangles.

3.3 – Evolutionary insights into the reversibility of queen specialization

Our recent work revealed that established ant queens that have been specialized for several years readily revert to expressing brood care upon the experimental removal of their workers (Figure 15B). We originally found such reversibility of the queen specialization in two species of ants that diverged more than 100 million years ago (Majidifar et al., 2024). However, on-going experiments suggest that it is not always the case, as there seems to be variation across ant species in the ability of established queens to revert to behavioral pluripotency. Our objective is to better understand this variation and to determine why the reversibility of queen specialization was maintained over evolutionary time.

Fitness benefits of the reversibility of queen specialization

Ongoing PhD thesis
Hugo Le Lay (2024 – present)

One hypothesis to explain why established ant queens conserve the ability to care for the brood is that it would provide them with an opportunity to produce workers again in the event that they lose all of them. This hypothesis makes two predictions. The first one is that some conditions result in queens losing all their workers, for example in young colonies with limited number of workers and/or if queens are more resistant to stressful conditions than workers. To test this first prediction, we will investigate whether queens show better resistance to stress than workers, which would support the hypothesis that established queens may be left without workers in some conditions. To do so, we will expose young *L. niger* colonies to either a pathogen, a drought treatment or a starvation treatment. We will then monitor the survival over time of queens and workers and determine whether one or several of the stress treatments kill

all workers but not the queen. The second prediction is that queens can start a new colony again upon losing their workers. To test it, we will use established *L. niger* colonies to experimentally remove all the workers, and isolate the queens with either no brood, some eggs, some larvae or some pupae. We will also investigate the effect of other factors, such as queen age and nutritional status. We will then monitor queen survival, brood development, and the production of workers. We will also film the queens three times per week to record their brood care behavior. These experiments will inform us on the potential benefits for established queens to express brood care behavior when isolated from their workers.

Variation across ant species in the reversibility of queen specialization

Ongoing PhD thesis
Maximilian Bolder (2022 – present)

Another, non-mutually exclusive hypothesis is that the reversibility of queen specialization is preferentially found in species where queens naturally express brood care behavior at some point in their life. For example, in species with independent colony foundation, queens need to express brood care to produce the first workers, while in species with dependent colony foundation, queens found their colony with the help of workers, and thus never have to express brood care (Peeters, 2020). According to this hypothesis, we would expect variation across ant species in the reversibility of queen specialization, and this variation would be associated with species-specific differences in foundation strategy and/or other colony-level or individual level life-history traits.

We originally reported the behavioral flexibility of queens in two species, *L. niger* and *T. nylanderi* (Majidifar et al., 2024), but both have single-queen colonies (monogyny), high morphological differentiation between queens and workers (caste polymorphism) and queens that must express brood care during colony foundation (independent colony founding). To better understand the evolutionary history of the reversibility of queen specialization, we investigated the brood care behavior of established queens, both with and without workers, in a larger, more diverse set of ant species. To gain access to these species, we implemented several strategies: we used ant species kept in our laboratory, we conducted dedicated field trips to Germany, France, Peru and the USA, and we initiated collaborations with Brendan Hunt (University of Georgia, USA) and Guojie Zhang (University of Copenhagen, Denmark). We quantified the effect of the presence of workers on the brood care behavior of a total of 466 queens from >65 ant species (the exact number will depend on pending confirmations of species determination). It is important to note that many of those species are represented by a limited number of independent replicates (>30 species with less than three queens). This mostly concerns the species that were observed directly in field research stations in Peru and in the USA, and that were not identified yet at the time of the observations. We are in the

process of curating and analyzing the dataset, which currently contains 37 ant species with at least three independent replicates (Table 2).

Table 2. Current list of 37 species with at least three independent replicates. One independent replicate corresponds to the quantification of the brood care behavior of one queen, with and without workers, from video recording. We used only one queen per colony, even in species with multiple-queen colonies. msp. = morphospecies.

Species	Genus	Subfamily	Number of replicates
Temnothorax longispinosus	*Temnothorax*	Myrmicinae	30
Temnothorax rugatulus	*Temnothorax*	Myrmicinae	30
Crematogaster msp.1	*Crematogaster*	Myrmicinae	28
Solenopsis Invicta	*Solenopsis*	Myrmicinae	26
Temnothorax americanus	*Temnothorax*	Myrmicinae	24
Stigmatomma pallipes	*Stigmatomma*	Amblyoponinae	21
Linepithema humile	*Linepithema*	Dolichoderinae	18
Cephalotes cf. dentidorsum	*Cephalotes*	Myrmicinae	17
Monomorium pharaonic	*Monomorium*	Myrmicinae	17
Anoplolepis gracilipes	*Anoplolepis*	Formicinae	15
Cardiocondyla obscurior	*Cardiocondyla*	Myrmicinae	15
Dolichoderus plagiatus	*Dolichoderus*	Dolichoderinae	15
Myrmica punctiventris	*Myrmica*	Myrmicinae	15
Pogonomyrmex barbatus	*Pogonomyrmex*	Myrmicinae	15
Paratrechina longicornis	*Paratrechina*	Formicinae	14
Aphaenogaster picea	*Aphaenogaster*	Myrmicinae	12
Cyphomyrmex cf. minutus	*Cyphomyrmex*	Myrmicinae	12
Formica rufibarbis	*Formica*	Formicinae	9
Lasius americanus	*Lasius*	Formicinae	7
Camponotus americanus	*Camponotus*	Formicinae	6
Tetramorium caespitum	*Tetramorium*	Myrmicinae	6
Camponotus lateralis	*Camponotus*	Formicinae	5
Carebara msp.1	*Carebara*	Myrmicinae	5
Formica fusca	*Formica*	Formicinae	5
Tetramorium bicarinatum	*Tetramorium*	Myrmicinae	5
Cephalotes cf. dentidorsum	*Cephalotes*	Myrmicinae	4
Crematogaster msp.2	*Crematogaster*	Myrmicinae	4
Pheidole pallidula	*Pheidole*	Myrmicinae	4
Crematogaster msp.3	*Crematogaster*	Myrmicinae	3
Diacamma rugosum	*Diacamma*	Ponerinae	3
Lasius emarginatus	*Lasius*	Formicinae	3
Lasius flavus	*Lasius*	Formicinae	3
Odontomachus msp.1	*Odontomachus*	Ponerinae	3
Pheidole msp.1	*Pheidole*	Myrmicinae	3
Plagiolepis msp.1	*Plagilepis*	Formicinae	3
Rhytidoponera metallica	*Rhytidoponera*	Ectatomminae	3
Solenopsis cf. corticalis	*Solenopsis*	Myrmicinae	3

With our ongoing cross-species comparative analyses, we aim to map the reversibility of queen specialization on the ant phylogeny to identify the factors and species-specific life-history traits that underlie variation among species. Although analyses are currently in progress, we have already identified variation among species in the behavioral response of queens to the experimental manipulation of the presence of workers. This variation can broadly be categorized into three distinct patterns.

First, queens in most species expressed no or very little brood care in presence of workers but showed an increase in brood care behavior upon the experimental removal of workers, as exemplified by *Myrmica punctiventris* (Figure 17A). This confirms that the social control of queen specialization that we reported in *L. niger* (Majidifar et al., 2024) is common in ants.

Second, we found that queens in a few species never expressed brood care behavior, irrespective of the presence of workers, as illustrated by *Linepithema humile* (Figure 17B). This suggests a constitutive specialization of queens in egg production that is not dependent on the social context.

The third pattern, found in a few species as well, was characterized by queens always expressing brood care, even in presence of workers, and was best exemplified by *Cardiocondyla obscurior* (Figure 17C). This interesting finding indicates that established queens in mature colonies of some ant species are not specialized in egg production and contribute to non-reproductive tasks as well. We now aim to associate this interspecific variation in the expression of brood care behavior in established queens with species-specific traits and the phylogenetic signal to retrace the evolutionary history of queen specialization in ants.

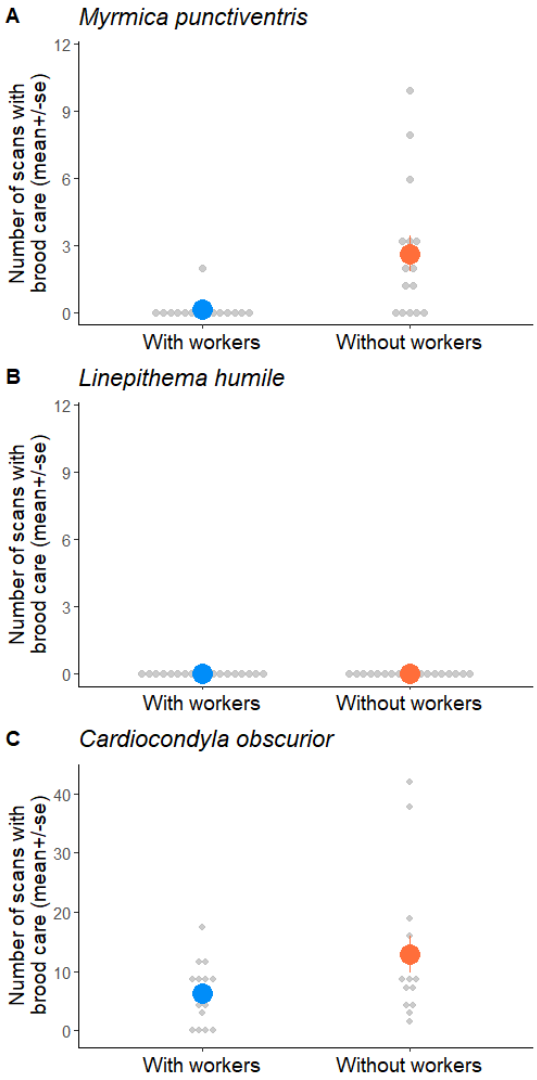

Figure 17. The effect of worker presence on queen brood care behavior differed across ant species. (A) *Myrmica punctiventris*. The removal of workers stimulated brood care behavior in queens (n=15, P=0.006). (B) *Linepithema humile*. Queens never expressed brood care, irrespective of worker presence (n=18, P=1). (C) *Cardiocondyla obscurior*. Queens always expressed brood care, with weak evidence for an effect of workers (n=15, P=0.05).

3.4 – Conclusion of Part 3

Our study of the factors controlling the behavioral specialization of ant queens in the process of colony foundation demonstrated that the presence of workers is necessary and sufficient to inhibit brood care, and thus to initiate the queen specialization in egg production (Majidifar et al., 2024). Interestingly, we found the queen specialization to be reversible, as established queens revert to expressing behavioral pluripotency upon removal of their workers, even after having been specialized for several years (Majidifar et al., 2024). These results have opened exciting avenues of research on the social and environmental factors that regulate the ontogenic process of colony foundation, the mechanisms that control the behavioral specialization of queens, and the evolutionary forces that maintain their behavioral flexibility.

The ongoing behavioral analyses of queens in multiple species of ants indicate that the reversibility of queen specialization is commonly found across the ant phylogeny. Furthermore, our mechanistic investigations of how social partners affect queen behavior in *L. niger* may reveal that the queen behavioral specialization is a mere passive consequence of queens and workers having different response thresholds to larval cues, *i.e.*, different positions on a continuous scale of likelihood to express brood care. This would stand in contrast with the typical view of queens as being fundamentally different from workers in terms of behavior. More generally, our research on the social control of queen specialization in ants overturns the long-standing notion of social insect queens as intrinsically specialized egg-laying machines.

Additional lines of research

In recent years, I have started to complement my main research agenda on the regulation and evolution of division of labor in insect societies with additional, more applied lines of research, which I briefly present in this part.

Ants are ecosystem engineers due to their large biomass, extensive interactions with plants and animals, and their impact on the physicochemical properties of the soil (Cammeraat & Risch, 2008; Chomicki & Renner, 2017; Folgarait, 1998; Holec & Frouz, 2006; Schultheiss et al., 2022). Because ants are central to ecosystem functioning, any environmental changes that affect the density and/or distribution of ant populations have the potential to negatively impact critical ecological processes. Ants can either be the target or the cause of such impacts, as some environmental modifications may produce a decrease in the density of native populations of ants, while others may consist in human-driven introductions of invasive ant species.

To address these questions, since my recruitment as a CNRS researcher at IRBI, I have initiated two projects building on previous research efforts. First, integrating my research on ant colony foundation within the broader framework of the ontogeny of biological systems led me to consider how the highly sensitive foundation stage may be affected by human-driven environmental changes. Notably, environmental pollutants have been shown to impact the development of both multicellular organisms (Ko et al., 2019) and superorganisms (Seidenath et al., 2021). To further investigate the effects of human perturbations on colony foundation in social insects, I secured a PhD grant from the SSBCV doctoral school (Universities of Tours and Orléans) to study the impact of heat stress on colony foundation in *Lasius niger*. This project is conducted in collaboration with Marlène Goubault (PU, University of Tours) and Irene Villalta (MCU, University of Tours) and involves a PhD student under our joint supervision.

Second, my research leveraged the unique characteristics of invasive ant species to conduct experimental manipulations of the social environment and controlled crosses in the laboratory, revealing the extreme specialization that underlie the functioning of these highly populous insect societies. Using invasive ants as study organisms to address fundamental questions in social insect biology has uncovered intrinsic components of caste determination (Libbrecht et al., 2011), sterile workers that have lost the typical physiological and molecular response to queen loss (Lenhart et al., 2025; Majoe et al., in preparation), and constitutively specialized queens that never engage in brood care, regardless of social context (section 3.3). These findings demonstrate that division of labor in invasive ant colonies relies on the irreversible commitment of colony members to their specialized roles. This highly structured social organization likely contributes to the ecological success, high productivity and invasive potential of these species

(Holway et al., 2002). These insights have fueled my interest in understanding the impact and expansion of invasive ants in newly colonized areas. Together with Jean-Luc Mercier (MCU, University of Tours), I co-lead the FIVALO project (*Mise en place d'un réseau de surveillance des espèces de fourmis invasives dans les régions du Val de Loire*), funded by the French Biodiversity Office (OFB). This project aims to study the distribution of invasive ants in the Loire valley.

Impact of thermal stress on the success of colony foundation in ants

Ongoing PhD thesis
Alice Roux (2024 – present)

Climate change not only increases average temperatures but also the frequency and the intensity of extreme thermal events, such as heat waves (Meehl & Tebaldi, 2004). These thermal perturbations have the potential to negatively impact the reproduction and survival of living organisms (Cinto Mejía & Wetzel, 2023; Filazzola et al., 2021; Pilakouta et al., 2023), and thus the functioning of entire ecosystems. These impacts are amplified when the perturbations affect species that are ecosystem engineers, which are characterized by the many interactions with their biotic and abiotic environment. It is therefore important to understand the impact of heat stress on ant populations.

So far, this question has been primarily addressed in mature colonies of ants (Diamond et al., 2012; Perez & Aron, 2020; Roeder et al., 2021), which are relatively protected against thermal perturbations by the architecture of the nest and thus the ability of ants to move more or less deep underground to buffer their exposure to temperature variation. However, very little is known on the effect of heat stress on founding queens, the newly mated queens that initiate a new colony. These queens are much more exposed to temperature changes, for example during the nuptial flight, which generally takes place in late spring or summer, but also in the following hours, when the queens walk around looking for a nest site to settle in. Then, they may experience very high temperatures, especially in urban areas. To produce their first cohort of workers, the founding queens must produce eggs and care for the brood to ensure its proper development, all of which could be affected by an early exposure to high temperatures. Therefore, we ask the question whether an exposure to heat stress affects the success of colony foundation in ants.

To start addressing this question, we used the black garden ant (*L. niger*). We collected founding queens after the nuptial flight and exposed them to either an acute heat stress of 43°C for 4 hours, or a control temperature of 25°C for 4 hours. After the treatment, all queens were kept at 21°C for 50 days to monitor the survival of the queens, their foundation success, their production of eggs and the development of their brood. We found that the heat treatment increased the mortality of founding queens. While no control queen died during the experiment, 8 out of 25 treated queens died within 50 days after the heat stress (Figure 18A). For the rest of the analyses, we only considered the queens that were still alive at the end of the experiment. We used the ability of queens to produce workers within 50 days after the treatment as a proxy for the success of colony foundation. While 17 of the 25 control queens had produced workers by day 50, we found that this proportion was drastically reduced in treated queens, as only one of them managed to produce workers (Figure 18B).

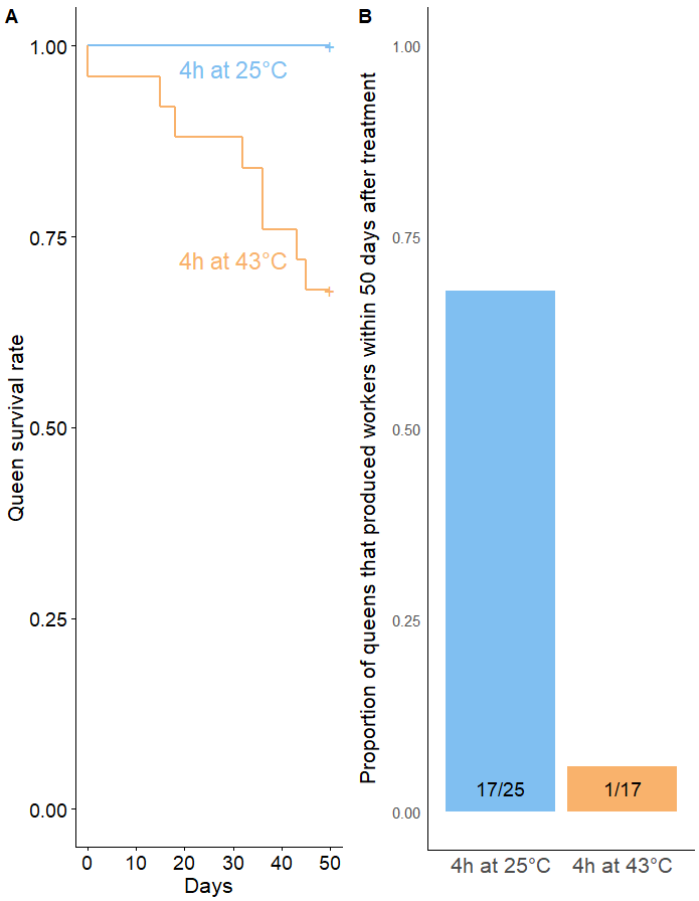

Figure 18. The heat treatment reduced the success of colony foundation. (A) The heat treatment (n=25) increased queen mortality compared to the control treatment (n=25, P<0.0001). (B) The heat treatment (n=25) reduced the proportion of queens that produced workers within 50 days after treatment compared to the control treatment (n=17, P<0.0001).

To better understand the impact of heat stress on colony foundation, we analyzed its effects on brood production and development. We found that the change over time in the number of eggs, larvae and pupae differed between the control and treated groups (Figure 19). Control queens roughly produced twice as many larvae as treated queens, consistent with our estimate of the hatching rate being reduced by half in treated queens compared to control queens. This difference resulted in control queens producing nearly three times as many pupae as treated queens, ultimately affecting the success of colony foundation.

Our data indicates that the impact of heat stress on worker production likely stems from effects on egg production and brood development, including a delayed egg production and a lower hatching rate resulting in less larvae and pupae. We suspect that the exposure to heat stress alters the ability of founding queens to lay viable eggs. This could come from a direct impact on the queen physiology and/or the sperm stored in the spermatheca, which could later affect egg quality. It may also be that the oocytes that are already present in the ovarioles of the founding queens at the time of the treatment are directly impacted. Another hypothesis is that the heat stress affects the ratio of viable eggs and trophic eggs produced by the founding queens. We are currently in the process of investigating these different hypotheses to better understand the impact of thermal perturbations on the foundation of ant colonies.

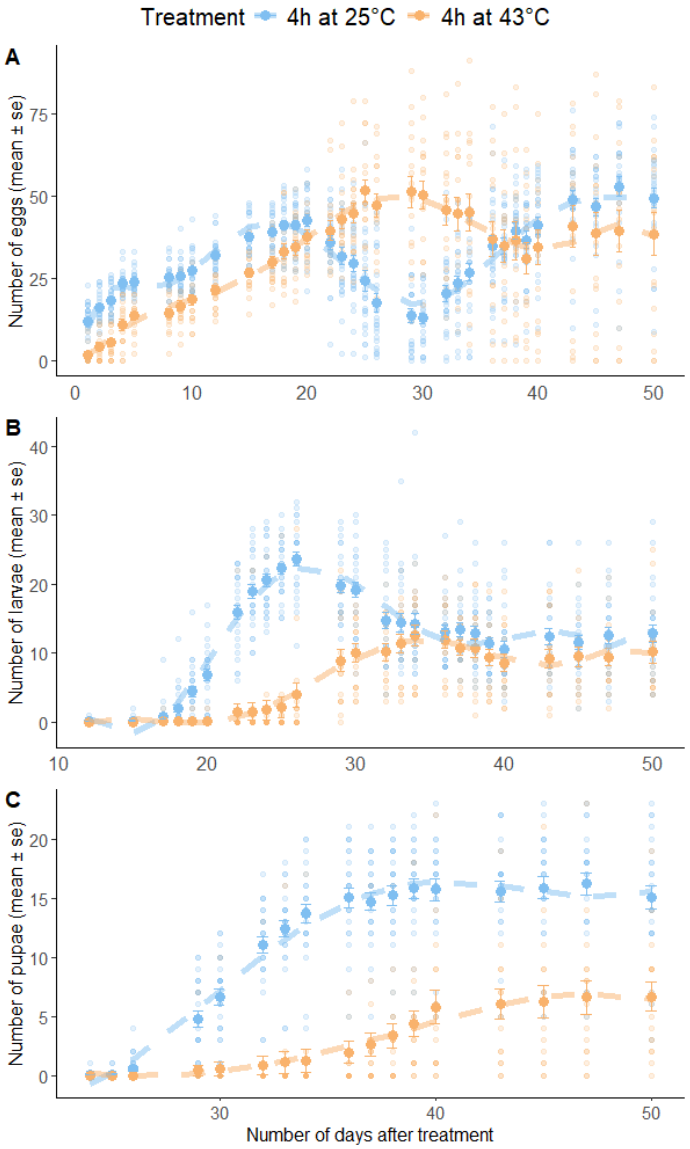

Figure 19. The heat treatment affected the time dynamics of brood production and development. Founding queens exposed to the heat (n=17) and control (n=25) treatments showed different non-linear changes over time in the number of (A) eggs (P<0.0001), (B) larvae (P<0.0001) and (C) pupae (P<0.0001).

Monitoring the expansion of invasive ants in the Loire valley

Invasive ants disrupt the balance of natural ecosystems by rapidly colonizing vast territories (Holway et al., 2002). They compete with other organisms, act as predators, and interfere with mutualistic interactions between plants and insects. In particular, they negatively impact the diversity and abundance of native ant species. Beyond ecological consequences, invasive ants also have substantial socio-economic impacts, disrupting the management of urban environments, including household waste systems, electrical networks, construction and agricultural activities, as well as public and private gardens (Angulo et al., 2022; Bertelsmeier &

Courchamp, 2014; Wong et al., 2023). To mitigate the harmful effects of invasive ant species, it is crucial to detect their presence as early as possible. However, in many cases, differentiation between native and invasive species based solely on morphology can be extremely challenging and prone to misidentification errors.

In recent years, several invasive ant species (e.g., *Tapinoma magnum*, *Lasius neglectus*, *Linepithema humile*) have established themselves in the Loire valley (Lenoir et al., 2023), yet no monitoring network existed to track their presence and geographic expansion. This absence was primarily due to the recent nature of these invasions and the difficulty in identifying problematic species. The FIVALO project aims to establish and coordinate a surveillance network to identify, detect, map, and assess the invasion levels of invasive ant species in two French regions: Pays de la Loire (PDL) and Centre-Val de Loire (CVL).

The main objective of FIVALO is to develop and implement effective methods for sample collection and species determination. To collect samples, we rely on collaborations with institutional partners such as two *conservatoires d'espaces naturels* (CEN PDL and CEN CVL) and two *fédérations de défense contre les organismes nuisibles* (FDGDON 49 and FREDON CVL). We have also developed an innovative sampling method in collaboration with the energy provider ENEDIS, which consists in collecting ants from their equipment that is malfunctioning because of infestation. To develop robust methods of species determination, we develop protocols based on chemical ecology and molecular biology. It is essential to combine different types of tools alongside morphological determination, as the effectiveness of each method varies depending on the species, with some tools being more discriminating than others. We then use the data collected to map the distribution of invasive ant species, raise awareness among the general public, local authorities, and businesses across different sectors, and develop monitoring indicators to assess the invasive potential of the species involved.

Conclusion

Understanding the reproductive division of labor between queens and workers in insect societies is a fundamental challenge in the study of social evolution. Throughout my academic career, I have combined experimental manipulations, behavioral observations and molecular analyses to investigate key aspects of reproductive specialization, including the genetic and maternal influences on caste determination, the hormonal, transcriptomic and epigenetic regulation of reproduction, and the role of the social environment in shaping central aspects of division of labor and behavioral specialization.

In recent years, I have established collaborations, secured research funding and supervised multiple student projects to explore how and why social interactions influence individual phenotypes in ant colonies. Our findings demonstrated that reproductive division of labor is not merely a fixed outcome of caste determination but is actively shaped by the social environment, with workers playing a pivotal role in inducing the behavioral specialization of queens in egg production. By showing that this queen specialization is not constitutive, but rather controlled by the social environment, our work offers novel insights that advance our understanding of division of labor in insect societies.

Synthèse en français

Régulation moléculaire et sociale de la division du travail chez les fourmis

Un défi majeur en sciences de la vie est de comprendre comment les organismes ajustent leur reproduction, leur physiologie et leur comportement en réponse aux conditions environnementales. Cette capacité à produire plusieurs phénotypes à partir d'un même génotype, appelée plasticité phénotypique, est particulièrement bien illustrée par la diversité fascinante de morphologies et de comportements observée au sein des colonies d'Hyménoptères sociaux, tels que les fourmis, les abeilles et les guêpes (Wilson, 1971). Ces sociétés d'insectes présentent une division du travail entre les reines, qui monopolisent la production des œufs, et les ouvrières, qui se spécialisent dans l'ensemble des autres tâches nécessaires au maintien de la colonie. Ces tâches non reproductrices incluent la recherche de nourriture, la construction et la défense du nid, ainsi que le soin apporté aux œufs et aux larves (Wilson, 1971). Cette division du travail reproducteur entre les castes de reines et d'ouvrières est au cœur du fonctionnement, du succès écologique et de l'évolution des insectes sociaux.

Dans ce contexte, il est essentiel de comprendre les facteurs et les mécanismes qui régulent la division du travail reproducteur dans les colonies d'insectes sociaux. La recherche sur ce sujet peut être globalement classée en deux approches principales. La première, centrée sur la détermination et la différenciation des castes, a permis d'élucider les processus régulant les trajectoires développementales alternatives qui conduisent à la production de reines et d'ouvrières (Cameron et al., 2013; Collins et al., 2020; Corona et al., 2016; Genzoni et al., 2023; Libbrecht et al., 2011; Libbrecht et al., 2013a; Libbrecht et al., 2013b; Montagna et al., 2015; Mutti et al., 2011; Psalti & Libbrecht, 2020; Schultner et al., 2023; Schwander et al., 2008; Schwander & Keller, 2008; Wheeler et al., 2006). La seconde, basée sur la comparaison des reines et des ouvrières au stade adulte, a mis en évidence un ensemble de différences phénotypiques et moléculaires spécifiques aux castes (Bonasio et al., 2012; Chandra et al., 2018; Corona et al., 2007, 2013; Feldmeyer et al., 2014; Grozinger et al., 2007; Kronauer & Libbrecht, 2018; Libbrecht et al., 2013b; Patalano et al., 2015). La première partie de ce mémoire décrit comment j'ai mis en œuvre ces deux approches afin d'améliorer notre compréhension des effets génétiques et maternels sur la détermination des castes, ainsi que d'identifier les voies moléculaires régulant la variation reproductive entre les reines et les ouvrières chez les fourmis.

Bien que les différences morphologiques entre reines et ouvrières soient fixées au stade adulte chez les Hyménoptères sociaux, des modifications de l'environnement social peuvent influencer la physiologie et le comportement individuels, et ainsi affecter la division du travail au sein de la colonie. L'exemple le plus documenté concerne les effets profonds de la perte de la reine sur l'activité reproductrice et le comportement agressif des ouvrières (Choppin et al., 2021; Heinze, 2008; Holman et al., 2010; Holman, 2014; Holman et al., 2016; Negroni et al., 2021; Ronai et al., 2016; Van Oystaeyen et al., 2014). Toutefois, d'autres membres de la colonie peuvent également influencer leurs partenaires sociaux de diverses manières, comme en témoignent les effets des larves et/ou des nymphes sur la reproduction et le comportement des ouvrières (Ulrich et al., 2016; Villalta et al., 2015), ainsi que sur la survie et le développement du couvain (Santos et al., 2024; Snir et al., 2022). La deuxième partie expose mes travaux de recherche sur la manière dont le contexte social façonne la variation phénotypique en termes de reproduction, de comportement et de longévité, ainsi que sur la productivité des colonies de fourmis.

Alors que de nombreuses études se sont concentrées sur l'impact du contexte social sur les ouvrières, l'effet de ce dernier sur le comportement des reines reste relativement méconnu, leur rôle étant généralement réduit à la reproduction. Ce biais dans la littérature a renforcé l'idée largement acceptée selon laquelle les reines seraient intrinsèquement spécialisées dans la production d'œufs dès leur stade adulte, et que cette spécialisation ne dépendrait pas des conditions environnementales. Cependant, le processus de maturation des reines d'insectes sociaux n'est pas totalement achevé au moment de leur émergence en tant qu'adultes. Cela est particulièrement bien illustré par le comportement des reines lors de la fondation des colonies : durant cette phase, les reines fondatrices expriment un large répertoire de comportements à la fois reproductifs et non reproductifs pour produire la première génération d'ouvrières (Augustin et al., 2011; Brossette et al., 2019; Cassill, 2002; Majidifar et al., 2024; Norman et al., 2016; Walsh et al., 2018; Wheeler, 1933; Woodard et al., 2013). Ce n'est qu'à partir de ce moment que les reines cessent d'exprimer des comportements non reproductifs pour se spécialiser strictement dans la ponte (Chouvenc, 2022; Majidifar et al., 2024; Woodard et al., 2013). La troisième partie de ce mémoire développe mes recherches sur les mécanismes sociaux et moléculaires contrôlant la spécialisation des reines fondatrices pluripotentes ainsi que le maintien de cette spécialisation dans les colonies établies de fourmis.

Partie 1 – La formation des reines et des ouvrières

La division du travail reproducteur chez les Hyménoptères sociaux constitue un aspect fondamental de l'organisation des colonies et un exemple frappant de plasticité phénotypique. Comprendre la variation de l'activité reproductrice entre les reines et les ouvrières nécessite d'examiner à la fois les mécanismes qui déterminent le devenir des individus au cours du développement larvaire et les voies moléculaires qui régulent les différences reproductives entre les reines et les ouvrières adultes.

1.1 – Effets intergénérationnels sur la détermination de la caste

Comprendre la division du travail reproducteur dans les colonies d'insectes sociaux implique d'élucider les facteurs et les mécanismes qui déterminent si un œuf se développera en une reine reproductive ou en une ouvrière fonctionnellement stérile. Longtemps étudiée chez les abeilles et les fourmis, la détermination de la caste a d'abord été considérée comme un processus strictement environnemental, des facteurs tels que la nutrition et la température durant le développement larvaire étant supposés dicter le devenir de l'individu (Corona et al., 2016; Psalti & Libbrecht, 2020; Schwander et al., 2010). Ainsi, les œufs femelles des Hyménoptères sociaux étaient considérés comme totipotents, ayant une probabilité équivalente de se développer en reines ou en ouvrières. Cependant, depuis le début des années 2000, plusieurs études ont mis en évidence des effets génétiques (Hartfelder et al., 2006; Hughes & Boomsma, 2008; Libbrecht et al., 2011; Schwander & Keller, 2008; Smith et al., 2008) et maternels (Libbrecht et al., 2013a; Schultner et al., 2023; Schwander et al., 2008) sur la détermination des castes, en particulier chez les fourmis. Certains œufs semblent donc avoir, dès leur ponte, une probabilité plus élevée de se développer en reines que d'autres. Dans le cadre de mon doctorat à l'université de Lausanne (Suisse), j'ai étudié les mécanismes sous-jacents aux effets génétiques et maternels sur la détermination de la caste chez les fourmis.

Des effets génétiques complexes influencent la détermination de la caste

La majorité des études ayant mis en évidence des effets génétiques sur la détermination de la caste chez les insectes sociaux se sont concentrées sur des espèces dont les colonies ne contiennent qu'une seule reine, fécondée par plusieurs mâles (Weitekamp et al., 2017). Dans ces conditions, tous les œufs femelles partagent la même mère, mais pas nécessairement le même père. Un groupe d'œufs ou d'individus issus d'un même père est appelé une *patriline*. Des différences entre *patrilines* dans les proportions relatives de reines et d'ouvrières ont été interprétées comme une preuve d'effets génétiques sur la détermination de la caste (Hughes & Boomsma, 2008; Smith et al., 2008). Ces influences génétiques ont traditionnellement été considérées comme des effets additifs résultant de simples différences alléliques. Cependant,

les comparaisons entre *patrilines* ne permettent pas de distinguer ces effets additifs d'effets plus complexes, tels que ceux dépendant de l'origine parentale des allèles. Afin de mieux comprendre la nature de ces effets génétiques, il est essentiel de quantifier les contributions relatives des deux parents au caractère étudié (Schwander & Keller, 2008). Dans cet objectif, nous avons utilisé la fourmi d'Argentine (*Linepithema humile*), qui offre la rare opportunité de réaliser des croisements contrôlés en laboratoire, afin de tester l'influence des lignées paternelles et maternelles sur la proportion de reines et d'ouvrières produites (Libbrecht et al., 2011). Nos résultats montrent que la lignée paternelle influence la production relative de reines et d'ouvrières, tandis qu'aucun effet significatif de la lignée maternelle n'a été détecté. Ces résultats révèlent que les effets génétiques sur la détermination de la caste chez *L. humile* reposent sur une architecture complexe, car des effets additifs classiques impliqueraient une influence des deux lignées parentales. La mise en évidence d'effets spécifiques à l'origine parentale est donc incompatible avec de simples différences alléliques et suggère l'implication d'influences génétiques plus complexes, telles que des effets épigénétiques, dans la détermination de la caste.

L'hormone juvénile régule les effets maternels sur la détermination de la caste

Les colonies de fourmis ne commencent à produire des individus sexués (nouvelles reines et mâles) qu'après plusieurs années. Des expériences d'hibernation artificielle chez la fourmi moissonneuse *Pogonomyrmex rugosus* ont démontré que seules les colonies ayant subi au moins une hibernation produisent de nouvelles reines. De plus, cet impact de l'hibernation résulte d'effets maternels : l'exposition de la reine au froid s'est révélée à la fois nécessaire et suffisante pour stimuler la production de nouvelles reines, indépendamment du statut d'hibernation des ouvrières (Schwander et al., 2008). Afin de mieux comprendre ces effets maternels sur la détermination de la caste, nous avons mené une série d'expériences visant à étudier les modifications moléculaires chez les reines les rendant plus susceptibles de produire de nouvelles reines en réponse à l'hibernation (Libbrecht et al., 2013a). En combinant des traitements hormonaux, des expositions expérimentales au froid et des mesures d'expression génique (par RT-qPCR), nous avons démontré expérimentalement l'implication de l'hormone juvénile dans la régulation des effets maternels sur la détermination de la caste chez *P. rugosus*. Les traitements hormonaux à l'aide d'un analogue synthétique de l'hormone juvénile (méthoprène) ont permis de reproduire les effets de l'hibernation, tant sur le plan phénotypique (augmentation de la production de nouvelles reines) que moléculaire (effets similaires sur l'expression génique et la composition protéomique des œufs). Sur la base de ces résultats, nous avons proposé un modèle impliquant l'hormone juvénile, la signalisation de l'insuline et les voies de la vitellogénine dans la régulation des effets maternels sur la détermination de la caste chez *P. rugosus*.

1.2 – Voies moléculaires régulant la reproduction des reines

Comprendre la division du travail reproducteur implique d'examiner les mécanismes moléculaires qui régulent la variation de l'activité reproductrice entre les reines et les ouvrières. L'approche la plus couramment utilisée pour aborder cette question repose sur la comparaison des reines et des ouvrières adultes dans des colonies matures d'insectes sociaux (Bonasio et al., 2012; Chandra et al., 2018; Corona et al., 2007, 2013; Feldmeyer et al., 2014; Grozinger et al., 2007; Kronauer & Libbrecht, 2018; Libbrecht et al., 2013b; Patalano et al., 2015). Ces investigations ont révélé le rôle central des voies de l'hormone juvénile, de la vitellogénine et de la signalisation de l'insuline, non seulement dans la régulation des variations d'activité reproductrice au sein des colonies, mais aussi dans l'évolution de la division du travail reproducteur chez les insectes sociaux (Chandra et al., 2018; Corona et al., 2007, 2013; Grozinger et al., 2007; Kronauer & Libbrecht, 2018; Libbrecht et al., 2013b). Mes travaux de recherche ont inclus de telles comparaisons entre reines et ouvrières, contribuant ainsi aux avancées dans la compréhension mécanistique de la division du travail chez les fourmis. Tout d'abord, durant mon doctorat, j'ai étudié la fonction de la vitellogénine dans la régulation de la reproduction et du comportement (Corona et al., 2013). Ensuite, en tant que chercheur postdoctoral à l'université Rockefeller (New York, USA), j'ai contribué à un projet explorant le rôle central de la voie de signalisation de l'insuline dans le contrôle des variations reproductives chez les fourmis (Chandra et al., 2018).

Les gènes de la vitellogénine remplissent des fonctions spécifiques aux castes

La vitellogénine est une protéine clé de la reproduction des insectes, servant principalement de source d'énergie pour l'embryon au sein de l'œuf en développement (Hagedorn & Kunkel, 1979). Des recherches menées chez l'abeille domestique ont montré que cette protéine joue également un rôle dans le comportement des ouvrières adultes (Amdam et al., 2003; Corona et al., 2007). Chez les fourmis, le gène codant pour cette protéine a subi des duplications, et de nombreuses espèces possèdent plusieurs copies, dont les fonctions restent en grande partie inconnues. Afin d'évaluer si ces copies de vitellogénine remplissent des fonctions distinctes chez les fourmis, nous avons étudié l'expression de deux gènes de la vitellogénine (*PbVg1* et *PbVg2*) chez les reines, les nourrices et les fourrageuses de la fourmi moissonneuse *Pogonomyrmex barbatus* (Corona et al., 2013). Nous avons observé que l'expression de *PbVg1*, plus élevée chez les reines que chez les ouvrières, correspond au rôle ancestral de la vitellogénine dans la reproduction. En revanche, nos résultats indiquent que *PbVg2*, dont l'expression était plus importante chez les fourrageuses que chez les nourrices ou les reines, a été co-opté pour remplir des fonctions comportementales non reproductives. Ces résultats suggèrent qu'après duplication, le gène de la vitellogénine a subi une sous-fonctionnalisation

chez *P. barbatus* (Lynch & Force, 2000), menant à une expression spécifique aux castes et aux comportements associés aux fonctions reproductives et non reproductives. Cette sous-fonctionnalisation et l'acquisition de nouveaux rôles dans un contexte social sont appuyées par la reconstruction des relations phylogénétiques entre les copies des gènes de vitellogénine chez différentes espèces de fourmis. En effet, nos analyses phylogénétiques ont révélé qu'une première duplication du gène ancestral de la vitellogénine est survenue après la divergence entre les clades ponéroïdes et formicoïdes, suivie de duplications ultérieures dans différentes lignées de fourmis. Cet article a posé les bases de plusieurs autres articles qui ont confirmé et approfondi la diversité des fonctions exercées par les gènes de la vitellogénine chez les fourmis (Kohlmeier et al., 2018, 2019; Morandin et al., 2014; Oxley et al., 2014).

Rôle de l'insuline dans l'évolution de la division du travail reproducteur

Chez les fourmis, la division du travail reproducteur entre les castes de reine et d'ouvrière a une origine unique et résulte d'une évolution à partir d'un ancêtre subsocial qui alternait entre phases de reproduction et phases de soins au couvain (Hunt, 2007; West-Eberhard, 1987). Pour mieux comprendre comment ces phases ont été modifiées en une asymétrie fixe entre les reines et les ouvrières, il est essentiel d'examiner la régulation de la division du travail reproducteur, c'est-à-dire les mécanismes permettant aux reines de pondre des œufs tout en empêchant les ouvrières de le faire. À cette fin, nous avons réalisé une étude comparative impliquant du séquençage ARN afin d'analyser l'expression génique dans le cerveau d'individus reproducteurs et non reproducteurs appartenant à sept espèces réparties dans la phylogénie des fourmis (Chandra et al., 2018). Nos comparaisons ont révélé qu'un gène candidat, *insulin-like peptide 2* (*ilp2*), codant pour un peptide de type insuline, était systématiquement surexprimé chez les individus reproducteurs dans toutes les espèces étudiées. Ce résultat suggère un rôle ancestral de *ilp2* dans la régulation de la reproduction chez les fourmis. L'hypothèse la plus parcimonieuse pour expliquer cette observation est que *ilp2* était déjà impliqué dans la reproduction chez l'ancêtre commun des fourmis. Nous avons ainsi formulé l'hypothèse que ce gène, et plus largement la voie de signalisation de l'insuline, a joué un rôle clé dans l'émergence de la division du travail reproducteur.

Pour tester cette hypothèse, nous avons utilisé *Ooceraea biroi*, une espèce de fourmi clonale dans laquelle les signaux larvaires inhibent la reproduction des adultes en supprimant l'expression de *ilp2*, entraînant ainsi une alternance de phases de reproduction et de soins au couvain au sein du cycle colonial, ce qui rappelle la subsocialité ancestrale. Nous avons montré que l'injection du peptide produit par *ilp2* produisait des individus dont la reproduction n'était plus inhibée par la présence de larves. Ce résultat confirme expérimentalement le rôle de *ilp2* dans la régulation de la reproduction en réponse au contexte social et suggère un rôle

potentiel dans l'évolution des castes de reines et d'ouvrières. En augmentant artificiellement *ilp2*, nous avons généré des fourmis qui se reproduisaient en continu, indépendamment de la présence des larves, et qui, par conséquent, ressemblaient à des reines. Des expériences complémentaires ont montré qu'une augmentation naturelle de *ilp2* pouvait être induite par une augmentation de la quantité de nourriture reçue pendant le développement larvaire. Dans l'ensemble, ces résultats suggèrent qu'une variation interindividuelle de l'apport nutritionnel au stade larvaire, et donc de l'expression de *ilp2*, pourrait être à l'origine évolutive de la division du travail reproducteur chez les fourmis parce qu'elle aurait produit une variation entre individus dans leur réponse physiologique à la présence de larves (Chandra et al., 2018). Une telle variation dans l'effet des larves sur la reproduction aurait favorisé l'émergence des castes de reines et d'ouvrières. Nous avons synthétisé ces résultats dans un modèle impliquant *ilp2*, et plus largement la voie de signalisation de l'insuline, dans l'évolution de la division du travail reproducteur chez les fourmis (Chandra et al., 2018).

Conclusion de la Partie 1

La division du travail reproducteur est un élément central du fonctionnement et de l'évolution des sociétés d'insectes. Pour mieux comprendre sa régulation, il est essentiel d'étudier ce qui fait une reine, c'est-à-dire les mécanismes qui sous-tendent la détermination de la caste et qui contrôlent l'activité reproductrice des adultes. Cette première partie décrit comment mes travaux de recherche chez les fourmis ont contribué à notre compréhension des effets génétiques et maternels sur la détermination de la caste, ainsi qu'à l'identification du rôle des voies de signalisation de l'hormone juvénile, de la vitellogénine et de l'insuline dans la régulation et l'évolution de la division du travail reproducteur. Ces études ont été menées relativement tôt dans ma carrière académique, puisque toutes, sauf une, faisaient partie de mon doctorat. Les résultats obtenus ont ouvert des perspectives thématiques et techniques qui ont influencé mes recherches de plusieurs manières.

Le premier impact a été la mise en évidence d'effets génétiques sur la détermination de la caste (Libbrecht et al., 2011) et la spécialisation comportementale (Libbrecht & Keller, 2012) qui ne correspondaient pas à de simples différences alléliques, suggérant ainsi des influences génétiques plus complexes, telles que des effets épistatiques et/ou spécifiques à l'origine parentale. Plus précisément, ces résultats ont identifié les mécanismes épigénétiques comme des candidats potentiels à la régulation de la plasticité phénotypique, ce qui a initié mon intérêt pour le rôle des processus épigénétiques dans la division du travail reproducteur et comportemental chez les insectes sociaux (Libbrecht et al., 2016, 2020).

Deuxièmement, ces travaux m'ont sensibilisé aux limites des comparaisons entre les castes morphologiquement distinctes de reines et d'ouvrières dans l'étude des mécanismes

moléculaires de la division du travail (Kronauer & Libbrecht, 2018). Une première limite est que les castes diffèrent par de nombreux traits interdépendants (e.g., reproduction, comportement, morphologie, nutrition, génétique), rendant difficile l'association des différences moléculaires à un phénotype spécifique. Une seconde limite tient au fait que les castes chez les Hyménoptères sont généralement fixées au stade adulte, ce qui rend ces études corrélatives et empêche la validation fonctionnelle des mécanismes candidats par des manipulations expérimentales.

Enfin, j'ai découvert qu'une approche alternative pour étudier la division du travail reproducteur consiste à utiliser des systèmes d'étude où la reproduction est plus plastique et manipulable expérimentalement (Kronauer & Libbrecht, 2018). *O. biroi* offre cette flexibilité, en permettant des manipulations fines de la présence de larves et, par conséquent, de l'activité reproductrice (Libbrecht et al., 2016, 2018; Oxley et al., 2014; Ravary et al., 2006; Ulrich et al., 2016). J'ai également réalisé que ces manipulations expérimentales de l'environnement social ne se limitaient pas à *O. biroi* et pouvaient être plus largement appliquées pour explorer l'influence de l'environnement social sur le comportement et la reproduction chez les insectes sociaux.

Partie 2 – L'importance de l'environnement social

Bien que la division du travail entre les reines et les ouvrières soit souvent considérée comme le résultat fixe de la détermination de la caste lors du développement larvaire, le contexte social peut influencer de manière dynamique les phénotypes individuels à l'âge adulte, entraînant une plasticité remarquable du comportement et de la reproduction. Cette partie explore comment les interactions sociales régulent la variation phénotypique chez les fourmis, depuis les mécanismes moléculaires sous-jacents à la plasticité reproductive jusqu'aux conséquences plus larges sur la longévité individuelle et la productivité de la colonie.

2.1 – Régulation moléculaire du contrôle social de la reproduction

Au cours de la dernière décennie, la fourmi clonale *Ooceraea biroi* s'est imposée comme un système d'étude puissant pour examiner les facteurs et mécanismes qui contrôlent la variation de l'activité reproductrice dans les colonies de fourmis (Libbrecht et al., 2016, 2018; Oxley et al., 2014; Ulrich et al., 2016). Cette espèce a perdu la caste des reines, et toutes les ouvrières se reproduisent par parthénogenèse. Leur reproduction est régulée par la présence de larves, qui inhibent la ponte (Ravary et al., 2006). Cet effet de l'environnement social est particulièrement intéressant pour plusieurs raisons. Premièrement, il entraîne un cycle de vie phasique dans lequel chaque colonie alterne entre une phase reproductive (en l'absence de larves) et une phase de soins au couvain (en présence de larves). Étant donné que ce cycle régulé par l'environnement social est similaire au cycle subsocial de l'ancêtre des fourmis (Kelstrup et al., 2018; Kronauer & Libbrecht, 2018; West-Eberhard, 1987), *O. biroi* constitue un modèle unique pour l'étude de l'évolution de la division du travail reproducteur (Chandra et al., 2018). Deuxièmement, nous avons découvert que les larves de *O. biroi* inhibent l'activité reproductrice des ouvrières adultes de manière dose-dépendante (Ulrich et al., 2016), permettant ainsi des manipulations expérimentales précises de l'activité reproductrice. Enfin, *O. biroi* offre la possibilité de contrôler des facteurs confondants généralement associés aux comparaisons entre castes (par exemple, l'âge, l'expérience individuelle, le patrimoine génétique) (Kronauer & Libbrecht, 2018). Pour toutes ces raisons, en tant que chercheur postdoctoral à l'université Rockefeller (New York, États-Unis), j'ai utilisé ce système d'étude pour examiner les changements transcriptomiques et les processus épigénétiques qui régulent l'activité reproductrice chez les fourmis.

Régulation transcriptomique du contrôle social de la reproduction

Pour étudier les modifications de l'expression génique sous-jacentes à l'activation ou à l'inhibition de la reproduction, nous avons développé un protocole chez *O. biroi* permettant de collecter des fourmis génétiquement identiques et du même âge à différents points dans le

temps lorsqu'elles modifient leur activité reproductrice en réponse à la présence de larves (Libbrecht et al., 2018). Tout d'abord, nous avons produit des clones du même âge qui soit se reproduisaient (en l'absence de larves), soit ne se reproduisaient pas (en présence de larves). Nous avons ensuite manipulé la présence de larves afin d'induire soit la stimulation (par le retrait de toutes les larves), soit l'inhibition (par l'ajout de larves) de la reproduction. Pour chacune de ces deux transitions, nous avons collecté des individus à différents moments et analysé l'évolution de l'expression des gènes dans le cerveau (via le séquençage ARN) au cours du temps suivant ces manipulations de l'environnement social.

Nos analyses ont révélé des différences entre les deux transitions, tant dans la nature des gènes impliqués que dans la dynamique temporelle des changements d'expression génique (Libbrecht et al., 2018). Nous avons observé que l'introduction de larves inhibant la reproduction entraînait des modifications beaucoup plus rapides de l'expression des gènes que leur retrait. Cette observation, selon laquelle la levée du signal larvaire s'accompagne d'un délai dans l'expression génique et les ajustements physiologiques, est cohérente avec l'hypothèse selon laquelle les signaux larvaires agissent comme un signal de renforcement de l'inhibition de la reproduction. Un tel délai est nécessaire chez *O. biroi* afin d'éviter une transition prématurée vers la reproduction, notamment lorsque certains individus quittent le nid pendant la phase de soins au couvain et ne sont donc exposés que de manière sporadique aux signaux larvaires. L'analyse des modifications de l'expression génique au cours du temps a également permis d'identifier des gènes candidats pour le contrôle neuronal des effets de l'environnement social sur la reproduction, incluant des gènes codant pour des neurotransmetteurs, des neurohormones et des neuropeptides. Récemment, nous avons utilisé une version modifiée du protocole expérimental pour étudier les changements transcriptomiques associés à l'activation de la reproduction dans deux autres tissus impliqués dans la traduction des signaux larvaires en variation reproductive : les antennes et les ovaires (Coulm et al., in preparation). En plus de fournir des éléments généraux sur la régulation du cycle reproductif en réponse à la présence de larves chez *O. biroi*, ces études ont identifié des gènes candidats spécifiques impliqués dans le contrôle de la reproduction, contribuant ainsi à notre compréhension moléculaire de la régulation de la division du travail reproducteur chez les fourmis.

Régulation épigénétique du contrôle social de la reproduction

Afin de mieux comprendre les mécanismes moléculaires régulant les modifications de l'expression génique associées à la variation reproductive en réponse aux modifications du contexte social chez *O. biroi*, nous avons étudié le rôle des processus épigénétiques. L'une des modifications épigénétiques les plus courantes est la méthylation de l'ADN, qui consiste en l'ajout d'un groupe méthyle à certaines bases nucléotidiques, en particulier les cytosines

(Allis et al., 2007). La découverte de gènes codant pour les enzymes responsables de ce processus épigénétique dans le génome de l'abeille domestique (Weinstock et al., 2006) a fait de la méthylation de l'ADN un mécanisme candidat pour l'évolution et la régulation de la vie sociale chez les insectes. Des études ultérieures ont mis en évidence des différences de méthylation de l'ADN entre reines et ouvrières chez l'abeille et plusieurs espèces de fourmis (Bonasio et al., 2012; Foret et al., 2012; Glastad et al., 2014; Lyko et al., 2010). Cependant, les difficultés liées à la comparaison de castes morphologiquement distinctes (Kronauer & Libbrecht, 2018), ainsi que l'absence de réplication biologique dans la plupart de ces études, nous ont incités à examiner le rôle de la méthylation de l'ADN dans la régulation de la variation reproductive chez *O. biroi* (Libbrecht et al., 2016).

Nous avons réalisé un séquençage du génome après traitement au bisulfite afin d'identifier les différences de méthylation à l'échelle du génome entre les cerveaux d'individus reproducteurs et non reproducteurs de même âge et génétiquement identiques. Nous n'avons trouvé aucune preuve que les schémas de méthylation de l'ADN étaient associés à la variation de la reproduction ou à l'expression des gènes, malgré l'utilisation de plusieurs approches statistiques. De plus, nous avons observé que la méthylation de l'ADN était soit très robuste (de nombreuses cytosines étaient systématiquement méthylées dans tous les échantillons ou alors méthylées dans aucun échantillon), soit hautement variable (de nombreuses cytosines étaient méthylées dans un seul échantillon, correspondant à une méthylation spécifique à l'échantillon). Ces résultats étant en contradiction avec les études précédentes rapportant une méthylation spécifique aux castes (Bonasio et al., 2012; Foret et al., 2012; Glastad et al., 2014; Lyko et al., 2010), nous avons émis l'hypothèse que l'absence de réplication biologique dans ces études pourrait avoir conduit à une confusion entre méthylation spécifique à l'échantillon et méthylation spécifique à la caste. Pour tester cette hypothèse, nous avons simulé les résultats que nos données auraient produits en utilisant le plan expérimental et les analyses statistiques des études antérieures (c'est-à-dire en l'absence de réplication biologique). Nos simulations ont montré que nous aurions en effet détecté des différences de méthylation dans les quatre comparaisons entre un échantillon reproducteur et un échantillon non reproducteur (une comparaison par colonie source), mais que ces différences de méthylation de l'ADN n'auraient pas été cohérentes entre les colonies sources. Ce résultat suggère que, dans les études antérieures dépourvues de réplication biologique, la méthylation spécifique à l'échantillon a pu être interprétée à tort comme une méthylation spécifique à la caste. Notre étude a eu un impact fort et positif sur le domaine émergent de l'épigénétique des insectes sociaux en soulignant la nécessité de contrôles rigoureux, d'un niveau de réplication adéquat et d'analyses statistiques appropriées. Depuis, d'autres travaux ont confirmé les préoccupations soulevées dans notre

publication quant au rôle de la méthylation de l'ADN dans la régulation de la division du travail reproducteur (Herb et al., 2012; Patalano et al., 2015; Standage et al., 2016).

2.2 – Rôle des interactions sociales chez d'autres espèces et dans d'autres contextes

L'étude de l'impact des partenaires sociaux sur la reproduction chez la fourmi clonale *O. biroi* a suscité mon intérêt pour comprendre si et comment les interactions sociales régulent la variation phénotypique dans d'autres contextes et chez d'autres espèces de fourmis. En tant que professeur assistant à l'université de Mainz (Allemagne), j'ai conçu et supervisé plusieurs projets de recherche menés par des étudiants de licence, de master et de doctorat. Ces projets exploitent le potentiel des manipulations expérimentales de l'environnement social dans les colonies de fourmis afin d'examiner le rôle des interactions sociales dans la régulation de la variation phénotypique en termes de reproduction, de longévité, d'agressivité et de production de couvain.

Les larves stimulent la reproduction des ouvrières après la perte de la reine

Dans la plupart des espèces d'Hyménoptères sociaux, les ouvrières des colonies qui perdent leur reine développent leurs ovaires et pondent des œufs haploïdes donnant naissance à des mâles (Holman et al., 2010; Van Oystaeyen et al., 2014). Bien que cet effet de la présence de la reine sur la reproduction des ouvrières soit bien documenté, il reste incertain si d'autres modifications du milieu social, comme la présence de couvain, influencent la production d'œufs par les ouvrières. Une hypothèse est que la présence de larves inhiberait la production d'œufs, car le soin apporté au couvain implique des coûts énergétiques, et les ouvrières orphelines privilégieraient alors l'allocation des ressources aux soins des larves existantes plutôt qu'à l'investissement dans la production d'œufs. Des études antérieures ont appuyé cette hypothèse en rapportant des effets inhibiteurs des larves sur la production d'œufs par les ouvrières chez les bourdons et les fourmis (Ebie et al., 2015; Starkey et al., 2019; Ulrich et al., 2016; Villalta et al., 2015). Une autre hypothèse est que la présence du couvain stimulerait la production d'œufs, les larves apportant divers bénéfices nutritionnels, passifs et actifs, aux membres adultes de la colonie (Schultner et al., 2017). Bien qu'un tel impact positif du couvain sur la reproduction ait été documenté dans des colonies matures contenant une reine (Schultner et al., 2017), aucune preuve n'indique que les larves stimulent la reproduction des ouvrières en l'absence de reine.

Afin de mieux comprendre l'effet des larves sur la reproduction des ouvrières chez les fourmis, nous avons étudié cette question chez *Temnothorax nylanderi* (Majidifar et al., in preparation). Nous avons observé que la suppression expérimentale de la reine stimulait la production

d'œufs chez les ouvrières, mais que l'augmentation du nombre d'œufs pondus au fil du temps était plus importante lorsque les ouvrières étaient maintenues avec des larves que lorsqu'elles en étaient privées. Cet effet s'est révélé dose-dépendant : un ratio élevé de larves par ouvrière conférait un bénéfice plus faible en termes de ponte qu'un ratio plus faible. Nos analyses indiquent que les larves ne stimulaient pas la production d'œufs lorsqu'elles avaient été affamées au préalable, suggérant que cet effet stimulant pourrait être d'origine nutritionnelle. Cependant, les ouvrières maintenues avec des larves présentaient une teneur en glycogène plus faible que celles privées de larves, ce qui pourrait refléter une mobilisation des réserves énergétiques pour le soin des larves et/ou l'investissement dans la ponte. Nos résultats mettent ainsi en évidence un effet positif et dose-dépendant de la présence des larves sur la reproduction des ouvrières orphelines chez *T. nylanderi*. Cette découverte illustre comment la présence et le nombre de partenaires sociaux distincts (reine et larves) interagissent pour influencer la reproduction et la physiologie des ouvrières. De manière plus générale, en contraste avec les études antérieures rapportant un effet inhibiteur des larves sur la reproduction des ouvrières dans d'autres espèces de fourmis (Ebie et al., 2015; Ulrich et al., 2016; Villalta et al., 2015), cette étude souligne que l'impact des interactions sociales sur la division du travail dépend de l'écologie et des caractéristiques spécifiques à chaque espèce.

Les manipulations du contexte social influencent la longévité des ouvrières

Les colonies d'insectes sociaux présentent une variation fascinante non seulement en termes d'activité reproductrice, mais aussi en matière de longévité. Le compromis typique entre longévité et fécondité, observé chez la plupart des organismes vivants (Gems & Partridge, 2013; Partridge et al., 1987; Westendorp & Kirkwood, 1998), semble absent chez les insectes sociaux : les reines fertiles vivent plus longtemps que les ouvrières non reproductrices (Heinze et al., 2013; Keller & Genoud, 1997; Kramer et al., 2015), et les ouvrières qui deviennent fertiles prolongent leur durée de vie (Kohlmeier et al., 2017; Kuszewska et al., 2017; Lopes et al., 2020; Majoe et al., 2021; Negroni et al., 2021). Cependant, comparer les reines aux ouvrières ou manipuler expérimentalement la reproduction des ouvrières par la suppression de la reine ne permet pas de dissocier l'effet de l'activité reproductrice de facteurs confondants tels que l'âge, la morphologie, l'expérience individuelle ou le patrimoine génétique (Kronauer & Libbrecht, 2018).

Dans cette étude, nous avons utilisé la fourmi clonale *O. biroi* pour examiner l'impact de la reproduction sur la longévité, ainsi que les mécanismes transcriptomiques sous-jacents, tout en contrôlant ces facteurs confondants (Lenhart et al., in preparation). Nous avons manipulé expérimentalement des ouvrières monomorphes, génétiquement identiques et du même âge, afin d'inhiber ou de stimuler leur reproduction immédiatement après leur émergence et

pendant une période de cinq mois. Pour ce faire, nous avons soit forcé les ouvrières à se reproduire en retirant régulièrement les œufs (phase de reproduction forcée), soit empêché leur reproduction en ajoutant régulièrement des larves (phase de soins au couvain forcée). Nous avons également inclus un traitement témoin, où les colonies expérimentales suivaient leur cycle naturel. Nous avons suivi la survie des ouvrières sur toute la durée de l'expérience et analysé les changements transcriptomiques liés à l'âge dans le cerveau et le corps gras.

Notre principal résultat est que l'inhibition expérimentale de la reproduction des ouvrières entraîne une réduction de leur longévité : fécondité et longévité ne sont pas négativement associées chez *O. biroi*. Les ouvrières maintenues en phase de soins au couvain présentaient une survie réduite par rapport aux ouvrières forcées à se reproduire en continu ou aux ouvrières témoins, alternant entre phases de reproduction et de soins du couvain. De plus, nous avons identifié des variations d'expression génique liées à l'âge qui différaient entre les ouvrières des traitements de reproduction forcée et de soins au couvain forcé. Nous avons trouvé 13 gènes dans le cerveau et 54 gènes dans le corps gras dont l'expression variait selon ces conditions, constituant ainsi des mécanismes moléculaires candidats pour expliquer la réduction de la survie en réponse à l'inhibition expérimentale de la reproduction. Dans cette étude, nous avons démontré, grâce à des manipulations expérimentales du contexte social, que le compromis habituel entre longévité et fécondité est absent chez *O. biroi*. Ces résultats complètent nos investigations récentes sur l'impact de la présence de la reine sur la survie des ouvrières dans d'autres espèces de fourmis (Lenhart et al., 2025; Majoe et al., in preparation). Ensemble, ces études soulignent l'importance des manipulations expérimentales de l'environnement social pour mieux comprendre l'association inhabituelle entre longévité et fécondité observée chez les insectes sociaux.

Les colonies de fourmis avec une force ouvrière plus diversifiée produisent plus de couvain

Une question centrale pour comprendre l'évolution sociale chez les insectes est de savoir pourquoi les fourmis, les abeilles et les guêpes ont évolué à plusieurs reprises vers des stratégies de reproduction réduisant l'apparentement génétique au sein de leurs colonies, comme l'accouplement des reines avec plusieurs mâles et/ou la fondation de colonies par plusieurs reines (Hughes et al., 2008). La sélection de telles stratégies est contre-intuitive, car elle semble entraver l'évolution de l'altruisme par la sélection de parentèle. Une hypothèse propose que l'augmentation de la diversité génétique associée à ces stratégies facilite la division du travail entre les ouvrières et améliore la performance des colonies. Plusieurs études ont testé cette hypothèse, mais les preuves d'un effet bénéfique de la diversité génétique restent limitées. Une grande partie de ces travaux ont rapporté des corrélations entre les

niveaux de diversité génétique et la performance des colonies (Cole & Wiernasz, 1999; Fjerdingstad et al., 2002; Fjerdingstad & Keller, 2004; Goodisman et al., 2007; Modlmeier et al., 2012), sans démontrer un lien de causalité. Les rares études expérimentales publiées jusqu'à présent se sont principalement appuyées sur l'insémination artificielle des reines avec le sperme d'un ou de plusieurs mâles (Cole & Wiernasz, 1999; Fjerdingstad et al., 2002; Fjerdingstad & Keller, 2004; Goodisman et al., 2007; Modlmeier et al., 2012). Or, ces manipulations affectent non seulement la diversité de la descendance produite, mais aussi les reines elles-mêmes. Ainsi, les effets observés sur la performance des colonies pourraient également résulter d'effets maternels (par exemple, sur la production d'œufs, la survie du couvain et son développement).

Dans cette étude, nous avons manipulé la composition sociale des colonies expérimentales afin de tester si une plus grande diversité au sein de la force ouvrière améliore la performance des colonies chez la fourmi noire des jardins *Lasius niger* (Psalti et al., 2021). Chez cette espèce, les colonies sont dirigées par une seule reine qui s'accouple généralement avec un seul mâle (Boomsma & Van der Have, 1998). Nous avons constitué des colonies expérimentales à faible diversité (en mélangeant des ouvrières issues d'une même colonie) et à forte diversité (en mélangeant des ouvrières issues de plusieurs colonies). Pour contrôler tout effet maternel, nous avons attribué aléatoirement une reine non apparentée à chaque colonie expérimentale. Notre principal résultat est que les colonies expérimentales plus diversifiées ont produit davantage de larves au fil du temps. En outre, nous avons constaté que l'augmentation de la diversité se traduisait par une plus grande variation de taille parmi les ouvrières. Nous suggérons que les bénéfices de la diversité résultent probablement d'une division du travail plus efficace, facilitant le développement et la survie du couvain. Notre étude apporte ainsi une preuve expérimentale d'un effet positif de l'augmentation de la diversité ouvrière sur la performance des colonies dans une espèce où la diversité génétique est naturellement faible. De tels bénéfices pourraient expliquer l'évolution des stratégies de reproduction réduisant l'apparentement génétique au sein des colonies d'insectes sociaux.

Rôle central de la fécondité dans les associations pléométrotiques des reines fourmis

Un exemple classique de coopération entre individus non apparentés est la pléométrose chez les fourmis (Bernasconi & Strassmann, 1999). La pléométrose définit l'association de plusieurs reines fondatrices après le vol nuptial pour établir ensemble une nouvelle colonie. Bien que ce comportement coopératif favorise la croissance initiale de la colonie (Madsen & Offenberg, 2017; Rissing & Pollock, 1987; Sommer & Hölldobler, 1995), les mécanismes expliquant ses bénéfices restent mal compris. Nous avons induit expérimentalement des associations pléométrotiques afin d'en analyser les bénéfices et avons proposé qu'ils résultent d'un apport nutritionnel accru pour les larves (Teggers et al., 2021). Toutefois, ces bénéfices sont conditionnels, car les

associations pléométrotiques sont transitoires : les reines cohabitent pour pondre des œufs et élever les premières larves, mais dès l'émergence des ouvrières, elles s'engagent dans des combats (parfois initiés et/ou rejoints par les ouvrières) jusqu'à ce qu'une seule reine survive (Bernasconi & Strassmann, 1999). Il est donc crucial pour les reines fondatrices de décider si elles doivent s'engager dans une association et de sélectionner leurs partenaires pléométrotiques en fonction de leur probabilité de survie à cette compétition. Par exemple, si un trait phénotypique est positivement corrélé avec la probabilité de survivre à l'association, une pression de sélection favoriserait le choix de partenaires présentant de faibles valeurs pour ce trait. Plusieurs traits, tels que la taille, la masse et la fécondité, sont en effet associés à la survie des reines dans ces associations (Aron et al., 2009; Nonacs, 1990, 1992). Cependant, ces traits sont souvent confondus, et on ignore encore quel facteur détermine spécifiquement l'issue de la pléométrose. De plus, on ignore si les reines fondatrices choisissent leurs partenaires en fonction de ces traits.

Dans cette étude, nous avons manipulé expérimentalement le contexte social pour répondre à ces questions. Nous avons formé des paires de reines différant en fécondité mais non en taille, et inversement, afin de dissocier l'effet de ces facteurs sur la survie des reines. Nous avons également fourni aux paires pléométrotiques des ouvrières non apparentées (via un remplacement du couvain) afin d'éviter toute confusion entre la fécondité des reines et la proportion de filles dans le pool d'ouvrières lorsque la coopération prend fin. Nos résultats montrent que les reines les plus fécondes survivent plus fréquemment aux associations, même après contrôle de la taille et de la parenté des ouvrières. Par ailleurs, pour étudier les traits associés au choix du partenaire, nous avons offert aux reines fondatrices le choix entre deux reines sélectionnées aléatoirement. Nous avons observé que les reines choisies comme partenaires pléométrotiques étaient moins fécondes que celles qui n'étaient pas sélectionnées, mais qu'elles ne différaient pas en taille (Teggers et al., 2021). Ainsi, lors de la sélection de leurs partenaires de coopération, les reines fondatrices de *L. niger* expriment une préférence qui augmente leur probabilité de survie à l'association. Cette étude révèle que la fécondité des partenaires sociaux joue un rôle central à la fois dans l'établissement et l'issue des associations pléométrotiques chez les fourmis.

2.3 – Conclusion de la Partie 2

Les colonies d'insectes sociaux sont des systèmes biologiques complexes et intégrés, qui fonctionnent grâce à la coopération entre composants spécialisés (Wheeler, 1911). En plus des différences intrinsèques entre castes morphologiquement distinctes, les interactions constantes entre partenaires sociaux génèrent une variation phénotypique à plusieurs niveaux, de la physiologie au comportement. Si l'influence de la présence de la reine sur la

reproduction et le comportement des ouvrières a été largement étudiée (Heinze, 2008; Holman et al., 2010; Lenhart et al., 2025; Van Oystaeyen et al., 2014), le rôle d'autres types d'interactions sociales dans la structuration de la division du travail et/ou l'optimisation du fonctionnement des colonies demeure relativement moins exploré.

La Partie 2 décrit comment mes travaux de recherche ont contribué à une meilleure compréhension mécanistique de l'impact des larves sur la reproduction (Libbrecht et al., 2016, 2018; Majidifar et al., in preparation; Ulrich et al., 2016) et la longévité (Lenhart et al., in preparation) des ouvrières, ainsi que des bénéfices coloniaux d'une plus grande diversité de la force ouvrière (Psalti et al., 2021). Ces études ont confirmé l'importance des manipulations expérimentales de l'environnement social pour élucider comment les interactions sociales assurent le bon fonctionnement des colonies. Elles ont également éveillé mon intérêt pour l'influence, encore peu étudiée, du contexte social sur la spécialisation comportementale des reines dans la production d'œufs, en particulier via les interactions avec les ouvrières et les larves.

Partie 3 – Perspectives de recherche sur la spécialisation comportementale des reines

Le fonctionnement des systèmes biologiques repose sur la coopération de composants spécialisés, et comprendre l'émergence et l'évolution de cette spécialisation constitue un défi majeur en biologie. Parmi les exemples typiques de ces systèmes biologiques figurent les organismes multicellulaires, composés de cellules spécialisées, ainsi que les sociétés d'insectes, constituées d'individus spécialisés (Szathmary & Maynard Smith, 1995). En effet, les colonies d'insectes sociaux (également appelées superorganismes) sont analogues aux organismes multicellulaires, dans la mesure où elles possèdent des reines qui monopolisent la reproduction (semblables aux cellules germinales) et des ouvrières fonctionnellement stériles qui effectuent toutes les tâches non reproductives, jouant ainsi le rôle de cellules somatiques (Boomsma & Gawne, 2018; Wheeler, 1911). Ces deux types de systèmes biologiques ont évolué à partir d'ancêtres solitaires et non spécialisés, dans le cadre de transitions majeures de l'évolution (Szathmary & Maynard Smith, 1995) : les organismes multicellulaires à partir d'organismes unicellulaires, et les superorganismes à partir d'insectes solitaires.

Fait intéressant, dans les deux cas, cette spécialisation doit également être établie à chaque génération au cours de l'ontogénie de ces systèmes biologiques, c'est-à-dire le processus développemental qui conduit à l'auto-assemblage et à la spécialisation de leurs composants à partir d'une unité unique. D'importants efforts de recherche ont été consacrés à l'étude de l'ontogénie des organismes multicellulaires, donnant naissance à l'ensemble du domaine de la biologie du développement. Ces travaux ont démontré que l'étude de l'ontogénie constitue une approche puissante pour comprendre l'évolution et l'émergence de la spécialisation (Brunet & King, 2017; Sogabe et al., 2019). Cependant, cette approche n'a pas été appliquée aux insectes sociaux, et les investigations expérimentales sur l'ontogénie des sociétés d'insectes restent rares (Chouvenc, 2022; Majidifar et al., 2024; Woodard et al., 2013). Ces dernières années, j'ai développé un programme de recherche visant à combler cette lacune et à fournir une compréhension approfondie de l'ontogénie des superorganismes. Mon objectif est d'identifier les mécanismes qui induisent et maintiennent la spécialisation individuelle émergeant au cours du processus ontogénique des sociétés d'insectes, afin d'apporter un éclairage nouveau sur son histoire évolutive.

Chez la plupart des espèces d'insectes sociaux, les reines fécondées fondent leur colonie de manière indépendante (Peeters, 2020) et sont comparables aux zygotes puisqu'elles constituent le premier stade de développement des superorganismes. Le développement des colonies à partir des reines fondatrices correspond ainsi à l'ontogénie des superorganismes. Ces reines pluripotentes expriment un large répertoire de comportements et remplissent

plusieurs fonctions essentielles à la production des premières ouvrières. Ce n'est qu'une fois les colonies établies que les reines deviennent strictement spécialisées dans la ponte (Wilson, 1971). La spécialisation des reines constitue un processus central dans l'ontogénie des superorganismes, tout comme la différenciation cellulaire dans l'ontogénie des organismes multicellulaires. Toutefois, les études sur la fondation des colonies se sont jusqu'à présent limitées à des descriptions naturalistes, des observations écologiques et l'examen de cas spécifiques (Bernasconi & Strassmann, 1999; Hölldobler & Wilson, 1990; Peeters, 2020; Sommer & Hölldobler, 1992; Wilson, 1971). Il est donc nécessaire de mener des investigations expérimentales pour identifier les facteurs et mécanismes qui contrôlent la spécialisation des reines fondatrices pluripotentes.

La pluripotence des reines fondatrices se manifeste de manière particulièrement évidente dans leur comportement envers le couvain. Les soins au couvain ont évolué à de multiples reprises chez les insectes et ont constitué une étape critique dans les différentes origines évolutives de la socialité chez ces organismes (Kronauer & Libbrecht, 2018). De plus, les variations individuelles dans la réponse au couvain ont probablement joué un rôle clé dans l'évolution de la division du travail reproducteur chez les fourmis (Chandra et al., 2018). Le soin apporté au couvain en développement est une tâche essentielle qui est généralement assurée par les ouvrières, mais que les reines doivent remplir lors de la phase de fondation. Cela est d'autant plus nécessaire que, chez les Hyménoptères sociaux, le développement des œufs jusqu'à l'âge adulte requiert des soins prodigués par des adultes (Schultner et al., 2017).

Afin d'étudier l'ontogénie des superorganismes, j'ai établi la fourmi noire des jardins (*Lasius niger*) comme modèle pour l'étude de la pluripotence et de la spécialisation comportementale des reines. Dans cette partie, je présente nos travaux récents, en cours et à venir sur l'étude du comportement de soins au couvain chez les reines de fourmis. J'ai initié cette ligne de recherche en tant que professeur assistant à l'université de Mainz (Allemagne) et je l'ai développée davantage depuis mon recrutement en 2023 comme chercheur CNRS au sein de l'Institut de Recherche sur la Biologie de l'Insecte (IRBI, UMR 7261) de l'université de Tours (France). Les travaux présentés ci-dessous s'inscrivent dans le cadre de projets menés par des étudiants en licence, master et doctorat sous ma supervision, et ont été financés par des subventions de la *Deutsche Forschungsgemeinschaft* (DFG) ainsi que de l'Agence Nationale de la Recherche (ANR), notamment via le projet ANR JCJC « ANTOGENY » (2024-2028).

3.1 - Contrôle social de la spécialisation des reines

Afin de mieux comprendre la transition comportementale des reines au cours du processus de fondation des colonies, nous étudions si et comment la présence des ouvrières

– et plus généralement les interactions sociales entre les membres de la colonie – régulent la spécialisation des reines dans la production d'œufs.

La présence des ouvrières initie et maintient la spécialisation des reines

La spécialisation comportementale des reines de *L. niger* est associée à la présence des ouvrières, comme le démontre notre observation selon laquelle les reines fondatrices, avant l'émergence des ouvrières, prodiguent davantage de soins aux larves que ces mêmes reines – devenues alors établies – après l'émergence des ouvrières (Majidifar et al., 2024). Il serait tentant d'attribuer ces changements comportementaux à un effet causal de la présence des ouvrières, mais d'autres facteurs confondants pourraient également les expliquer, tels que l'âge des reines (les reines fondatrices étant plus jeunes que les reines établies) ou leur statut nutritionnel (les reines fondatrices étant affamées, tandis que les reines établies sont nourries par les ouvrières). Pour démontrer l'effet de la présence des ouvrières, il est nécessaire d'introduire expérimentalement des ouvrières auprès de reines fondatrices n'en ayant pas encore produit. Pour ce faire, nous avons prélevé du couvain dans des colonies de *L. niger* en milieu naturel et l'avons maintenu en laboratoire, où nous avons suivi l'émergence des ouvrières. Nous avons ainsi collecté des ouvrières nouvellement écloses, qui ne suscitaient aucune agressivité de la part d'individus étrangers et étaient immédiatement acceptées par les reines fondatrices, probablement en raison de l'absence d'un profil chimique colonie-spécifique sur leur cuticule (Dahbi et al., 1998).

Ce protocole nous a permis de manipuler expérimentalement la présence des ouvrières auprès de reines fondatrices du même âge, tout en contrôlant leur statut nutritionnel. Nous avons ainsi confirmé que l'ajout expérimental d'ouvrières entraînait une diminution du comportement de soins au couvain des reines. Cette expérience démontre que la présence des ouvrières est nécessaire et suffisante pour initier la spécialisation comportementale des reines de *L. niger*. Nous avons ensuite cherché à savoir si cette présence était également nécessaire au maintien de la spécialisation des reines dans les colonies établies. Pour cela, nous avons utilisé des colonies de *L. niger* fondées en laboratoire plusieurs années avant le début de l'expérience. Nous avons d'abord mesuré le comportement de soins au couvain des reines après avoir retiré toutes les ouvrières sauf cinq. Nous avons ensuite retiré ces cinq dernières ouvrières et quantifié le comportement de soins au couvain des reines au cours des deux jours suivants. Nous avons constaté que les reines exprimaient des niveaux élevés de soins au couvain après le retrait expérimental des ouvrières. Ce résultat montre que la présence des ouvrières initie non seulement la spécialisation des reines lors de la fondation des colonies, mais la maintient également en permanence dans les colonies établies.

Après avoir démontré que la présence des ouvrières contrôle la spécialisation des reines, nous avons cherché à déterminer si des indices chimiques signalant leur présence suffisaient à inhiber le comportement de soins au couvain chez les reines, ou si la présence physique des ouvrières était nécessaire. Les insectes sociaux détectent leurs partenaires sociaux grâce au mélange d'hydrocarbures présents sur leur cuticule (Sprenger & Menzel, 2020). Nous avons donc extrait les hydrocarbures cuticulaires (CHC) d'ouvrières de *L. niger* et les avons appliqués sur des billes de verre fournies à des reines fondatrices du même âge. Nous n'avons observé aucun effet du traitement aux CHC sur l'expression du comportement de soins au couvain chez les reines, indiquant que celles-ci ne modifient pas leur comportement en réponse à la simple détection des CHC d'ouvrières. Ce résultat a été confirmé par trois expériences supplémentaires (Majidifar et al., 2024), dont l'une consistait à tester si le comportement des reines était affecté par la présence d'ouvrières séparées d'elles et du couvain par une grille métallique. Ce dispositif permettait aux ouvrières d'entrer en contact antennaire avec la reine et le couvain, mais empêchait toute interaction plus rapprochée, comme l'échange de fluides via la trophallaxie (LeBoeuf, 2021). Nous avons constaté que les reines maintenues avec des ouvrières séparées par une grille exprimaient autant de soins au couvain que celles maintenues sans ouvrières. Ces expériences montrent que les indices chimiques seuls ne suffisent pas à déclencher la spécialisation des reines et suggèrent que les ouvrières doivent interagir étroitement avec les reines et/ou les larves pour inhiber leur comportement de soins au couvain.

Compréhension mécanistique de l'effet des ouvrières sur la spécialisation des reines

L'un de nos objectifs actuels est de tester différentes hypothèses pouvant expliquer pourquoi la présence physique des ouvrières – mais pas leurs signaux chimiques – inhibe le comportement de soins au couvain des reines (Majidifar et al., 2024).

Une première hypothèse est que l'effet des ouvrières sur le comportement des reines nécessite des interactions rapprochées, notamment via l'échange de fluides lors de la trophallaxie. En effet, les fluides trophallactiques ne contiennent pas uniquement de la nourriture, mais aussi des molécules influençant le comportement (LeBoeuf et al., 2016). Nous allons déterminer si l'échange de fluides entre les reines et les ouvrières déclenche la spécialisation comportementale des reines. Pour cela, nous utiliserons des enregistrements vidéo en continu de reines fondatrices afin d'examiner si la trophallaxie est nécessaire à l'initiation de leur spécialisation comportementale. Nous collecterons également les fluides trophallactiques des ouvrières et les fournirons expérimentalement à des reines fondatrices pour tester leur effet sur le comportement de soins au couvain. Si nos expériences révèlent que l'échange de fluides par trophallaxie est responsable de l'effet des ouvrières sur le

comportement des reines, nous entreprendrons une caractérisation protéomique du contenu des fluides trophallactiques (Hakala et al., 2021; LeBoeuf et al., 2016).

Une seconde hypothèse est que l'effet des ouvrières sur la spécialisation des reines passe par leur influence sur les larves. En prenant soins des larves, les ouvrières réduiraient les besoins de soins de celles-ci, jusqu'au point où elles recevraient tous les soins nécessaires des ouvrières, et où les reines cesseraient alors d'exprimer le comportement de soins au couvain. Cette hypothèse implique que les reines fondatrices ajustent leur comportement en fonction des besoins des larves. Nous observerons donc le comportement des reines envers des larves ayant des besoins de soins plus ou moins élevés, obtenus par des manipulations de leur statut nutritionnel ou sanitaire.

Les larves d'insectes sociaux communiquent leur présence et leurs besoins aux ouvrières via des signaux chimiques (Schultner et al., 2017). Nous émettons l'hypothèse que les reines fondatrices répondent également à ces signaux. Ces signaux peuvent être fixes et simplement indiquer la présence du couvain, ou dynamiques et refléter les besoins des larves. Nous chercherons à déterminer si et comment les larves signalent leur présence et/ou leurs besoins en soins. Pour tester si ces signaux sont véhiculés par leurs hydrocarbures cuticulaires (CHC), nous analyserons les CHC larvaires afin d'identifier des composés chimiques spécifiques aux larves et/ou corrélés à leurs besoins. Nous étudierons également la réponse comportementale des reines fondatrices aux CHC extraits des larves. Enfin, nous prendrons en compte l'importance d'autres signaux chimiques (e.g., composés volatils) produits par les larves, ainsi que de signaux physiques, tels que le comportement de quémande et la posture corporelle (Schultner et al., 2017).

3.2 – Régulation moléculaire de la spécialisation des reines

Si nous avons accumulé des preuves solides montrant que l'environnement social influence le comportement de soins au couvain chez les reines *L. niger* (Majidifar et al., 2024), les mécanismes moléculaires traduisant la présence des ouvrières en modifications comportementales restent à élucider. Nous combinons des analyses transcriptomiques à des manipulations fonctionnelles de mécanismes candidats afin de construire un modèle mécanistique intégré de la spécialisation comportementale des reines.

Bases transcriptomiques de la spécialisation comportementale des reines

Les analyses transcriptomiques par RNA-seq constituent une approche puissante pour identifier les changements d'expression génique impliqués dans la plasticité comportementale. Dans mes travaux, j'ai utilisé cet outil pour révéler l'implication de certaines voies moléculaires (Kohlmeier et al., 2019; Libbrecht et al., 2018) et/ou identifier des gènes candidats pour des études

fonctionnelles ultérieures (Chandra et al., 2018). Ces dernières années, nous avons généré 277 transcriptomes de reines de *L. niger* à partir de tissus impliqués dans la régulation du comportement des insectes (cerveau et corps gras) ou la détection des signaux environnementaux (antennes). Ces tissus ont été disséqués chez des reines à divers stades du développement des colonies, ainsi que chez des reines fondatrices ayant été manipulées expérimentalement pour induire des variations de comportement de soins au couvain (Majidifar et al., 2024). Nous exploitons actuellement ces ressources pour réaliser des analyses transcriptomiques à grande échelle afin d'identifier des gènes et/ou des voies moléculaires corrélés à la spécialisation comportementale des reines.

Développement d'un protocole pour la validation fonctionnelle des mécanismes candidats

Bien que les analyses transcriptomiques soient puissantes, elles restent de nature corrélative et ne permettent pas de démontrer un lien causal entre les gènes et/ou les voies moléculaires candidats et la variation phénotypique. C'est pourquoi nous développons actuellement un protocole pour valider fonctionnellement le rôle des mécanismes candidats. Nous avons déjà mis en place un protocole de micro-injection permettant d'injecter jusqu'à 0,5 µL dans la capsule céphalique des reines de *L. niger*, avec une mortalité très limitée (<5 %). Cette technique ouvre la possibilité d'administrer des molécules de petite taille afin de manipuler des voies spécifiques dans le cerveau ou les glandes associées, par exemple en injectant des activateurs ou des inhibiteurs de ces voies (e.g., méthoprène ou précocène pour manipuler la voie de la JH). Nous pouvons également utiliser des ARN double-brin pour inhiber l'expression de gènes spécifiques via l'interférence par ARN (RNAi). Nous sommes actuellement en train d'optimiser notre protocole RNAi afin d'obtenir une inhibition robuste et hautement reproductible de l'expression des gènes cibles. Notre objectif est d'utiliser cette approche pour tester si l'inhibition de l'expression des gènes candidats affecte la spécialisation comportementale des reines en réponse à la présence des ouvrières. Nous développons actuellement un dispositif expérimental permettant l'enregistrement en gros plan de la tête et des antennes de reines immobilisées sur un support fixe. Cette méthode permettra la détection et le suivi automatisés de parties spécifiques du corps (e.g., tête, segments antennaires). Grâce à ce protocole, nous serons en mesure d'exposer les reines aux manipulations expérimentales par micro-injection tout en les filmant, ce qui permettra d'identifier les premières réponses comportementales et de quantifier l'évolution des modifications comportementales au cours du temps après traitement.

3.3 – Compréhension évolutive de la réversibilité de la spécialisation des reines

Nos travaux récents ont révélé que les reines établies de certaines espèces de fourmis, bien qu'ayant été spécialisées pendant plusieurs années, retrouvent rapidement la capacité d'exprimer des soins au couvain après le retrait expérimental de leurs ouvrières. Nous avons initialement observé cette réversibilité de la spécialisation des reines chez deux espèces de fourmis ayant divergé il y a plus de 100 millions d'années (Majidifar et al., 2024). Toutefois, des expériences en cours suggèrent que cette réversibilité n'est pas systématique, certaines espèces de fourmis semblant présenter une variabilité dans leur capacité à retrouver une pluripotence comportementale. Notre objectif est de mieux comprendre cette variabilité et de déterminer pourquoi la réversibilité de la spécialisation des reines a été maintenue au cours de l'évolution.

Avantages sélectifs de la réversibilité de la spécialisation des reines

Une hypothèse expliquant pourquoi les reines établies conservent la capacité de s'occuper du couvain est que cette aptitude leur permettrait de produire à nouveau des ouvrières en cas de perte totale de celles-ci. Cette hypothèse repose sur deux prédictions. La première est que certaines conditions entraînent la perte de toutes les ouvrières, par exemple dans les jeunes colonies où leur nombre est limité et/ou si les reines résistent mieux aux conditions stressantes que les ouvrières. Pour tester cette prédiction, nous examinerons si les reines montrent une meilleure résistance au stress que les ouvrières, ce qui appuierait l'hypothèse selon laquelle des reines établies pourraient se retrouver sans ouvrières dans certaines conditions. La seconde prédiction est que les reines peuvent recommencer à fonder une colonie après avoir perdu leurs ouvrières. Pour la tester, nous retirerons expérimentalement toutes les ouvrières de colonies établies de *L. niger* pour isoler les reines. Nous suivrons ensuite leur survie, le développement du couvain et la production d'ouvrières. Nous filmerons également les reines trois fois par semaine afin d'enregistrer leur comportement de soins au couvain. Ces expériences nous informeront sur les bénéfices potentiels pour les reines établies à exprimer un comportement de soins au couvain lorsqu'elles sont isolées de leurs ouvrières.

Variation interspécifique dans la réversibilité de la spécialisation des reines

Une autre hypothèse, non exclusive, est que la réversibilité de la spécialisation des reines est préférentiellement présente dans les espèces où les reines doivent naturellement exprimer un comportement de soins au couvain à un moment donné de leur vie. Par exemple, dans les espèces à fondation indépendante, les reines doivent s'occuper du couvain pour produire les premières ouvrières, tandis que dans les espèces à fondation dépendante, les reines fondent leur colonie avec l'aide des ouvrières et n'ont donc jamais besoin d'exprimer de soins au couvain (Peeters, 2020). Selon cette hypothèse, nous nous attendons à observer une variation

interspécifique dans la réversibilité de la spécialisation des reines, en lien avec des différences spécifiques de stratégie de fondation et/ou d'autres traits de vie au niveau de la colonie ou de l'individu.

Nous avons initialement rapporté la flexibilité comportementale des reines chez deux espèces, *L. niger* et *Temnothorax nylanderi* (Majidifar et al., 2024), mais ces deux espèces partagent plusieurs caractéristiques : elles forment des colonies monogynes (une seule reine par colonie), présentent une forte différenciation morphologique entre reines et ouvrières (polymorphisme de caste), et leurs reines doivent exprimer des soins au couvain lors de la fondation de la colonie (fondation indépendante). Afin de mieux comprendre l'histoire évolutive de la réversibilité de la spécialisation des reines, nous avons étudié le comportement de soins au couvain chez des reines établies, avec et sans ouvrières, dans un ensemble plus large et plus diversifié d'espèces de fourmis. Pour accéder à ces espèces, nous avons mis en place plusieurs stratégies : nous avons utilisé des espèces élevées dans notre laboratoire, réalisé des expéditions de terrain en Allemagne, en France, au Pérou et aux États-Unis, et initié des collaborations avec Brendan Hunt (université de Georgia, États-Unis) et Guojie Zhang (université de Copenhague, Danemark). Nous avons quantifié l'effet de la présence des ouvrières sur le comportement de soins au couvain chez un total de 466 reines appartenant à plus de 65 espèces de fourmis (le nombre exact dépendra des confirmations en cours de la détermination des espèces). Il est important de noter que de nombreuses espèces sont représentées par un nombre limité de réplicats indépendants (>30 espèces avec moins de trois reines). Cela concerne principalement les espèces observées directement dans des stations de recherche au Pérou et aux États-Unis, et qui n'étaient pas encore identifiées au moment des observations. Nous sommes en train de finaliser la curation et l'analyse du jeu de données, qui comprend actuellement 37 espèces de fourmis avec au moins trois réplicats indépendants.

Nos analyses comparatives interspécifiques en cours visent à intégrer la réversibilité de la spécialisation des reines dans la phylogénie des fourmis et à identifier les facteurs et traits spécifiques des espèces sous-jacents à cette variation. Bien que les analyses soient en cours, nous avons déjà identifié des variations interspécifiques dans la réponse comportementale des reines à la manipulation expérimentale de la présence des ouvrières. Ces variations peuvent être regroupées en trois grands types de réponses. Premièrement, dans la plupart des espèces, les reines expriment peu ou pas de soins au couvain en présence d'ouvrières mais montrent une augmentation de ce comportement après leur retrait expérimental. Cela confirme que le contrôle social de la spécialisation des reines, que nous avons mis en évidence chez *L. niger* (Majidifar et al., 2024), est répandu chez les fourmis. Deuxièmement, dans quelques espèces, les reines n'expriment jamais de soins au couvain, indépendamment de la

présence d'ouvrières. Cela suggère une spécialisation complète des reines dans la production d'œufs, non modulée par le contexte social. Troisièmement, nous avons identifié des espèces où les reines expriment systématiquement des soins au couvain, même en présence d'ouvrières. Ce résultat indique que, dans certaines espèces de fourmis, les reines établies dans des colonies matures ne sont pas strictement spécialisées dans la ponte et contribuent également aux tâches non reproductives. Nous cherchons maintenant à associer cette variation entre espèces à des traits spécifiques et au signal phylogénétique pour retracer l'histoire évolutive de la spécialisation des reines fourmis.

3.4 – Conclusion de la Partie 3

Nos recherches sur les facteurs contrôlant la spécialisation comportementale des reines de fourmis lors de la fondation des colonies ont démontré que la présence d'ouvrières est nécessaire et suffisante pour inhiber les soins au couvain et initier ainsi la spécialisation des reines dans la ponte (Majidifar et al., 2024). De manière surprenante, nous avons montré que cette spécialisation est réversible, les reines établies retrouvant une pluripotence comportementale après la perte de leurs ouvrières, même après plusieurs années de spécialisation (Majidifar et al., 2024). Ces résultats ouvrent de nouvelles perspectives passionnantes sur les facteurs sociaux et environnementaux régulant l'ontogénie des colonies, les mécanismes sous-jacents à la spécialisation comportementale des reines et les forces évolutives qui maintiennent leur flexibilité comportementale.

Les analyses comportementales en cours sur les reines de plusieurs espèces de fourmis indiquent que la réversibilité de la spécialisation des reines est un phénomène largement répandu à travers la phylogénie des fourmis. De plus, nos investigations mécanistiques sur l'influence des partenaires sociaux sur le comportement des reines *L. niger* pourraient révéler que leur spécialisation comportementale n'est qu'une conséquence passive de différences de seuils de réponse aux stimuli larvaires entre reines et ouvrières, c'est-à-dire de leur position respective sur un continuum de propension à exprimer des soins au couvain. Cette hypothèse remettrait en question la vision classique des reines comme étant fondamentalement distinctes des ouvrières sur le plan comportemental. Plus généralement, nos recherches sur le contrôle social de la spécialisation des reines chez les fourmis renversent l'idée établie de longue date selon laquelle les reines des insectes sociaux seraient intrinsèquement spécialisées dans la production d'œufs.

Autres axes de recherche

Ces dernières années, j'ai commencé à compléter mon programme de recherche principal sur la régulation et l'évolution de la division du travail dans les sociétés d'insectes par des axes de recherche plus appliqués, que je présente brièvement dans cette section.

Les fourmis jouent un rôle clé dans les écosystèmes en raison de leur biomasse importante, de leurs interactions multiples avec les plantes et les animaux, ainsi que de leur influence sur les propriétés physico-chimiques des sols (Cammeraat & Risch, 2008; Chomicki & Renner, 2017; Folgarait, 1998; Holec & Frouz, 2006; Schultheiss et al., 2022). Parce qu'elles sont centrales au fonctionnement des écosystèmes, toute modification environnementale affectant la densité et/ou la répartition des populations de fourmis est susceptible d'avoir un impact négatif sur des processus écologiques essentiels. Ces impacts peuvent concerner directement les fourmis elles-mêmes ou être causés par leur action : certaines modifications environnementales peuvent entraîner une diminution des populations natives, tandis que d'autres résultent d'introductions anthropiques d'espèces de fourmis invasives.

Depuis mon recrutement en tant que chercheur CNRS à l'IRBI, j'ai initié deux projets pour aborder ces questions. Premièrement, j'ai obtenu un financement doctoral de l'école doctorale SSBCV (universités de Tours et d'Orléans) pour étudier l'impact du stress thermique sur la fondation des colonies de *Lasius niger*. Ce projet implique une doctorante que je co-encadre avec Marlène Goubault (PU, université de Tours) et Irene Villalta (MCU, université de Tours). Deuxièmement, en collaboration avec Jean-Luc Mercier (MCU, université de Tours), nous avons lancé le projet FIVALO (*Mise en place d'un réseau de surveillance des espèces de fourmis invasives dans les régions du Val de Loire*), financé par l'Office Français de la Biodiversité (OFB). Ce projet vise à étudier la répartition des espèces de fourmis invasives dans la vallée de la Loire.

Impact du stress thermique sur le succès de fondation des colonies de fourmis

Le changement climatique entraîne non seulement une augmentation des températures moyennes, mais aussi une intensification de la fréquence et de la sévérité des événements thermiques extrêmes, tels que les vagues de chaleur (Meehl & Tebaldi, 2004). Ces perturbations thermiques peuvent avoir des effets négatifs sur la reproduction et la survie des organismes vivants (Cinto Mejía & Wetzel, 2023; Filazzola et al., 2021; Pilakouta et al., 2023) et, par conséquent, sur le fonctionnement des écosystèmes dans leur ensemble. Ces impacts sont d'autant plus marqués lorsque les espèces affectées sont ingénieures des écosystèmes, caractérisées par leurs nombreuses interactions avec leur environnement biotique et abiotique. Il est donc essentiel de mieux comprendre l'effet du stress thermique sur les populations de fourmis.

Jusqu'à présent, cette question a été principalement étudiée chez les colonies matures (Diamond et al., 2012; Perez & Aron, 2020; Roeder et al., 2021). Celles-ci bénéficient d'une certaine protection contre les variations thermiques grâce à l'architecture de leur nid et à la capacité des ouvrières à ajuster la profondeur à laquelle elles se trouvent pour limiter leur exposition aux températures extrêmes. En revanche, l'effet du stress thermique sur les reines fondatrices reste largement méconnu. Ces reines sont beaucoup plus exposées aux variations de température, notamment lors du vol nuptial, qui a généralement lieu à la fin du printemps ou en été, ainsi que dans les heures qui suivent, lorsqu'elles cherchent un site pour fonder leur colonie. Elles peuvent ainsi être confrontées à des températures très élevées, en particulier en milieu urbain. Pour assurer le succès de la fondation, les reines doivent pondre des œufs et prendre soin du couvain jusqu'à l'émergence des premières ouvrières, un processus qui pourrait être compromis par une exposition précoce à des températures élevées. Nous avons donc cherché à déterminer si le stress thermique affecte le succès de la fondation des colonies chez les fourmis.

Pour aborder cette question, nous avons étudié la fourmi noire des jardins *L. niger*. Nous avons collecté des reines fondatrices après leur vol nuptial et les avons soumises à un stress thermique aigu (43°C pendant 4 heures) ou à une température contrôle (25°C pendant 4 heures). Après le traitement, toutes les reines ont été maintenues à 21°C pendant 50 jours afin de suivre leur survie, leur succès de fondation, leur production d'œufs et le développement de leur couvain. Nos résultats montrent que le stress thermique augmente la mortalité des reines fondatrices. Alors qu'aucune reine du groupe contrôle n'est morte au cours de l'expérience, 8 des 25 reines traitées sont décédées dans les 50 jours suivant l'exposition à la chaleur. Pour les analyses suivantes, nous nous sommes concentrés sur les reines encore en vie à la fin de l'expérience. Nous avons utilisé la production d'ouvrières dans les 50 jours suivant le traitement comme indicateur du succès de la fondation. Alors que 17 des 25 reines contrôles avaient produit des ouvrières à la fin de l'expérience, ce nombre chutait drastiquement chez les reines traitées, où une seule reine sur 25 avait réussi à produire des ouvrières. Afin de mieux comprendre l'impact du stress thermique sur la fondation des colonies, nous avons analysé ses effets sur la production et le développement du couvain. Nous avons constaté que l'évolution du nombre d'œufs, de larves et de nymphes différait entre les groupes contrôle et traité. Les reines contrôles ont produit environ deux fois plus de larves que les reines traitées, ce qui concorde avec une diminution de moitié du taux d'éclosion des œufs dans le groupe traité. Cette différence s'est ensuite traduite par une production de nymphes trois fois plus faible chez les reines exposées au stress thermique, impactant ainsi directement le succès de la fondation des colonies.

Nos données suggèrent que l'effet négatif du stress thermique sur la production d'ouvrières résulte principalement d'une réduction de la ponte et d'un retard dans le développement du couvain, notamment une production d'œufs retardée et un taux d'éclosion plus faible. Nous soupçonnons que l'exposition au stress thermique altère la capacité des reines fondatrices à pondre des œufs viables. Cette altération pourrait être due à un effet direct sur la physiologie de la reine et/ou sur le sperme stocké dans la spermathèque, ce qui impacterait la qualité des œufs fécondés. Il est également possible que les ovocytes déjà présents dans les ovarioles des reines au moment du traitement aient été directement affectés. Une autre hypothèse est que le stress thermique modifie le ratio entre les œufs viables et les œufs trophiques produits par les reines fondatrices. Nous sommes actuellement en train d'explorer ces différentes pistes afin de mieux comprendre l'impact des perturbations thermiques sur la fondation des colonies de fourmis.

Surveillance de l'expansion des fourmis invasives dans le Val de Loire

Les fourmis invasives perturbent l'équilibre des écosystèmes naturels en colonisant rapidement de vastes territoires (Holway et al., 2002). Elles entrent en compétition avec d'autres organismes, agissent comme des prédatrices et interfèrent avec les interactions mutualistes entre les plantes et les insectes. En particulier, elles ont un impact négatif sur la diversité et l'abondance des espèces de fourmis indigènes. Au-delà des conséquences écologiques, les fourmis invasives génèrent également d'importantes perturbations socio-économiques, affectant la gestion des environnements urbains, notamment les systèmes de traitement des déchets ménagers, les réseaux électriques, les infrastructures de construction et d'agriculture, ainsi que les jardins publics et privés (Angulo et al., 2022; Bertelsmeier & Courchamp, 2014; Wong et al., 2023). Afin de limiter ces effets néfastes, il est essentiel de détecter la présence de ces espèces invasives le plus tôt possible. Cependant, l'identification de ces espèces uniquement sur la base de critères morphologiques peut être extrêmement difficile et sujette à des erreurs d'identification.

Au cours des dernières années, plusieurs espèces de fourmis invasives (*Tapinoma magnum*, *Lasius neglectus*, *Linepithema humile*) se sont établies dans le Val de Loire (Lenoir et al., 2023). Pourtant, aucun réseau de surveillance n'existait pour suivre leur présence et leur expansion géographique. Cette absence s'explique principalement par la nature récente de ces invasions et par la difficulté d'identifier précisément les espèces problématiques. Le projet FIVALO a été mis en place afin d'établir et de coordonner un réseau de surveillance visant à identifier, détecter, cartographier et évaluer le niveau d'invasion des espèces de fourmis invasives dans deux régions françaises : Pays de la Loire (PDL) et Centre-Val de Loire (CVL).

L’objectif principal du projet FIVALO est de développer et d’implémenter des méthodes efficaces pour le prélèvement d’échantillons et la détermination des espèces. Pour collecter les échantillons, nous collaborons avec des partenaires institutionnels, notamment deux *Conservatoires d’Espaces Naturels* (CEN PDL et CEN CVL) ainsi que deux *Fédérations de Défense contre les Organismes Nuisibles* (FDGDON 49 et FREDON CVL). Nous avons également développé une méthode innovante de prélèvement en collaboration avec le fournisseur d’énergie ENEDIS, qui consiste à récupérer les équipements défectueux en raison d’une infestation par les fourmis.

Afin de garantir une détermination fiable des espèces, nous développons des protocoles combinant la morphologie, l’écologie chimique et la biologie moléculaire. Il est en effet crucial d’associer plusieurs approches, car leur efficacité varie selon les espèces, certaines méthodes étant plus discriminantes que d’autres. Les données collectées nous permettent ensuite de cartographier la distribution des espèces invasives, de sensibiliser le grand public ainsi que les acteurs locaux et professionnels de divers secteurs, et de développer des indicateurs de surveillance afin d’évaluer le potentiel invasif des espèces concernées.

Conclusion

Comprendre la division du travail reproducteur entre les reines et les ouvrières dans les sociétés d'insectes représente un défi fondamental dans l'étude de l'évolution sociale. Tout au long de ma carrière académique, j'ai combiné des manipulations expérimentales, des observations comportementales et des analyses moléculaires afin d'examiner des aspects clés de la spécialisation reproductive, notamment les influences génétiques et maternelles sur la détermination de la caste, la régulation hormonale, transcriptomique et épigénétique de la reproduction, ainsi que le rôle de l'environnement social dans la structuration de la division du travail et de la spécialisation comportementale.

Ces dernières années, j'ai établi des collaborations, obtenu des financements de recherche et encadré plusieurs projets étudiants afin d'explorer comment et pourquoi les interactions sociales influencent les phénotypes individuels au sein des colonies de fourmis. Nos résultats ont démontré que la division du travail reproducteur n'est pas simplement une conséquence figée de la détermination de la caste, mais qu'elle est activement modulée par l'environnement social, les ouvrières jouant un rôle central dans l'induction de la spécialisation comportementale des reines dans la production d'œufs. En montrant que cette spécialisation des reines n'est pas constitutive, mais contrôlée par l'environnement social, nos travaux apportent de nouvelles perspectives qui enrichissent notre compréhension de la division du travail dans les sociétés d'insectes.

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Annexes

Appendix 1

Genetic components to caste allocation in a multiple-queen ant species

Libbrecht R, Schwander T & Keller L

2011

Evolution

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GENETIC COMPONENTS TO CASTE ALLOCATION IN A MULTIPLE-QUEEN ANT SPECIES

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Reproductive division of labor and the coexistence of distinct castes are hallmarks of insect societies. In social insect species with multiple queens per colony, the fitness of nestmate queens directly depends on the process of caste allocation (i.e., the relative investment in queen, sterile worker and male production). The aim of this study is to investigate the genetic components to the process of caste allocation in a multiple-queen ant species. We conducted controlled crosses in the Argentine ant *Linepithema humile* and established single-queen colonies to identify maternal and paternal family effects on the relative production of new queens, workers, and males. There were significant effects of parental genetic backgrounds on various aspects of caste allocation: the paternal lineage affected the proportion of queens and workers produced whereas the proportions of queens and males, and females and males were influenced by the interaction between parental lineages. In addition to revealing nonadditive genetic effects on female caste determination in a multiple-queen ant species, this study reveals strong genetic compatibility effects between parental genomes on caste allocation components.

KEY WORDS: **Caste determination, *Linepithema humile*, Queen specialization, sex ratio, social insects.**

One of the major transitions in evolution is the shift from solitary organisms to societies with reproductive division of labor (Maynard Smith and Szathmary 1995; Szathmary and Smith 1995). In eusocial Hymenoptera (ants, bees and wasps), reproductive division of labor is associated with morphological differences between the reproductive queens and the nonreproductive workers (Wilson 1971; Holldobler and Wilson 1990; Bourke and Franks 1995). These morphological differences, which can be extremely marked in some ant species, arise from a developmental switch during the larval stage (Wilson 1971; Holldobler and Wilson 1990).

For several decades, it was assumed that social insect female brood are fully totipotent, and that environmental factors alone determine whether an individual becomes a reproductive queen, or a functionally sterile worker. However, several recent studies have revealed that genetic factors can, and often do, play an important role in queen and worker caste determination (see Smith et al. [2008b] and Schwander et al. [2010] for review). These genetic influences range from plastic genotypes that are biased toward queen or worker development (e.g. *Pogonomyrmex rugosus* [Schwander and Keller 2008], *Acromyrmex echinatior* [Hughes and Boomsma 2008]) to a strictly genetic determination (e.g. *Pogonomyrmex* lineages [Helms Cahan et al. 2002; Julian et al. 2002; Volny and Gordon 2002], *Solenopsis xyloni* [Helms Cahan and Vinson 2003], *Wasmannia auropunctata* [Fournier et al. 2005]).

The occurrence of a genetic component to caste determination has important implications in species where colonies contain several queens, as this may influence each queen's relative reproductive success. Several studies have shown that queens within a colony may differ in their relative contribution to worker and queen production (Ross 1988; Bourke et al. 1997; Fournier et al.

2004). However, it is unknown whether these differences arise from competitive interactions among queens and other social effects or whether intrinsic genetic differences among queens and/or their mates directly bias the developmental trajectories of their female brood.

Another important factor affecting queen reproductive success in multiple-queen colonies is their relative contribution to male production. Social Hymenoptera have a haplodiploid sex determination system whereby diploid females develop from fertilized eggs whereas haploid males develop from unfertilized eggs (Crozier 1977). Both queens and workers have been shown to influence the proportion of new queens and males produced in their colonies. Queens may influence the sex ratio produced by altering the relative proportion of haploid and diploid eggs laid (Passera et al. 2001; Rosset and Chapuisat 2006) whereas workers may later affect sex ratio by selectively killing males or preferentially rearing females into queens rather than workers (Pamilo 1991; Aron et al. 1995; Passera et al. 1995; Keller et al. 1996; Sundstrom et al. 1996; Hammond et al. 2002). Accordingly, in multiple-queen colonies of ants such as *Linepithema humile*, *Pachycondyla* sp., *Pheidole pallidula* and *Formica exsecta*, queens vary in their relative contribution to male and female (queens and workers) production (Fournier and Keller 2001; Heinze et al. 2001; Fournier et al. 2004; Kummerli and Keller 2007b). Similarly, a skew in the production of males or new queens has been reported in *L. humile* and *Leptothorax acervorum* (Bourke et al. 1997; Fournier and Keller 2001). However, it remains unknown whether these contribution differences among queens have genetic components and, if so, whether these components are additive or result from epistatic and pleiotropic effects.

The aim of this study is to investigate genetic effects on the process of caste allocation (the relative investment in queen, worker and male production) in an ant species with multiple queens per colony. For this purpose, we conducted controlled crosses in the Argentine ant *L. humile.* Colonies of this species contain numerous reproductive queens (Newell 1909; Markin 1970) and, in contrast to most other ants, it is possible to obtain both males and queens, as well as induce mating, in the laboratory (Keller and Passera 1992). After conducting controlled crosses, we established single-queen colonies to study the effects of maternal and paternal genetic backgrounds, as well as the interaction between parental genomes on caste allocation.

Methods

PRODUCTION OF PARENTAL LINEAGES

We collected *L. humile* colonies on 11 February 2008 in Port-Leucate (3°2′20″E, 42°51′22″N), southern France and set up 13 single-queen colonies with 2.5 cm^3 (ca. 1000 workers). To ensure that colonies contained only brood from the mother queen, we removed all the brood present during the first two weeks. The queens were then allowed to lay eggs during eight weeks before being removed so as to stimulate the production of sexuals (new queens and males; Keller and Passera 1992, 1993). Colonies were then regularly checked to transfer all male and queen pupae to queenless and broodless recipient colonies, set up to receive the pupae of only a single sex and colony. This allowed us to obtain large numbers of unmated queens and males of the same lineage (i.e., produced by the same mother queen). These individuals were used to conduct the controlled crosses.

CONTROLLED CROSSES

Of the 26 recipient colonies, six produced enough new queens and four produced enough males to conduct replicate crosses between these maternal and paternal lineages. Mating was obtained by placing one unmated queen with four to six males overnight in a 6.5-cm-diameter vial (Keller and Passera 1992). In *L. humile*, queens are inseminated by only one male even if they mate multiple times (Keller et al. 1992; Krieger and Keller 2000). These crosses allowed us to obtain between two and eight singly inseminated queens for 22 of the 24 possible maternal-by-paternal lineage combinations (Table 1). The 110 newly mated queens were then overwintered with ca. 1000 workers for three months in the dark at 10 ± 2°C, 60% humidity to trigger the production of sexual offspring (Vargo and Passera 1992).

After overwintering, each mated queen was placed with a new set of ca. 600 workers (collected randomly in the same stock colony composed of a mix of several field colonies collected on 16 February 2009) and no brood in 20 × 14 × 5 cm transparent plastic boxes under a 12h:12h artificial light:dark cycle at 25°C, 60% humidity. Colonies were fed a mixture of mealworms, eggs, honey, and vitamins three times a week. Queens were allowed to lay eggs during six weeks before being removed. Under field conditions, 90% of the queens are killed by the workers before the beginning of the reproductive season (Markin 1970; Keller et al.

Table 1. The number of singly mated queens obtained per parental lineages combination. Each of these singly mated queens is a new queen from one of the maternal lineages (rows) inseminated by a male from one of the paternal lineages (columns).

		Paternal lineages			
		Pat1	**Pat2**	**Pat9**	**Pat13**
Maternal lineages	**Mat3**	4	6	5	-
	Mat5	4	3	8	7
	Mat6	2	4	6	4
	Mat7	-	6	2	5
	Mat8	6	6	5	8
	Mat12	2	6	5	6

1989). Thus, queen removal mimicked the conditions leading to the production of males and new queens in the field (Keller and Passera 1992, 1993). Colonies were then monitored weekly to remove all pupae produced. As the pupal stage lasts more than seven days at 25°C in *L. humile* (R. Libbrecht, pers. obs.), this allowed us to count all queen, workers, and male pupae produced, and estimate the worker/queen, male/queen, and male/female ratios.

STATISTICAL ANALYSIS

Among the 110 colonies that overwintered successfully, 20 were removed from the analysis: five queens died during the experiment, four colonies did not produce any offspring, and 11 colonies did not produce any female offspring suggesting that the queens were not inseminated. To test for the effect of maternal and paternal lineages (taken as random variables) on colony-level offspring production, we conducted two-way analyses of variance (ANOVAs) on models optimized to fit our data. The numbers of offspring, females and males were analyzed using a generalized linear model (GLM) with Poisson distributed errors. The worker/queen, male/queen, and male/female proportions were analyzed using a GLM with binomial errors. The models were checked for overdispersion and corrected when needed using quasi-likelihood to specify an appropriate variance function. Correlation tests were carried out using Spearman rank correlation tests. All statistical analyses were performed with *R* (http://www.R-project.org).

Results

Every component of caste allocation varied considerably among the single-queen colonies. Both the proportion of the female offspring that developed into queens (female caste ratio) and the proportion of queens among the sexual offspring (sex ratio) ranged from 0 to 1 (female caste ratio: 0.091 ± 0.16 and sex ratio: 0.38 ± 0.36, mean $\pm$ SD) whereas the proportion of females among all the offspring produced ranged from 0.008 to 1 (0.78 ± 0.26, mean $\pm$ SD).

For each component of caste allocation, we found significant effects of either the paternal lineage or the interaction between parental lineages. The female caste ratio was significantly influenced by the paternal lineage ($F_{3,12} = 6.44$, $P = 0.007$, Fig. 1) whereas there was no significant effect of the maternal lineage ($F_{5,12} = 1.77$, $P = 0.19$) and no significant interaction between maternal and paternal lineages ($F_{12,89} = 0.75$, $P = 0.69$). The sex ratio and the proportion of females among the offspring were not significantly influenced by the paternal (queen/male proportion: $F_{3,12} = 1.12$, $P = 0.38$; female/male proportion: $F_{3,12} = 1.82$, $P = 0.2$) or the maternal lineage (queen/male proportion: $F_{5,12} = 1.18$, $P = 0.38$; female/male proportion: $F_{5,12} = 0.70$, $P = 0.63$). By contrast, there were significant interactions between maternal and paternal lineages on both of these proportions (queen/male proportion: $F_{12,88} = 2.06$, $P = 0.032$, Fig. 2; female/male proportion: $F_{12,89} = 3.29$, $P < 0.001$, Fig. 3). The effect sizes for the different components of caste allocation are summarized in Table 2.

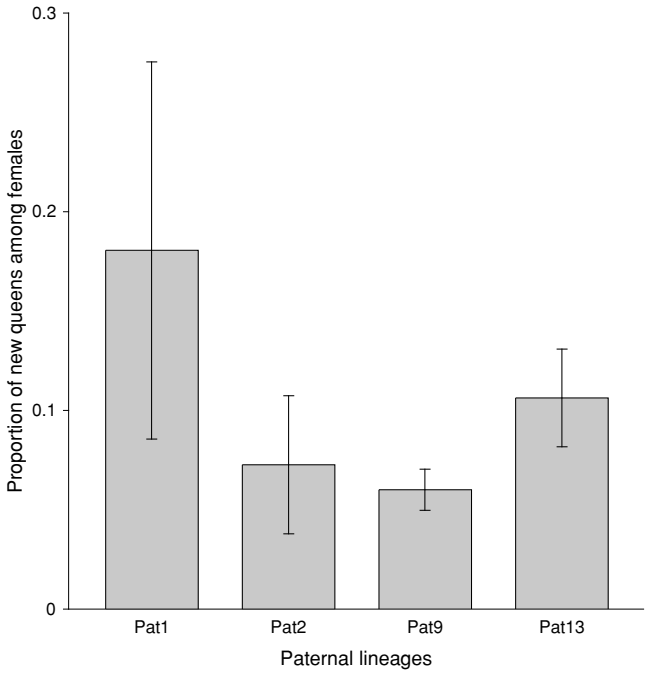

Figure 1. The proportion of new queens among female offspring is significantly affected by the paternal lineage (mean $\pm$ SE for each paternal lineage).

Because there appeared to be extreme variation among colonies in the total number of offspring produced (range 23–234; 120.3 ± 45.6, mean $\pm$ SD), we also tested for parental lineage effects on numbers of different offspring produced. The total number of offspring was significantly affected by the maternal lineage ($F_{5,12} = 3.33$, $P = 0.04$) but not by the paternal lineage ($F_{3,12} = 1.35$, $P = 0.30$) or by the interaction between maternal and paternal lineages ($F_{12,89} = 1.74$, $P = 0.076$). By contrast, we found a different pattern when separately analyzing the numbers of males and females produced: neither the paternal (number of males: $F_{3,12} = 1.26$, $P = 0.33$; number of females: $F_{3,12} = 2.21$, $P = 0.14$) nor the maternal lineage (number of males: $F_{5,12} = 0.81$, $P = 0.56$; number of females: $F_{5,12} = 1.52$, $P = 0.25$) significantly affected these numbers, whereas there was a significant interaction between parental lineages (number of males: $F_{12,89} = 3.24$, $P < 0.001$; number of females: $F_{12,89} = 2.45$, $P = 0.01$). The effect sizes for the numbers of male and female offspring produced are summarized in Table 2.

Finally, to test for possible allocation trade-offs, we analyzed correlations between caste numbers produced. Significant negative correlations revealed trade-offs between queen and male ($n = 90$, rho $= -0.24$, $P = 0.024$, Fig. 4), and female and male productions ($n = 90$, rho $= -0.34$, $P = 0.001$, Fig. 5). There appeared

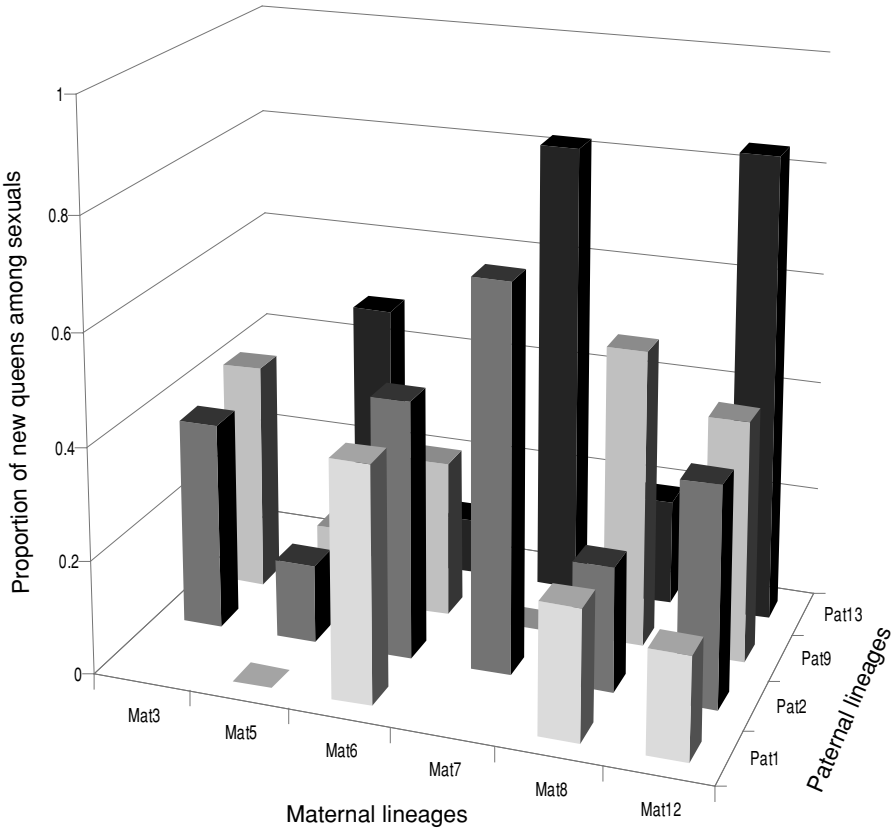

Figure 2. The proportion of new queens among sexual offspring is significantly affected by the interaction between parental lineages (each bar depicts the mean for all queens per combination of parental lineages).

to be no trade-off between queen and worker production as the number of queens and workers were positively correlated ($n = 90$, rho $= 0.32$, $P = 0.002$, Fig. 6).

Discussion

This study demonstrates strong effects of the maternal and paternal lineages on offspring production and caste allocation (i.e., the proportion of queens, workers and males produced) in the Argentine ant *L. humile*. The maternal lineage had a significant effect on the number of offspring produced and the paternal lineage influenced the proportion of females developing into queens or workers (i.e., the process of caste determination). There were also significant interactions between parental lineages for the two other components of caste allocation, namely the proportion of offspring being queens or males and the relative production of males and females.

Several lines of evidence suggest that these parental lineage effects have genetic components. First, all the experiments were conducted under highly controlled laboratory conditions, with colonies containing similar numbers of workers, thus largely removing possible environmental effects. Second, the workers that reared the new queens and the males were unrelated to them and all came from the same stock colonies hence ensuring a uniform social environment. Finally, after mating, queens of all lineages were placed in new colonies containing a new set of workers coming from the same stock colonies. Again, the number of workers was standardized in all colonies. As a result, mothers, fathers, and grandmothers of the broods considered in the analyses were kept under similar environmental and social conditions. This design thus makes it highly likely that parental lineage effects on caste allocation and brood production stem from genetic variation among lineages, even if some environmental influences cannot completely be ruled out. Importantly, a genetic component on sex ratio and caste allocation may stem from both direct and indirect effects. Direct influences could originate from genetic differences in offspring survival and/or development whereas indirect effects could stem from workers altering brood care and/or rearing allocations in response to changes in brood composition (Linksvayer 2006). A combination of direct and indirect effects is also possible. For example, a direct genetic effect inducing a larger proportion of females to develop into queens may reduce the resources available and lead workers to eliminate a greater proportion of males. In the following sections, we discuss in more details the effect of parental lineages on each component of offspring production and caste allocation.

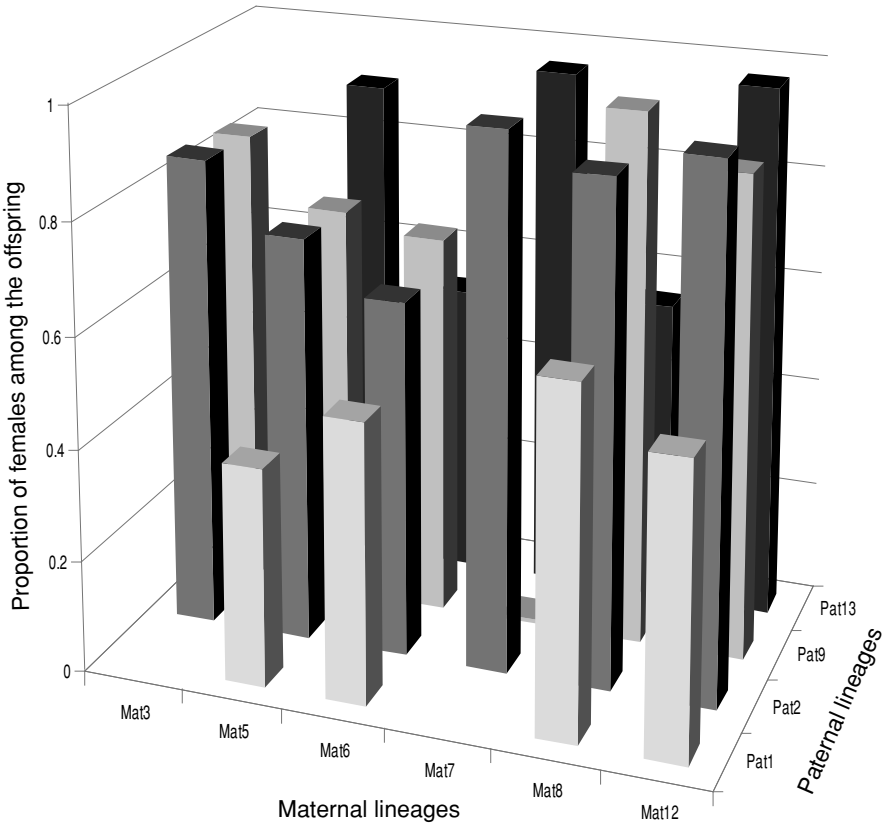

Figure 3. The proportion of females among offspring is significantly affected by the interaction between parental lineages (each bar depicts the mean for all queens per combination of parental lineages).

The first interesting finding of our study is that the paternal lineage affected the relative production of queens and workers while no significant effect of the maternal lineage was detected. This pattern reveals that the genetic effects on female caste determination in *L. humile* have a complex architecture, as classic additive effects would imply an influence of both parental lineages. This result is unlikely to stem from little statistical power for detecting maternal lineage effects. Because of haplodiploidy, the proportion of within-lineage additive genetic variation is smaller for the maternal than the paternal lineages (this is because full sisters share a larger portion of their genome than full brothers). As a consequence, additive genetic factors are more likely to generate significant effects of the maternal than the paternal lineage. The influence of only the paternal lineage on female caste fate is thus best consistent with parent of origin-specific effects and/or other epigenetic factors. Thus, caste-biasing genes could be expressed in the female brood only if paternally inherited. Alternatively, heritable epigenetic changes that affect the likelihood for the female offspring to develop into queens could be triggered by male-dependent conditions.

Table 2. Reduction of deviance obtained when the maternal lineage, the paternal lineage, or the interaction between parental lineages is added to the model. These percentages thus represent the extent to which an explanatory variable improves the model's ability to account for the empirical data. The significance of maternal and paternal lineage effects and of their interaction is also notified (*P < 0.05; **P < 0.01; ***P < 0.001).

	Maternal lineage (%)	Paternal lineage (%)	Interaction between maternal and paternal lineages (%)
Relative proportion of new queens and workers	7.08	15.43**	9.58
Relative proportion of new queens and males	11.12	6.35	22.68*
Relative proportion of females and males	7.86	12.24	26.9***
Number of offspring	22.48*	5.46	16.18
Number of females	13.09	11.46	20.68**
Number of males	9.68	8.99	28.49***

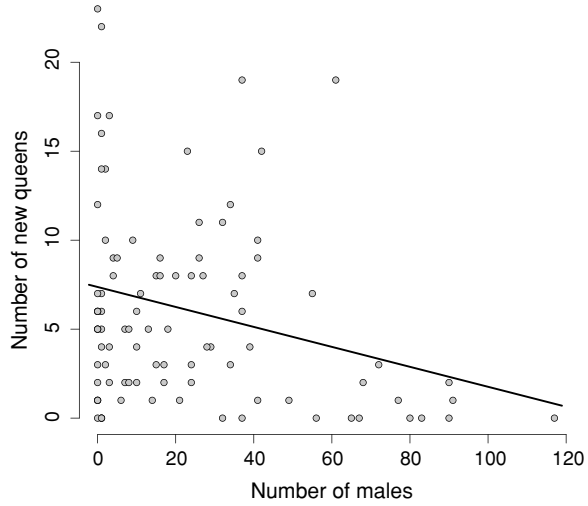

Figure 4. Across colonies the number of new queens produced is negatively correlated with the number of males produced (Spearman rank correlation test; $n = 90$, rho $= -0.24$, $P = 0.024$).

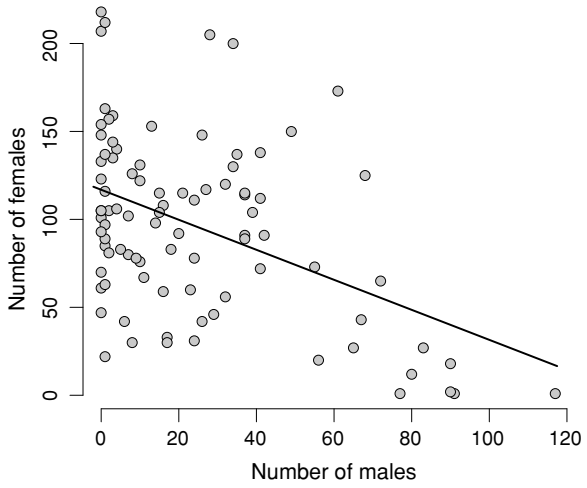

Figure 5. Across colonies the number of females produced is negatively correlated with the number of males produced (Spearman rank correlation test; $n = 90$, rho $= -0.34$, $P = 0.001$).

One important issue discussed by previous studies that reported genetic effects on female caste determination is the maintenance of genetic variation for the trait, as alleles biasing caste development toward queens should quickly go to fixation (Crozier and Pamilo 1996). Four hypotheses have been proposed to account for the maintenance of genetic variation. First queen-biasing alleles are associated with costs such as decreased colony productivity (Bourke and Ratnieks 1999; Wenseleers and Ratnieks 2004). Second, queen-biasing alleles are deleterious when in the homozygous form (Keller and Ross 1998; Hayashi et al. 2007). Third, under sexual antagonism (Rice 1984), queen-biasing alleles, which are favored in females, decrease male fitness (Moritz et al. 2005). Finally, genetic influences on female caste can be maintained if the genetic architecture underlying caste biasing is complex (Schwander et al. 2010). The finding of imprinting and/or epigenetic effects on caste determination is very interesting in this perspective because it reveals a new type of genetic influences that are more complex than additive genetic effects. The traditional method used to test for genetic components to caste determination is to compare the relative representations of patrilines among new queens and workers in species with only one multiply mated queen per colony (Hughes and Boomsma 2008; Schwander and Keller 2008; Smith et al. 2008a; Frohschammer and Heinze 2009). However, this experimental design does not allow inferring the genetic architecture underlying caste bias. We call for more studies on the influence of genetic architecture on the developmental fate of female brood to get a better understanding of the maintenance of genetic effects on caste determination in social insect species.

Our study also revealed significant interactions between parental lineages on sex ratio (proportion of queens and males) while neither the maternal nor the paternal lineage affected this proportion. The lack of maternal and paternal lineage effects are difficult to interpret given that the source colonies used for setting up the parental lineages already showed a biased sex ratio (male bias for the paternal and queen bias for the maternal lineages). This may have altered the distribution of genetic variability for sex ratio between the maternal and paternal lineages. By contrast, several mechanisms could explain the interaction between parental lineages on sex ratio. Sex ratio could be influenced directly by interactions between parental lineages if compatibility between parental genomes affects the viability of female broods or influences the likelihood of egg fertilization. Alternatively, sex ratio may be influenced indirectly via changes in the proportion of females that develop into queens rather than workers. The latter

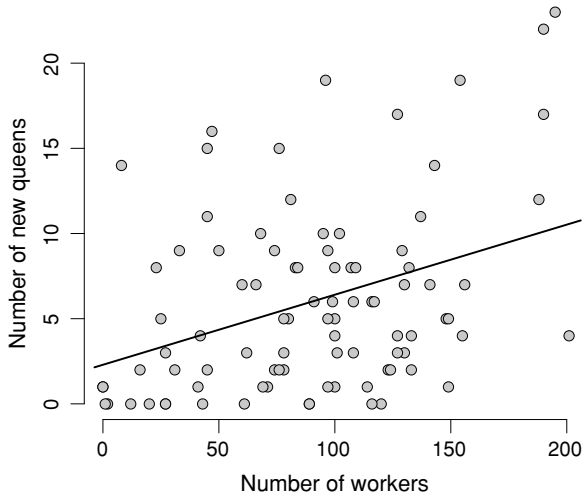

Figure 6. Across colonies the number of new queens produced is positively correlated with the number of workers produced (Spearman rank correlation test; $n = 90$, rho $= 0.32$, $P = 0.002$).

explanation is unlikely in *L. humile* because, contrary to the proportion of males and females, the proportion of new queens and workers produced was not significantly affected by the interaction between parental lineages. Our analyses of brood numbers are best consistent with compatibility affecting fertilization probability, although additional effects on female brood viability or the worker propensity to preferentially raise new queens and/or eliminate males remain possible. Indeed, the numbers of males and females produced were negatively correlated and both were significantly affected by interactions between the maternal and paternal lineages. This is the expected pattern if queens laid a fixed number of eggs independently of the ratio of haploid to diploid eggs among them, thereby generating a trade-off in the numbers of males and diploid brood produced. Queens may actively change sex allocation depending on qualities of the sperm transferred by their mate (Fjerdingstad and Boomsma 1997; Fjerdingstad 2004) or fertilization success may be passively influenced by compatibilities between parental genomes. Whatever the detailed mechanism, our results reveal that interactions between queens and males can affect the colony sex ratio. Previous studies showed that the queen influences the colony sex ratio in *S. invicta*, *F. selysi*, and *Cardiocondyla kagutsuchi* (Passera et al. 2001; Rosset and Chapuisat 2006; Frohschammer and Heinze 2009). However, because these studies were not designed to detect potential effects of the interaction between the queens and their mates, it is impossible to infer whether the reported queen influences also stem from interaction effects or between-queen differences.

The finding that interactions between the queen and her mate may affect the relative production of new queens and males has important implications for sex ratio and conflict theory in social insects. Because of the haplodiploid mechanism of sex determination, there is a potential conflict between queens and their mates over the sex ratio produced (Haig 1998; Helantera and Ratnieks 2009), as males have all their genes in their daughters but none in the males produced. There is thus strong pressure on males to bias the sex ratio toward females (Haig 1998) while queens should favor balanced sex ratios because they are equally related to their daughters and sons. The finding that the interaction between queens and males can influence the sex ratio produced should be added to the traditional queen/worker framework when studying intracolonial conflicts in social insects.

Our study also provides a new explanation for why queen in multiqueen societies often tend to specialize in the production of a single caste. For example, queens producing more males produce fewer queens in *L. humile* (Fournier and Keller 2001) and *F. exsecta* (Kummerli and Keller 2007a, b). A trade-off between the contribution to worker and queen production has been reported in *P. pallidula* (Fournier et al. 2004) and a trade-off between worker and male production in *L. acervorum* (Hammond et al. 2006). In these studies, it was not possible to determine whether the queen specializations (Kummerli and Keller 2007a,b) resulted from competition and social interactions between queens or from intrinsic differences between the broods produced by queens. In our single-queen colonies, we found similar trade-offs for the relative investment into males and queens, as well as males and females, revealing that competition and social interactions between queens are not required to generate specializations. In addition, given that caste allocation in our colonies was also influenced by the interaction between parental lineages, queen specialization reported in the previous studies may at least partly stem from genetic differences. More generally, queen specialization is likely to be affected by complex genetic interactions between the queen and her mate. Such interaction effects between queens and males may help to explain the maintenance of queen specialization in social insect species with multiple queens per colony.

Finally, we also found that the total number of offspring produced in our experimental colonies was affected exclusively by the maternal lineage. This is not surprising given that both the number of eggs produced and the nutrients in the eggs depend on the mother queens. Because males only contribute their sperm to offspring production, paternal effects in this case would have to occur mainly via some type of chemical manipulation of the females, possibly in combination with effects on diploid brood viability. The effect of the maternal lineage on the number of offspring produced thus stems most likely from between-lineage variation in fecundity and/or egg viability. This variation may derive from genetic differences between queens from different lineages (Frohschammer and Heinze 2009), maternal effects (Schwander et al. 2008), and/or different environmental conditions experienced by different lineages during their development. As explained above, all colonies were maintained under highly controlled conditions so that differences in environmental conditions should only have a minor contribution to differences between lineages compared to genetic or maternal effects.

In conclusion, the use of controlled crosses in the laboratory allowed us to demonstrate widespread effects of both parental genetic backgrounds on several components of caste allocation. Our study provides evidence that nonadditive genetic effects account for between-queen and between-colony variations in the caste and sex ratios produced. Such diverse influences of nonadditive genetic effects demonstrate overall complex architectures of the genetic components to caste allocation. More such studies are strongly needed to develop insights into the genetics of phenotypic plasticity and caste allocation in social insects.

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Appendix 2

Interplay between insulin signaling, juvenile hormone and vitellogenin regulates maternal effects on polyphenism in ants

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Interplay between insulin signaling, juvenile hormone, and vitellogenin regulates maternal effects on polyphenism in ants

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Polyphenism is the phenomenon in which alternative phenotypes are produced by a single genotype in response to environmental cues. An extreme case is found in social insects, in which reproductive queens and sterile workers that greatly differ in morphology and behavior can arise from a single genotype. Experimental evidence for maternal effects on caste determination, the differential larval development toward the queen or worker caste, was recently documented in *Pogonomyrmex* seed harvester ants, in which only colonies with a hibernated queen produce new queens. However, the proximate mechanisms behind these intergenerational effects have remained elusive. We used a combination of artificial hibernation, hormonal treatments, gene expression analyses, hormone measurements, and vitellogenin quantification to investigate how the combined effect of environmental cues and hormonal signaling affects the process of caste determination in *Pogonomyrmex rugosus*. The results show that the interplay between insulin signaling, juvenile hormone, and vitellogenin regulates maternal effects on the production of alternative phenotypes and set vitellogenin as a likely key player in the intergenerational transmission of information. This study reveals how hibernation triggers the production of new queens in *Pogonomyrmex* ant colonies. More generally, it provides important information on maternal effects by showing how environmental cues experienced by one generation can translate into phenotypic variation in the next generation.

Many plants and animals can express specific adaptive responses to their environment through phenotypic plasticity, whereby a given genotype can develop into different phenotypes depending on environmental conditions (1, 2). Maternal effects, through which the environmental conditions experienced by the mother are translated into phenotypic variation in the offspring (3, 4), contribute to many phenotypic traits in a wide variety of taxa (5, 6) and have important ecological and evolutionary consequences (7, 8). Investigating the mechanisms of cross-generational transmission of information underlying maternal effects is needed to better understand the optimization of phenotypes in changing environments (6) and, more generally, the evolution of life history strategies (9).

In many insect species, maternal effects are known to affect polyphenism (3, 10), an extreme form of phenotypic plasticity characterized by the production of alternative and discrete phenotypes from a single genotype (1, 11–13). Such maternal effects allow adequate responses to environmental cues such as temperature, photoperiod, nutrition, and population density in many species (10). Examples of maternal effects on insect polyphenism include the production of sexual versus parthenogenetic morphs in aphids (14, 15), winged versus wingless morphs in firebugs (16), and dispersal versus solitary morphs in locusts (17, 18). The endocrine system was found to play a role in the regulation of some maternal effects on insect polyphenisms (19–21), but the nature of the physiological and genetic pathways interacting with the hormonal system to translate environmental cues into offspring polyphenism remains mostly unknown (22).

The most striking example of polyphenism is found in insect societies (23), where a reproductive division of labor leads to the coexistence of fertile queens and sterile workers that greatly differ in morphology and behavior (24, 25). Even though recent studies revealed genetic influences on caste determination in social insects (reviewed in ref. 26), female caste fate is primarily influenced by environmental factors in most species studied (27–39). In ants, several studies suggested that maternal factors such as temperature or queen age may affect caste determination (40–44). However, it is only recently that the first example of maternal effects on female caste polyphenism was documented experimentally (45). Cross-fostering of eggs between hibernated and nonhibernated *Pogonomyrmex* colonies revealed strong maternal effects on caste production, as only eggs produced by a hibernated queen were able to develop into queens, irrespective of the hibernation status of the rest of the colony (45). Such maternal effects on the caste fate of the female offspring require that the hibernation triggers changes in the queen that affect polyphenism in the offspring. Hormones may be involved in this process in *Pogonomyrmex* ants, as *Pogonomyrmex rugosus* queen- and worker-destined eggs differed in their ecdysteroid content (45) and *Pogonomyrmex barbatus* mature queens treated with juvenile hormone (JH) were recently found to produce larger workers (46).

Studies on the mechanisms regulating insect polyphenisms (reviewed in ref. 10) suggest that the insulin/insulin-like growth factor signaling (IIS), JH, and vitellogenin (Vg) pathways, known to regulate reproduction in adult insects (47–51), play predominant roles in modulating larval development in response to environmental cues. A well-known example illustrating the role of these pathways is the caste fate of the female brood (queen or worker) in the honey bee *Apis mellifera* (52–58). In this species, worker-triggered differences in larval diet induce changes in IIS that affect JH (57), possibly through the release of neuropeptides (e.g., allatostatin and allatotropin) that influence JH production by the corpus allatum, as found in *Drosophila* (59). Changes in JH in turn affect the production of Vg (60–62), which may be involved in the process of caste determination (62, 63). Such effects of JH on Vg production, also reported in flies (64), locusts (65), and cockroaches (66), have been proposed to involve the action of ecdysteroids (62, 67–70). IIS, JH, and Vg may also play a role in the regulation of caste differentiation of larvae in ants, as caste-specific expressions

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of genes involved in the IIS pathway were documented in *Solenopsis invicta* (71) and *Diacamma* sp. (72). Interestingly, caste-specific differences in IIS, JH, and Vg were also documented in adult ants and bees (48, 73–78), suggesting further roles of these pathways in the regulation of social life (74, 79).

We propose that the interplay between IIS, JH, and Vg regulates maternal effects on caste polyphenism in ants by translating the environmental conditions experienced by the queen during hibernation into the production of alternative phenotypes in the offspring. Under this hypothesis, IIS would translate environmental cues into changes in JH, which would, in turn, affect the amount of Vg in queens and in eggs, thus possibly affecting the caste fate of the offspring (62, 63). This hypothesis makes four predictions. First, a pharmacological increase of JH in queens should mimic the effect of hibernation and stimulate the production of queens. Second, hibernation should affect IIS and the production of JH in queens. Third, both hibernation and a JH increase should stimulate the production of Vg in queens. Finally, Vg content should differ between queen- and worker-destined eggs. We tested these predictions by performing artificial hibernation, hormonal treatments, gene expression analyses, hormone measurements, and Vg quantification in *Pogonomyrmex rugosus*, an ant species in which temperature-triggered changes in the queen had previously been shown to affect the relative production of queens and workers. Each of the four predictions was confirmed by our experiments, thus revealing that the interplay between IIS, JH, and Vg regulates maternal effects on caste polyphenism in *P. rugosus*.

Results

To investigate the mechanisms of caste allocation, we compared the production of queens between control, hibernated, and methoprene-treated *P. rugosus* colonies. There was a great variation among colonies in the proportion of queens among the offspring produced, ranging from 0 to 0.47 (0.05 ± 0.11, mean ± SD). There was a significant effect of the treatments on the proportion of queens produced [$F_{(2,73)} = 40.51$, $P < 0.001$; Fig. 1]. Hibernation significantly increased the proportion of queens among the female offspring ($t = 2.06$, $P = 0.04$). The methoprene (JH analog) treatment had a similar—albeit stronger—effect, as the queen/worker ratio among the female offspring was significantly higher in colonies fed methoprene-treated food compared with control colonies ($t = 5.39$, $P < 0.001$). Interestingly, when only pupae that did not receive any treatment during larval development but were produced by treated queens (thus, those collected after week 11) were considered, there was also a significant difference between control and methoprene-treated colonies in the proportion of queens produced ($t = 5.56$, $P < 0.001$), showing that at least part of the observed effect of methoprene on caste determination was triggered by maternal effects.

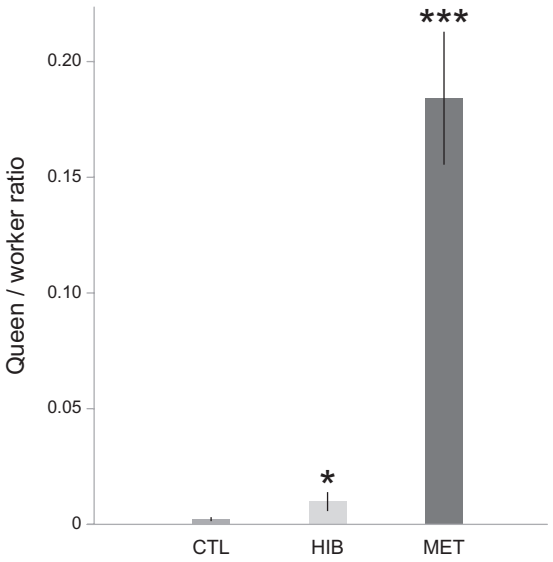

Fig. 1. The proportion of queens among the offspring produced (mean ± SE) was increased in hibernation (HIB) and methoprene (MET) treatments compared with control (CTL). *$P < 0.05$; ***$P < 0.001$.

Whole-body queen samples were used to measure the expression of genes involved in the IIS pathway (two insulin-like peptide genes: *ILP1* and *ILP2*), JH production (one gene coding for JH epoxidase: *JHepox*), and vitellogenesis (two Vg genes: *Vg1* and *Vg2*). The treatments significantly affected the expression of all of the genes tested [*ILP1*: $F_{(2,36)} = 5.30$, $P = 0.01$; *ILP2*: $F_{(2,36)} = 19.47$, $P < 0.001$; *JHepox*: $F_{(2,36)} = 4.12$, $P = 0.02$; *Vg1*: $F_{(2,36)} = 11.15$, $P < 0.001$; *Vg2*: $F_{(2,36)} = 7.93$, $P = 0.001$]. Compared with the control group, both hibernation and methoprene treatments up-regulated the expression of *ILP1* (hibernation: $t = 1.92$, $P = 0.06$; methoprene: $t = 3.24$, $P = 0.003$; Fig. 2), *ILP2* (hibernation: $t = 4.02$, $P < 0.001$; methoprene: $t = 6.14$, $P < 0.001$; Fig. 2), *JHepox* (hibernation: $t = 2.28$, $P = 0.03$; methoprene: $t = 2.65$, $P = 0.01$; Fig. 3), *Vg1* (hibernation: $t = 2.20$, $P = 0.03$; methoprene: $t = 4.72$, $P < 0.001$; Fig. 4), and *Vg2* (hibernation: $t = 2.15$, $P = 0.04$; methoprene: $t = 3.98$, $P < 0.001$; Fig. 4).

To determine whether ecdysteroids mediated the effect of JH on Vg genes expression, we compared the 20-hydroxyecdysone (20E) titer between queens from the control, hibernation, and methoprene groups. Although the 20E titer was lower in the methoprene group (3.38 ± 4.44 pg/mg) compared with the control (8.16 ± 8.47 pg/mg) and hibernation (8.18 ± 9.28 pg/mg) groups, the effect of the treatments was not significant (Kruskal–Wallis $\chi^2 = 2.76$, $P = 0.25$). However, there was a significant negative correlation between the 20E titer in queens and the proportion of queens in their brood (Spearman correlation test, $\rho = -0.40$, $P = 0.01$).

There was no significant difference between treatments in the number [$F_{(2,72)} = 1.35$, $P = 0.27$] and weight [$F_{(2,72)} = 1.09$, $P = 0.34$] of eggs produced. However, the treatments significantly affected the proportion of Vg among total proteins (Kruskal–Wallis $\chi^2 = 6.63$, $P = 0.04$; Fig. 5). The proportion of Vg in the protein content of eggs produced by both hibernated ($U = 42$, $P = 0.038$) and methoprene-treated ($U = 53.5$, $P = 0.026$) queens was significantly higher than in eggs produced by control queens. By contrast, this proportion did not differ significantly between eggs produced by hibernated and methoprene-treated queens ($U = 79$, $P = 0.93$).

Discussion

Each of the four predictions developed under the hypothesis that the interplay between IIS, JH, and Vg regulates maternal effects on caste polyphenism in *P. rugosus* was confirmed by this study. In line with the first prediction that an artificial increase of JH in queens should stimulate the production of queens, the feeding of *P. rugosus* colonies with a JH analog (methoprene) mimicked the effect of hibernation, with both hibernated and methoprene-treated colonies showing an increased production of queens. These results reveal a role of JH in the regulation of caste polyphenism in *P. rugosus*. In this species, maternal effects were previously found to stimulate the production of queens in response to hibernation, as only colonies headed by a hibernated queen produced queens, whether or not the workers had been exposed to cold (45). The exposure to cold therefore triggers changes in queens that make them more likely to lay queen-destined eggs. In this study, the methoprene treatment also targeted the queen, as evidenced by an increase in the proportion of queens among the offspring developing in a non–methoprene-treated environment from eggs laid by methoprene-treated queens. Similar results were found in *Pheidole pallidula*, in which direct topical application of JH on the queen stimulated the production of queens (80), and in

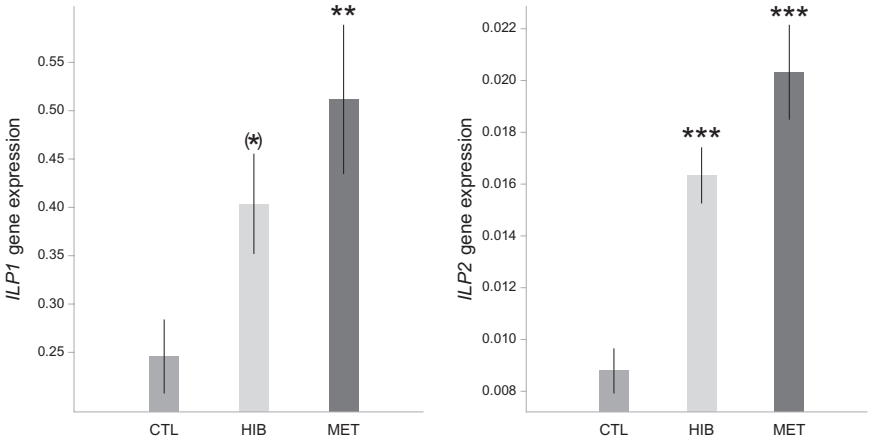

Fig. 2. *ILP1* and *ILP2* were up-regulated in hibernation and methoprene treatments. The *y* axis indicates the relative gene expression in queens, corresponding to the *ILP1* and *ILP2* mRNA levels relative to the *RP49* (control) mRNA level (mean ± SE). $^{(*)}P = 0.06$; $^{**}P < 0.01$; $^{***}P < 0.001$.

P. barbatus, in which it affected the size of the workers produced (46). Overall, the observed effects of hibernation and methoprene treatments show that hibernation-triggered JH changes in queens are involved in the production of queens in *P. rugosus*.

The second prediction was that hibernation should affect IIS and JH in queens. In line with this prediction, our results revealed that genes involved in IIS (*ILP1* and *ILP2*) were up-regulated in *P. rugosus* hibernated queens. This suggests that hibernation can translate into changes in the IIS pathway. Low temperature or the associated photoperiod changes could directly affect IIS, as reported in the regulation of insect diapause (81). Alternatively, the effect of exposure to cold could have been mediated by a change in the queen nutritional status due to decreased activity and metabolism (82) or lower food intake during hibernation. Such effects of nutrition on IIS have been reported in *Drosophila* (83–85). Changes in IIS usually result in the release of neuropeptides (e.g., allatostatin, allatotropin) that influence the production of JH by the corpus allatum (59, 64). Accordingly, the exposure to cold also up-regulated the expression of the *JHepox* gene, which encodes JH epoxidase, the enzyme that catalyzes the oxidation of methyl farnesoate into JH III (86, 87), the last step in the JH biosynthesis in most insects (88–91).

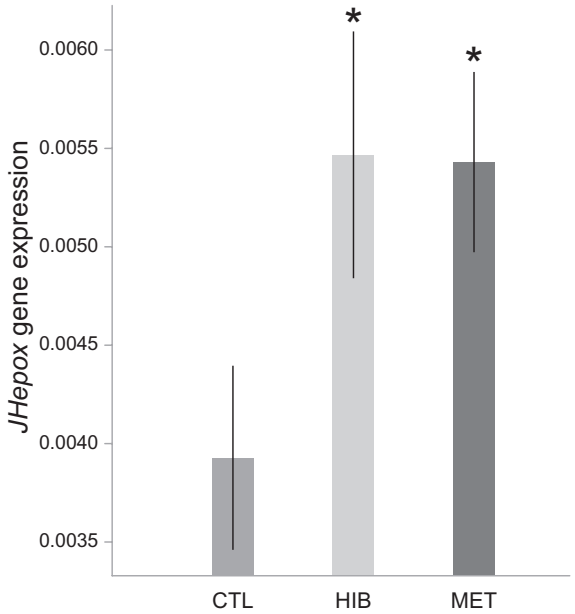

Fig. 3. *JHepox* was up-regulated in hibernation and methoprene treatments. The *y* axis indicates the relative gene expression in queens, corresponding to the *JHepox* mRNA level relative to the *RP49* (control) mRNA level (mean ± SE). $^{*}P < 0.05$.

The finding that the expression of IIS genes was also affected by the methoprene treatment could be explained by JH translating environmental cues into IIS changes rather than the opposite. This is consistent with the report that RNAi-mediated manipulation of JH production affects IIS in *Tribolium* beetles (49). However, the effect of methoprene on IIS is not incompatible with IIS regulating JH production, as it may have been mediated by the associated changes in Vg (48, 92), of which levels are known to affect IIS through the target-of-rapamycin pathway in bees (55, 57, 78). Furthermore, IIS is known to regulate the production of JH in flies (59, 64). Although our data and the available literature do not provide a definite answer on the directionality of the relationship between IIS and JH in ants, our results clearly show interactions between these pathways in response to environmental changes such as those experienced during hibernation.

The third prediction was that both hibernation and an artificial increase in JH should stimulate the production of Vg. In our experiments, both hibernation and methoprene treatments stimulated the production of queens and up-regulated the expression of Vg genes (*Vg1* and *Vg2*) in queens. The effect of hibernation on vitellogenesis is likely to have been triggered by the increase in JH production. This is supported by the finding that the methoprene treatment also up-regulated Vg expression. These results show that JH-regulated vitellogenesis in adult *P. rugosus* queens is involved in the regulation of caste polyphenism.

In insects, effects of JH on Vg production have been proposed to be mediated by the ecdysteroid pathway (62, 67–70). Our results do not provide evidence for such a role of ecdysteroids, as the 20E titer in queens did not differ significantly among treatments. Interestingly, the results revealed a trend toward a reduction of 20E titer in methoprene-treated queens and a significant negative relationship between the 20E titer in queens and the proportion of queens in their offspring. This suggests that ecdysteroids may be involved in the process of caste determination (45, 93).

Finally, the fourth prediction was that the Vg content in eggs should correlate positively with their likelihood of developing into queens. This prediction was also supported by our data. Although neither the number nor the weight of eggs produced differed between control, hibernated, and methoprene-treated queens, the proportion of Vg in the protein content was significantly higher in eggs produced by both hibernated and methoprene-treated queens than by control queens. It is likely that the increased production of

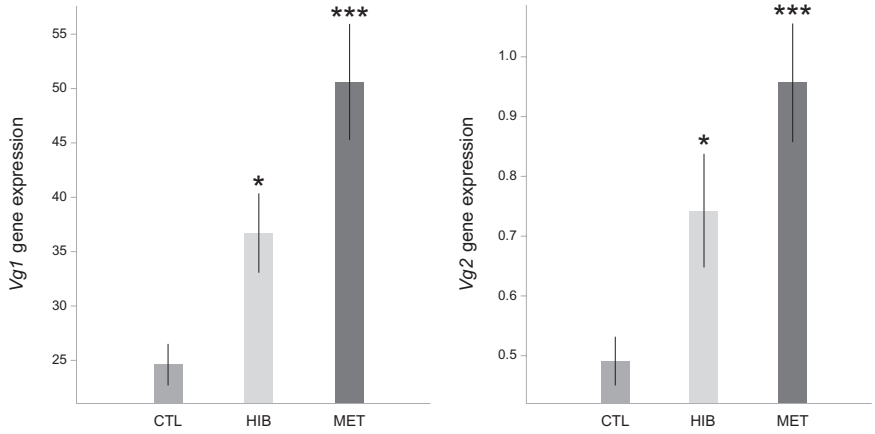

Fig. 4. *Vg1* and *Vg2* were up-regulated in hibernation and methoprene treatments. The *y* axis indicates the relative gene expression in queens, corresponding to the *Vg1* and *Vg2* mRNA levels relative to the *RP49* (control) mRNA level (mean ± SE). *$P < 0.05$; ***$P < 0.001$.

Vg in hibernated and methoprene-treated queens translated into a higher Vg content in the eggs, increasing their likelihood of developing into queens. How the Vg content in eggs alters the caste fate remains to be investigated, but, as Vg is thought to act as a nutritive source for the embryo (94), more Vg in the egg could result in more energy during early development, facilitating the path toward queen development. The finding of a higher proportion of Vg in eggs produced by queen-producing hibernated and methoprene-treated queens is consistent with our fourth prediction, and shows that the quantity of Vg injected in the eggs is involved in the early regulation of caste allocation and plays a role in the intergenerational transmission of information required for maternal effects on polyphenism to happen.

Overall, this study describes the mechanisms that allow the environmental cues experienced by one generation to be translated into phenotypic variation in the next generation. The interaction between IIS and JH in queens translates environmental cues into changes in Vg production. This affects the quantity of Vg injected into the eggs produced, influencing their development toward the queen or worker caste. In addition to the insights provided on the regulation of caste determination in social insects, this study raises the possibility that the interplay between IIS, JH, and Vg is also involved in the maternal regulation of other insect polyphenisms. More generally, this study provides routes to study the proximate mechanisms regulating maternal effects on any phenotypic traits.

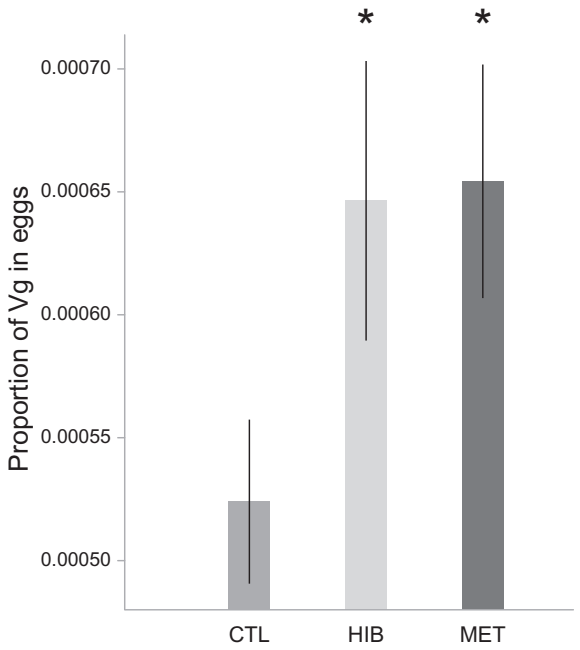

Fig. 5. The proportion of Vg among total proteins (mean ± SE) was increased in eggs produced in hibernation and methoprene treatments. *$P < 0.05$.

Methods

Pogonomyrmex rugosus founding queens were collected during nuptial flights on July 15, 2008, in Bowie, AZ (N32°18′54″/W109°29′03″). Although it is affected by genetic compatibility effects, caste determination in this species remains mostly environmental with very strong maternal effects (26, 32, 45). After worker eclosion, the colonies were kept in laboratory conditions (30 °C, 60% humidity, and 12-h/12-h light:dark cycle) in plastic boxes containing a nest, a foraging area, and water tubes, and were fed once a week with grass seeds and a mixture of eggs, honey, and crushed mealworms. The experiments were performed on 92 2.5-y-old colonies that had never been exposed to cold and never produced queens. The colonies were divided in three groups: control ($n = 26$), hibernation ($n = 25$), and methoprene ($n = 26$).

The experimental manipulations were divided in two phases (Fig. S1). The first phase was set up to test the effect of an exposure to cold. Colonies from the hibernation group were kept for 2.5 mo in a dark climate chamber at 13 °C ± 1 °C and 60% humidity. The transition to and out of hibernation was done over a period of 2 wk by progressively decreasing or increasing temperature in a 8-h/16-h light:dark cycle. All of the other colonies (control and methoprene groups) were kept in the usual laboratory conditions (30 °C, 60% humidity, and 12-h/12-h light:dark cycle). The first phase terminated at week 0, when the second phase started. The second phase was set up to test the effect of JH treatment. To do so, we used methoprene (Sigma-Aldrich), a synthetic analog of JH. The colonies from the methoprene group were fed four mealworms crushed with 0.1 mg of methoprene in 0.1 mL of acetone each week for 8 wk (from week 0 to week 7), whereas colonies from the hibernation and control groups received four mealworms crushed in 0.1 mL of acetone. There is a drawback to this whole-colony approach because it affects all of the individuals in the colony. To circumvent this problem and determine whether the methoprene treatment acted on the queens to affect caste determination, we performed an additional analysis restricted to the offspring that pupated after week 11. These individuals were produced by methoprene-treated queens, but were not exposed to methoprene during larval development. The experimental design also required to control for a potential effect of acetone: feeding colonies mealworms crushed in acetone had no significant effect on the proportion of queens produced (food with acetone: $n = 26$; food without acetone: $n = 15$; Mann–Whitney *U* test: $U = 204$, $P = 0.15$).

Samples were collected in each colony to assess the proportion of queens among the female offspring produced, the number, weight, and Vg content of eggs produced, the expression of candidate genes, and the ecdysteroid titers in queens. All of the pupae produced were collected from week 3 until no brood remained (Fig. S1) and observations of size and morphology allowed the assignation of each pupa to the queen or worker caste. The proportion of

queens among the offspring produced was then calculated for each colony (except one that did not produce enough offspring; control: $n = 26$; hibernation: $n = 25$; methoprene: $n = 25$). At week 4, the queen of each colony was isolated for 24 h in a 2-mL plastic tube closed with wire mesh and placed in the colony. Thus, the queen could still communicate with workers, reducing the stress of isolation. This method allowed us to collect and count the number of eggs produced by each queen in 24 h (control: $n = 26$; hibernation: $n = 25$; methoprene: $n = 25$). At week 5, a batch of eggs was collected in each colony (between 5 and 52 eggs per colony; 26.1 ± 8.9, mean $\pm$ SD) and weighed using a microbalance (Mettler Toledo MT5) to a precision of 1 μg (control: $n = 26$; hibernation: $n = 25$; methoprene: $n = 25$). The eggs were then stored at −80 °C for further measurement of Vg content, successfully performed on eggs produced by 40 colonies (control: $n = 15$; hibernation: $n = 11$; methoprene: $n = 14$). At week 7, the queen was collected in each colony. One-half of the queens were flash-frozen in liquid nitrogen and stored at −80 °C for later RNA extraction (control: $n = 13$; hibernation: $n = 13$; methoprene: $n = 13$), whereas the other half was used for ecdysteroid measurement (control: $n = 12$; hibernation: $n = 12$; methoprene: $n = 13$).

RNA extractions from whole-body queen samples were performed using a modified protocol including the use of TRIzol (Invitrogen) for the initial homogenization and the RNeasy Plus Micro extraction kit (Qiagen). For each individual queen, cDNAs were synthesized using 500 ng of total RNA, random hexamers, and Applied Biosystems reagents. Levels of mRNA were quantified by quantitative real-time PCR (qRT-PCR) using ABI Prism 7900 sequence detector and SYBR Green. All qRT-PCR assays were performed in triplicate and subjected to the heat-dissociation protocol following the final cycle of the qRT-PCR to check for amplification specificity. qRT-PCR values of each gene were normalized by using an internal control ribosomal protein 49 (*RP49*) gene. Paralog-specific primers (Table S1) were designed using sequence alignment (95) and primer analysis (96) programs. Primer sequences overlapped coding regions split by introns, allowing the specific amplification of cDNA levels over potential genomic DNA contaminations. Transcript quantification calculations were performed by using the ΔΔCT method (97).

The ecdysteroid titer in queens was determined using the liquid chromatography–mass spectrometry method developed by Westerlund and Hoffmann (98), with some minor modifications (see *SI Text* for details). The amount of Vg in eggs was measured by dot-blotting using *Ectatomma tuberculatum* (Formicidae: Ectatomminae) anti-Vg antibodies (99) (see *SI Text* for details).

To test for the effect of the treatments on the proportion of queens among the offspring, gene expression, and egg number and weight, we conducted ANOVAs on models optimized to fit our data. The proportion of queens was fit using a generalized linear model with quasi-binomial errors. The gene expression data were fit using a general linear model with normal errors. The ecdysteroid and Vg data could not be normalized and were analyzed using Kruskal–Wallis and Mann–Whitney nonparametric tests. The correlation between the ecdysteroid titer and the proportion of queens produced was tested using a Spearman rank correlation test. All statistical analyses were performed with R (www.R-project.org).

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Appendix 3

Vitellogenin underwent subfunctionalization to acquire caste and behavioral specific expression in the harvester ant *Pogonomyrmex barbatus*

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Vitellogenin Underwent Subfunctionalization to Acquire Caste and Behavioral Specific Expression in the Harvester Ant *Pogonomyrmex barbatus*

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Abstract

The reproductive ground plan hypothesis (RGPH) proposes that the physiological pathways regulating reproduction were co-opted to regulate worker division of labor. Support for this hypothesis in honeybees is provided by studies demonstrating that the reproductive potential of workers, assessed by the levels of vitellogenin (Vg), is linked to task performance. Interestingly, contrary to honeybees that have a single Vg ortholog and potentially fertile nurses, the genome of the harvester ant *Pogonomyrmex barbatus* harbors two Vg genes (*Pb_Vg1* and *Pb_Vg2*) and nurses produce infertile trophic eggs. *P. barbatus*, thus, provides a unique model to investigate whether Vg duplication in ants was followed by subfunctionalization to acquire reproductive and non-reproductive functions and whether Vg reproductive function was co-opted to regulate behavior in sterile workers. To investigate these questions, we compared the expression patterns of *P. barbatus* Vg genes and analyzed the phylogenetic relationships and molecular evolution of Vg genes in ants. qRT-PCRs revealed that *Pb_Vg1* is more highly expressed in queens compared to workers and in nurses compared to foragers. By contrast, the level of expression of *Pb_Vg2* was higher in foragers than in nurses and queens. Phylogenetic analyses show that a first duplication of the ancestral Vg gene occurred after the divergence between the poneroid and formicoid clades and subsequent duplications occurred in the lineages leading to *Solenopsis invicta*, *Linepithema humile* and *Acromyrmex echinatior*. The initial duplication resulted in two Vg gene subfamilies preferentially expressed in queens and nurses (subfamily A) or in foraging workers (subfamily B). Finally, molecular evolution analyses show that the subfamily A experienced positive selection, while the subfamily B showed overall relaxation of purifying selection. Our results suggest that in *P. barbatus* the Vg gene underwent subfunctionalization after duplication to acquire caste- and behavior- specific expression associated with reproductive and non-reproductive functions, supporting the validity of the RGPH in ants.

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Introduction

Division of labor is the cornerstone of insect societies and implies the coexistence of individuals that differ in morphology, reproduction and behavior [1,2]. There are usually two levels of division of labor among individuals in social insect colonies. The first relates to a reproductive division of labor, whereby reproduction is monopolized by one or several queens while sterile workers perform all the tasks related to colony maintenance. The second relates to a division of labor among the worker force, whereby workers perform different tasks in an age-dependent sequence: young workers usually perform tasks inside the colony (e.g. brood care) while old workers forage outside the nest [3,4]. The colony organization of advanced eusocial insects evolved independently in ants, bees, and wasps [5,6]. While the ecological constraints favoring social evolution are well studied [7], it remains largely unknown whether the genetic mechanisms regulating behavior are conserved among species [8–12].

The ovarian ground plan hypothesis (OGPH) is a theoretical framework that seeks to explain the evolution of reproductive division of labor in social insects [13,14]. The OGPH proposes that the physiological pathways regulating the reproductive and behavioral cycles of solitary ancestors have been co-opted and selected to evolve into the queen and worker castes of existing eusocial insects. This hypothesis is based on the observations that the ovarian cycle of alternate development and depletion phases of solitary wasps is associated with reproductive and non-reproductive behavioral traits that resemble the queen and worker castes of eusocial insects: females with developed ovaries lay eggs while females with undeveloped ovaries forage for food and defend the

Author Summary

One of the main features of social insects is the division of labor, whereby queens monopolize reproduction while sterile workers perform all of the tasks related to colony maintenance. The workers usually do so in an age-dependent sequence: young workers tend to nurse the brood inside the nest and older workers are more likely to forage for food. Previous studies revealed that vitellogenin, a yolk protein typically involved in the regulation of reproduction in solitary insects, has been co-opted to regulate division of labor in the honeybee. In this study, we investigate such a role of vitellogenin in another group of social insects: the ants. We first use phylogenetic analyses to reveal the existence of multiple vitellogenin genes in most of the sequenced ant genomes. Then we compare the expression of the two vitellogenin genes (*Pb_Vg1* and *Pb_Vg2*) among queens, nurses and foragers in the seed-harvester ant *Pogonomyrmex barbatus*. Our results suggest that, after the initial duplication in ants, the vitellogenin genes acquired caste and behavioral specific expression associated with reproductive and non-reproductive nutritionally related functions. This study also shows that ants and bees, despite having evolved sociality independently, have conserved similar mechanisms to regulate division of labor.

nest [13]. The same was likely true in the solitary ancestors of ants and bees. The reproductive ground plan hypothesis (RGPH) extends this concept to explain the evolution of worker division of labor in honeybees [15–17]. Indeed, honeybee worker subcastes have two distinctive phases of ovarian activity, with nurses having large ovaries and high titers of the yolk protein vitellogenin (Vg), and foragers small ovaries and low titers of Vg [15,18,19]. The RGPH suggests that the mechanisms controlling ovarian activity influence the behavioral development and the mechanisms of food collection in worker honeybees. Support for this hypothesis is provided from studies demonstrating that workers with larger ovaries and higher titers of Vg are more likely to forage at younger ages and show a pollen foraging bias compared to workers with smaller ovaries and lower titers of Vg, which are more likely to forage at older ages and show a nectar foraging bias [15,16]. These variations in reproductive traits have a genetically inherited component as strains with different ovarian activity and foraging bias have been selected from wild type populations [15,20].

Although it has been established that the pleiotropic mechanisms connecting reproduction and division of labor have a genetic component, three lines of evidence suggest that the two processes are linked by an additional nutritional factor. First, in honeybees, reproductive queens and potentially reproductive nurses (with large and medium-sized ovaries, respectively) [18,21,22] consume diets with higher protein content [23] compared to sterile foragers with smaller and presumably non-functional ovaries [18]. Second, pollen consumption in nurses [23] is associated with higher Vg protein levels [19], compared to foragers that only consume honey [23]. Finally, there is a causal relationship between nutrition, Vg levels and behavior as pollen consumption is required to induce Vg expression [24,25] and experimental reduction of Vg expression results in precocious foraging [26,27].

To determine whether the co-option of reproductive pathways plays a major role in social evolution would require to investigate the link between reproductive physiology and behavior in other social insects, preferentially in those, such as ants, that evolved sociality independently from bees [5,6,28]. Ants have two additional characteristics that make them a particularly interesting model to study the predictions of the RPGH. First, in contrast to the honeybee genome that contains a single Vg gene, ant genomes can harbor multiple Vg genes. Indeed, the genome of the fire ant *Solenopsis invicta* harbors four Vg genes, two of them preferentially expressed in queens (*Si_Vg2* and *Si_Vg3*) and the two others in foraging workers (*Si_Vg1* and *Si_Vg4*) [29]. Vg duplication and subsequent subfunctionalization to acquire caste-specific expression provides a unique opportunity to test whether the genes associated with reproduction were co-opted to regulate worker behavior. Second, also in contrast with bees and wasps, where workers are facultatively sterile, workers are fully sterile in a significant number of ant species, including *P. barbatus* and *S. invicta* [30–32], which allows one to test the hypothesis that Vg reproductive function was co-opted to regulate behavior in species with fully sterile workers.

The first aim of this study was to determine the number of Vg genes in *P. barbatus* and other ants and investigate their phylogenetic relationships. This analysis is expected to determine whether the occurrence of multiple Vg genes is a phenomenon specific to *S. invicta* [29] or shared with other ant species as well as provide information on the origin and evolution of Vg genes in ants. Our second objective was to test whether Vg genes in *P. barbatus* display caste-specific expression profiles similar to that observed in *S. invicta*, which will address the question whether Vg gene duplication and subfunctionalization to acquire caste-specific functions is a common feature in ant species. Finally, the third objective of this study was to investigate whether the expression of Vg genes *in P. barbatus* is associated with task performance as predicted by the RGPH. We carried out these objectives by annotating Vg genes, building a phylogenetic tree, measuring mRNA levels of *P. barbatus* Vg genes in queens, nurses and foragers and performing molecular evolution analyses.

Results

We identified two adjacent copies of Vg genes (*Pb_Vg1* and *Pb_Vg2*) in the genome of *Pogonomyrmex barbatus* [33] with predicted lengths of 1742 and 1654 amino acids, respectively (Table 1). The two genes are separated by a putative mariner-like transposon, a DNA transposable element that has been involved in duplication events [34]. The two Vg genes have an identical number of exons (Figure 1A) and share the three structural domains typical of vitellogenins: the lipoprotein N-terminal domain (LPD-N), the domain of unknown function 143 (DUF1943) and the von Willebrand factor type D domain (VWD) [35,36] (Figure 1B). However, the coding sequence of *Pb_Vg2* is truncated compared to *Pb_Vg1* because of an earlier stop codon in the last exon of *Pb_Vg2*.

To determine whether the presence of multiple Vg genes is a general feature of ants, we searched for Vg genes in the five additional recently published ant genomes. These are divided up into four different subfamilies: Myrmicinae (*Atta cephalotes* and *Acromyrmex echinatior*) [37,38], Formicinae (*Camponotus floridanus*) [39] Dolichoderinae (*Linepithema humile*) [40] and Ponerinae (*Harpegnathos saltator*) [39]. We found that numbers of Vg genes vary between one and five per species (Table 1), and that when a genome contains multiple Vg genes, they are always adjacent. To determine the evolutionary history of these genes, we subsequently constructed a phylogenetic tree using known hymenopteran Vg sequences.

The phylogenetic analysis (Figure 2) revealed that the first duplication of the ancestral Vg gene in ants resulted in two gene subfamilies with different predicted amino acid length (Table 1). The phylogenetic analysis also showed that additional duplications occurred in the lineages leading to *Acromyrmex echinatior*, *Solenopsis*

Table 1. Predicted protein length of Vg genes in ants.

Species	Gene	Length	Subfamily	Expression Bias	GI number
Atta cephalotes	*Ac_Vg1*	1747	A	?	ACEP11141-PA
Atta cephalotes	*Ac_Vg2*	?	B	?	ACEP22565-PA
Acromyrmex echinatior	*Ae_Vg1*	1738	A	?	AECH16709-PA
Acromyrmex echinatior	*Ae_Vg2*	1655	B	?	AECH16711-PA
Acromyrmex echinatior	*Ae_Vg3*	1650	B	?	AECH16710-PA
Camponotus floridanus	*Cf_Vg*	1845	A	?	CFLO22478-PA
Harpegnathos saltator	*Hs_Vg*	1754	A	?	HSAL13088-PA
Linepithema humile	*Lh_Vg1*	1738	A	?	LH17501-PA
Linepithema humile	*Lh_Vg2*	1758	A	?	LH17497-PA
Linepithema humile	*Lh_Vg3*	1749	A	?	None
Linepithema humile	*Lh_Vg4*	1792	A	?	LH17495-PA
Linepithema humile	*Lh_Vg5*	1673	B	?	LH25675-PA
Pogonomyrmex barbatus	*Pb_Vg1*	1742	A	Q>N>F	PB16966-PA
Pogonomyrmex barbatus	*Pb_Vg2*	1654	B	F>Q = N	PB16966-PA
Solenopsis invicta	*Si_Vg1*	1641	B	F>Q	SINV15084-PA
Solenopsis invicta	*Si_Vg2*	1808	A	Q>F	SINV14922-PA
Solenopsis invicta	*Si_Vg3*	1761	A	Q>F	SINV14925-PA
Solenopsis invicta	*Si_Vg4*	1650	B	F>Q	None

Vg paralogs are divided in two subfamilies with different phylogenetic origin and protein length. Subfamilies A and B, range from 1641 to 1673 and from 1738 to 1845 amino acids, respectively. Known expression bias and gene identification numbers of the automatic gene predictions from which our gene models were derived are specified. *Ac_Vg2* genomic sequence is uncompleted.
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invicta and *Linepithema humile*. Interestingly, the two *Pogonomyrmex barbatus* genes (*Pb_Vg1* and *Pb_Vg2*) respectively cluster with the *S. invicta* genes preferentially expressed in queens (*Si_Vg2* and *Si_Vg3*) and foraging workers (*Si_Vg1* and *Si_Vg4*) [29].

To test the prediction that *P. barbatus* Vg genes display caste-specific expression profiles similar to their closest *S. invicta* orthologs, we performed quantitative RT-PCR analysis of Vg genes in *P. barbatus* queens and workers in five independent colonies (Figure S1). On average, *Pb_Vg1* was 4.7 times more highly expressed in queens than in nurses (pMCMC <0.0001) and 908 times more highly expressed in queens than in foragers (pMCMC <0.0001). The expression of *Pb_Vg2* did not differ between queens and nurses (pMCMC = 0.98), but it was on average 5.7 times more highly expressed in foragers than in queens (pMCMC = 0.0028) (Figure 3).

Furthermore, we tested whether the expression of Vg genes in *P. barbatus* is associated to task performance as predicted by the RGPH. We found that *Pb_Vg1* was significantly more highly expressed in nurses than in foragers in 5 out of 5 colonies (Wilcoxon tests; col1: W = 56, p = 0.001; col2: W = 70, p = 0.0001; col3: W = 56, p = 0.0003; col4: W = 49, p = 0.0006; col5: W = 48, p = 0.0007), while *Pb_Vg2* was significantly more highly expressed in foragers than in nurses in 4 out of 5 colonies (Wilcoxon tests; col1: W = 28, p = 0.06; col2: W = 16, p = 0.005; col3: W = 0,

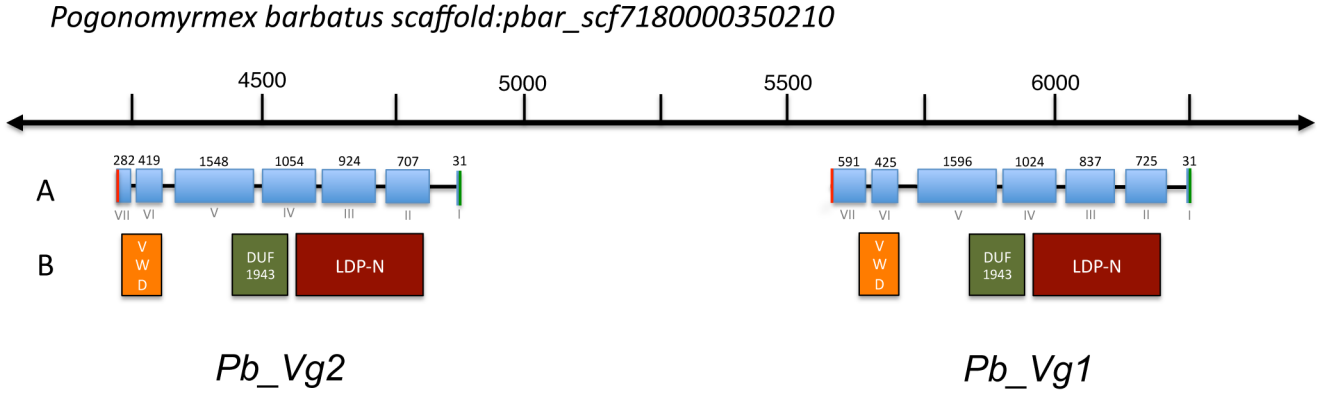

Figure 1. Genomic map of vitellogenin genes in *Pogonomyrmex barbatus*. A. Exon-intron organization of *Pb_Vg1* and *Pb_Vg2* genes. Blue boxes represent exons and joining lines introns. Green and red lines indicate start and stop codons respectively. Exon number (I–VII) and size (31–1596 bp) are indicated at the bottom and top of the figure. Exon VII in *Pb_Vg2* (282 bp) is truncated with respect to *Pb_Vg1* (591 bp). B. Localization of predicted protein structural domains: LPD-N (vitellogenin), DUF1943 and VDW.
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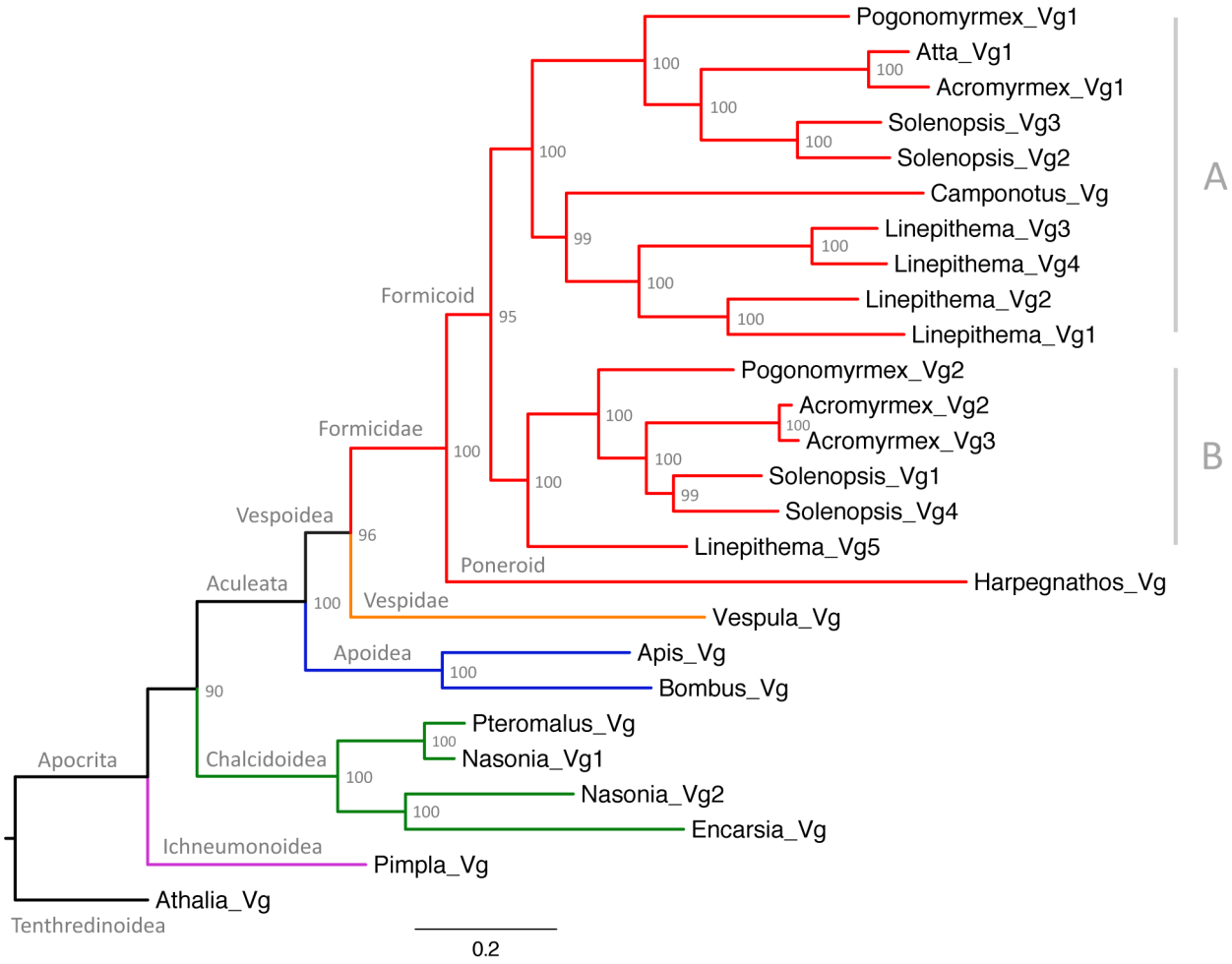

Figure 2. Maximum likelihood tree of ant vitellogenins. Vg sequences from species belonging to representative Hymenopteran groups are also included. Values at the nodes represent bootstrap support from 10,000 replicates. A and B represent different subfamilies of the formicoid clade.
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p = 0.0003; col4: W = 5, p = 0.02; col5: W = 0, p = 0.0007). On average, *Pb_Vg1* was 190 times more highly expressed in nurses than in foragers (pMCMC<0.0001) and *Pb_Vg2* 6.5 times more highly expressed in foragers than in nurses (pMCMC <0.0001) (Figure 3). *Pb_Vg1* is the predominant transcript in workers as it is expressed in strikingly high levels in nurses compared to *Pb_Vg2* in foragers (Figure 3); a pattern similar to that observed for the single Vg gene in honeybees.

Finally, we performed molecular evolution analyses to determine the relative contributions of selection for novel biochemical functions (i.e. positive selection), selection for the maintenance of existing biochemical functions (i.e. purifying selection) and neutral evolution in the evolution of ant Vg genes. In particular, we estimated selective pressures on two basal branches of Vg respectively leading to primitive ants and modern (Formicoid) ants, and on the two branches that followed the duplication of Vg in the ancestor of modern ants (Figure 2). Analyses of the branch common to the ancestor of all ants (Formicidae) and the branch common to all modern ants yielded identical results: 60.5% of codon sites evolved under purifying selection (dN/dS = 0.24), 39.5% neutrally (dN/dS = 1), and none had evidence for positive selection. The two branches that followed the duplication of Vg are interesting because one branch includes all Vg genes known to be more highly expressed in queens than in workers (hereafter referred to as subfamily A, which includes *Pb_Vg1*, *Si_Vg2* and *Si_Vg3*), while the other branch includes all Vg genes more highly expressed in foraging workers than in queens (hereafter referred to as subfamily B, which includes *Pb_Vg2*, *Si_Vg1* and *Si_Vg4*) (Figure 2 and Table 1). The branch leading to subfamily B shows overall relaxation of purifying selection and no significantly positively selected sites. However, the branch leading to subfamily A shows strong evidence for positive selection (p = 0.008), with a total of 7.1% of sites under positive selection. The three sites with the highest posterior probabilities of being under positive selection in this branch (S44, E382 and N456; pBEB>95%) are part of the main vitellogenin N-terminal lipoprotein domain (LPD-N) that is likely implicated in the uptake of vitellogenin to the ovary [41], providing further support that these changes affect the biochemical properties of the protein.

Discussion

This study reveals that the genome of *P. barbatus* harbors two Vg paralogs and that Vg underwent one or several rounds of duplication in other species, demonstrating that the existence of multiple Vg genes is a common phenomenon in ants. The phylogenetic analysis clarifies how Vg genes evolve in ants. First, it shows that the first duplication of the ancestral Vg gene occurred

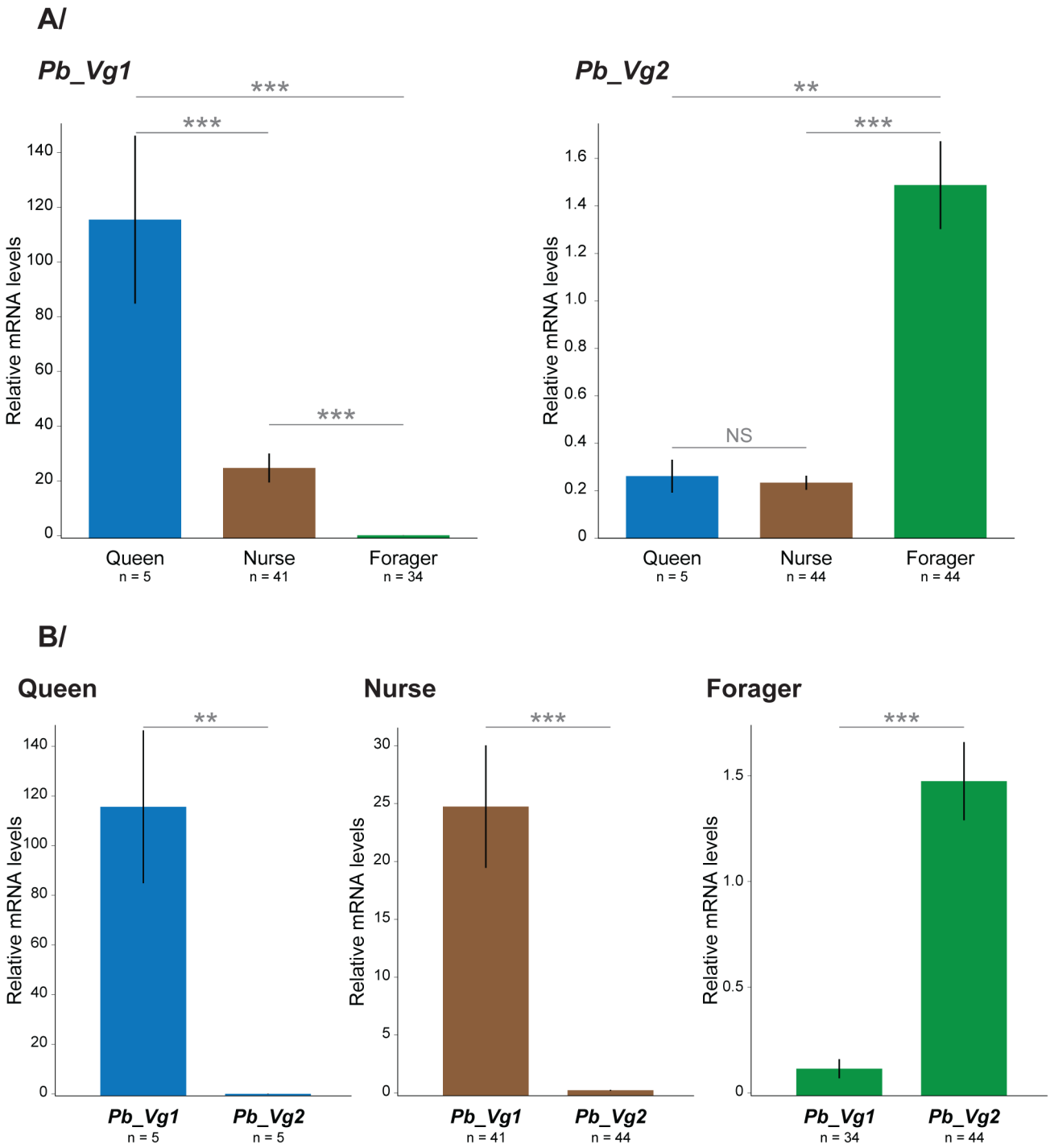

Figure 3. Relative mRNA levels of *Pb_Vg1* and *Pb_Vg2* in queens, nurses and foragers. Values were pooled from five independent colonies (Figure S1). The y axes indicate the relative gene expression, corresponding to the *Pb_Vg1* and *Pb_Vg2* mRNA levels relative to the ribosomal protein RP49 (control) gene mRNA level (mean ± se). The upper panel (A) compares the expression levels of each Vg gene among queens, nurses and foragers. The lower panel (B) compares the expression of both Vg genes separately in queens, nurses and foragers.
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after the divergence between the poneroid and formicoid clades. The poneroid clade includes primitive ants of the subfamily Ponerinae while the formicoid clade includes the three main subfamilies of modern ants: Myrmicinae, Dolichoderinae and Formicinae [28,42]. Because the divergence between primitive and modern ants apparently coincided with the duplication of Vg genes, it is tempting to speculate that this molecular event could have contributed to the evolution of modern ants. Second, the initial pair of Vg paralogs did not experience further duplication or losses in the lineages leading to *Pogonomyrmex barbatus* and *Atta cephalotes* but the ancestor of *Pb_Vg2* appears to be lost in *Camponotus floridanus*. Third, several rounds of duplications occurred independently in the lineages leading to *Solenopsis invicta*, *Linepithema humile* and *Acromyrmex echinatior*, giving rise to multiple

Vg genes in each of these species. Intriguingly, the *S. invicta* Vg genes more highly expressed in queens than in workers (*Si_Vg2* and *Si_Vg3*) cluster with *Pb_Vg1* on one branch of the tree, while the genes preferentially expressed in foraging workers (*Si_Vg1* and *Si_Vg4*) cluster with *Pb_Vg2* on the other branch of the tree, suggesting that Vg genes in *P. barbatus* might share a caste-specific expression pattern similar to their closest *S. invicta* orthologs.

This prediction was confirmed by our analyses showing that *Pb_Vg1* is preferentially expressed in queens and *Pb_Vg2* in forager. This suggests that Vg gene duplication and subfunctionalization to acquire caste-specific expression related to reproductive and non-reproductive functions may be a general feature of ant species with multiple Vg genes. Expression and functional analyses in additional species will need to be performed to determine the extent to which this is the case.

Three lines of evidence suggest that the first round of duplication of Vg genes facilitated the evolution of queen-worker specialization. First, the duplication occurred in the common ancestor of formicoids (modern ants). A key feature of these ants is a marked morphological, physiological and behavioral differentiation between queens and workers. This contrasts with non-modern ants that exhibit few or no differences between castes. Second, the gene expression differences we identified suggest that the two subfamilies of Vg paralogs evolved different functions, with genes from subfamily A predominantly playing roles in queens and subfamily B predominantly playing roles in workers. Finally, our molecular evolution analyses suggest that these two subfamilies are evolving differently since the duplication. Positive selection on the paralogs in subfamily A may stem from a process of neofunctionalization associated with the evolution of a dramatically higher reproductive output of queens in modern ant species. Furthermore, relaxation of purifying selection on the subfamily B is consistent with the loss of the reproductive constraints and evolution of new functions of Vg (i.e. subfunctionalization) in workers. These results are thus consistent with the proposal that gene duplication followed by caste-specific expression can circumvent the constraints of antagonistic pleiotropy, thus facilitating the evolution of new caste-specific functions in ants [43,44]. Subfunctionalization of duplicated genes has been described in other contexts. For example, the duplication of a single copy gene in a basal vertebrate gave rise to oxytocin and vasopressin neurotransmitter genes. These two genes have distinct physiological and behavioral roles in vertebrates while the single homologous gene in invertebrates has both vasopressin-like and oxytocin-like functions [45]. Similarly, duplication of estrogen receptor ER-β occurred in the lineage leading to teleost fish. These two copies are expressed in alternate parts of the brain suggesting that subfunctionalization occurred and affects behavior [46].

Interestingly, there is apparently a single copy of Vg in *Apis mellifera* and other bees. A comparative analysis of the evolution of Vg and seven other genes in *A. mellifera* and several closely related species showed evidence of positive selection for Vg [47]. In particular replacement polymorphisms were significantly enriched in parts of the protein involved in binding lipid, suggesting a link between the structure of the gene, its function, and its effects on fitness [47]. These data have been interpreted as social pleiotropy leading to only limited constraints on adaptive protein evolution [47,48]. This raises the question of why multiple duplications occurred in ants but not in bees. A possible reason lies in the much greater phenotypic differences between queens and workers in ants compared to bees. In many modern ants, queens and workers greatly differ in size and other morphological, physiological and behavioral traits [1,2]. For example ant workers have lost the ability to fly and in some species they are completely sterile. This higher differentiation in ants than bees may lead to greater selection for different roles of Vg in queens and workers, and thus greater potential benefits for Vg duplication and subfunctionalization in ants. Interestingly, the association between the strength of female caste differentiation and the presence of multiple Vg genes seems to hold in social wasps and termites. Although no genome has been sequenced so far in these two groups of social insects, the available data suggest that the social wasps *Vespula vulgaris* and *Polistes metricus* (low differentiation between castes) only have a single Vg gene [49,50] while the termite *Reticulitermes flavipes* (high differentiation between castes) have two Vg genes [51].

The pattern of expression of *Pb_Vg1* (high in queens, medium in nurses and low in foragers) is similar to that observed for Vg in the honeybee [25,52], suggesting an association between ovarian activity and the expression of this gene. In contrast to honeybee nurses which can produce fertile eggs in queenless conditions and sometimes even in the queen presence, workers are sterile in most *Pogonomyrmex* species [32]. However, *Pogonomyrmex* workers can produce trophic eggs which are thought to be the main method of nutrient redistribution because trophallaxis – mouth-to-mouth food transfer - has not been observed in this genus [32]. Interestingly, a recent study in *Pogonomyrmex californicus* showed that nurses had significantly increased ovarian activity compared to foragers [53], suggesting that trophic eggs are specifically produced by nurses. This pattern of nutrient sharing differs from the honeybee, where the proteins and lipids provided to the larvae, queen and foragers come from the hypopharyngeal and mandibular gland secretions of the nurses [53–56]. These results, together with our finding that *Pb_Vg1* is predominantly expressed in nurses, suggest that the primary role of this protein as a source of amino acids and lipids for the developing embryo has been co-opted to a novel nutritionally related role associated with the production of trophic eggs. In honeybees Vg expression and ovarian activity are linked with the genetic pathways associated with the regulation of behavior [15,16]. It remains to be tested if such relation is conserved in sterile ants with functional ovaries that produce trophic eggs.

Our results revealed that the level of expression of *Pb_Vg2* was much lower than that of *Pb_Vg1* in queens. By contrast *Pb_Vg2* was expressed at higher level than *Pb_Vg1* in sterile foragers suggesting that the function of this gene is not related to ovarian activity but likely has a different function in foragers. Our molecular evolution analysis showing overall relaxation of purifying selection and no positively selected sites on the paralogs in subfamily B suggests a new role of these proteins neither associated to its existing biochemical function nor novel functions derived of positively selected domains. Instead, the release of functional constrains on the LPD-N structural domain, implicated in lipid transport and Vg receptor binding [41], suggests that these proteins are not imported to the ovary and after having lost their lipid-binding capabilities, they may be primarily used as source of amino acids in foragers. In honeybees, earlier findings that Vg protein was present not only in fertile queens but also in functionally sterile workers [19,57] and drones [58] led to the proposal that this yolk protein could have both reproductive and non-reproductive functions [59]. Our results indicate that similar functional division of Vg took place in *P. barbatus* and other modern ant species via gene duplication and subfunctionalization.

The preferential expression of *Pb_Vg1* in nurses and of *Pb_Vg2* in foragers follow a general gene expression pattern observed in honeybees, in which egg-laying workers show up-regulation of genes associated with reproduction while non-reproductive workers overexpress genes involved in foraging-related functions [60]. This is consistent with the RGPH prediction that reproductive

pathways were co-opted to regulate worker behavior and supports the evolutionary theory prediction that potentially reproductive individuals are selected to carry out low-risk tasks, in order to not compromise their reproductive future [61,62]. In ants, this ancestral mechanism regulating division of labor has been conserved, even in species with workers having lost their reproductive potential.

Conclusion

The results of this study suggest that Vg has been co-opted to regulate worker behavior in the ant *P. barbatus* as in the honeybee. Support for RGPH in groups of insects that evolved sociality independently, demonstrates that the co-option of reproductive pathways to regulate behavior is a major director in the evolution of sociality in insects. On the other hand, the expression of one Vg paralogs in sterile workers reveals that Vg adaptation to regulate worker behavior is not necessarily linked to reproduction but maybe linked exclusively to nutritional functions. Our result suggests that, after the initial duplication in ants, *Vg* genes underwent neo- or subfunctionalization to acquire caste- and behavioral-specific functions. Overall, our results suggest that even though ants and bees evolved sociality independently, they have conserved similar mechanisms to regulate division of labor.

Materials and Methods

Gene prediction and annotation

To determine gene models, we first ran TBLASTN using known hymenopteran Vg sequences against ant genome sequences downloaded from the fourmidable database [63]. Subsequently, ruby/bioruby scripts [64] were used to extract relevant subsets of each genome. Gene predictions were generated on each subset using MAKER2 [65] s65mand subsequently manually refined using Apollo [66]. Conflicts gene predictions were resolved by using EST data when available, splice prediction algorithms (http://www.fruitfly.org/seq_tools/splice.html) and manual verification of splicing consensus sequences.)

Phylogenetic analyses

Inaccurate sequence alignment or phylogeny leads to misleading or incorrect results in molecular evolution analyses. We used an approach centered on the use of phylogenetically aware codon-level aligner PRANK, which is likely to minimize the risks of introducing errors [67,68]. This required several steps. We preliminarily aligned the 26 Vg protein sequences with MAFFT linsi [69] and removed ambiguous sections of the alignment using trimAl (option -gappyout) [70]. A first tree was built with RAxML (model GTRGAMMAI) [71] and rooted with "nw_root" (Newick Utilities package [72]). This tree was used as a guide tree in PRANK [73] to obtain a high-quality codon-level alignment of the 26 Vg coding sequences. Ambiguous sections of the alignment were removed using trimAl (option -gappyout) and a final tree was built with RAxML (GTRGAMMAI model); 10,000 bootstraps were generated to assess its confidence. Selective pressures (dN/dS) on different parts of the phylogenetic tree were estimated using the branch-site codon-substitution model from CodeML (PAML 4.6) [74]. Such dN/dS ratios are obtained by computing the number of non-synonymous changes (dN) over synonymous changes (dS) (see Table 2 for more details). Vg site coordinates (S44, E382, N456) are given as in *Apis mellifera* Vg (Uniprot identifier VIT_APIME).

Sample collection

Pogonomyrmex barbatus founding queens were collected during nuptial flights on July 15th, 2008 in Bowie, Arizona, USA (N32°18′54″//W109°29′03″). Colonies were then kept in laboratory conditions (30°C, 60% humidity and 12 h/12 h light:dark cycle) in 15×13×5 cm plastic boxes with water tubes, and were fed once a week with grass seeds and a mixture of eggs, honey and smashed mealworms. Thirty months later, five of these colonies were used to collect samples on December 16th, 2010. Task performance in workers is age related, thus nurses tend to be younger than foragers [2]. Young ants interacting with the brood in the nest tube were considered as nurses. To collect foragers, each colony was connected with a cardboard-made bridge to a foraging area composed of a plastic box containing grass seeds. Any ant handling a food item in the foraging area was considered as forager. Ant samples were flash-frozen in liquid nitrogen and kept at −80°C for further RNA extraction.

Gene expression analysis

Whole body worker samples were used to measure the expression of *Pb_Vg1* and *Pb_Vg2* genes. RNA extractions were performed using a modified protocol including the use of Trizol (Invitrogen) for the initial homogenization step, RNeasy extraction kit and DNAse I (Qiagen) treatment to remove genomic DNA traces. For each individual worker, cDNAs were synthesized using 500 ng of total RNA, random hexamers and Applied Biosystems reagents. Levels of mRNA were quantified by quantitative real-time polymerase chain reaction (qRT-PCR) using ABI Prism 7900 sequence detector and SYBR green. All qPCR assays were performed in triplicates and subject to the heat-dissociation protocol following the final cycle of the qPCR in order to check for amplification specificity. qRT-PCR values of each gene were normalized by using an internal control gene (RP49). Paralog-specific primers (Table S1) were designed using sequence

Table 2. Results of test of positive selection.

Branch	lnL0	lnL1	2*(lnL1-lnL0) [p-value, df = 1]	ω0	p0 (%)	ω1	p1 (%)	ω2	p2a + p2b(%)	Sites BEB>95%
Formicidae	−62600.98	−62600.98	0.00 [1.000]	0.24	60.5	1.00	39.5	1.00	0	None
Formicoid	−62600.98	−62600.98	0.00 [1.000]	0.24	60.5	1.00	39.5	1.00	0	None
Subfamily A	−62597.90	−62594.35	7.12 [0.008**]	0.24	56.5	1.00	36.4	15.15	7.1	S44 (87), E382 (761), N456 (963)
Subfamily B	−62600.29	−62598.65	3.29 [0.070]	0.24	59.5	1.00	38.9	17.80	1.5	None

Selective pressures are divided into three classes: ω0 (dN/dS<1, negative selection), ω1 (dN/dS = 1, neutral evolution) and ω2 (dN/dS ≥1, positive selection). p0, p1 and p2a+p2b are their respective proportion. lnL0 and lnL1 are the likelihood values of the null (ω2 = 1) and alternative (ω2≥1) models, respectively. The likelihood ratio test [2*(lnL1-lnL0)] allows discriminating between neutral evolution and positive selection. A p-value<0.01 presents a significant test (marked as **). Sites under positive selection with BEB score >95% are shown.

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alignment [75] and primer analysis [76] programs. Primer sequences overlapped coding regions split by introns, allowing the specific amplification of cDNA levels over eventual genomic DNA contaminations. Transcript quantification calculations were performed by using the ΔΔCT method [77].

Statistical analysis

All data were analyzed using R (http://www.r-project.org/) and the R packages lme4 [78] and language R [79]. The effect of caste on gene expression relative values was analyzed using linear mixed effects models. To avoid pseudoreplication, the colony was included as a random effect. We checked for normality and homogeneity by visual inspections of plots of residuals against fitted values. To assess the validity of the mixed effects analyses, we performed likelihood ratio tests to confirm that the models with fixed effects differed significantly from the null models with only the random effects. Throughout the paper, we present MCMC (Markov-chain Monte Carlo) estimated p-values that are considered significant below the 0.05 threshold (all significant results remained significant after Bonferroni correction).

Supporting Information

Figure S1 Relative mRNA levels of *Pb_Vg1* and *Pb_Vg2* in queens, nurses, and foragers. Experiments were performed in five independent colonies of *Pogonomyrmex barbatus*. The y axes indicate the relative gene expression, corresponding to the *Pb_Vg1* (panel A) and *Pb_Vg2* (panel B) mRNA levels relative to the ribosomal protein RP49 (control) gene mRNA level (mean ± se).
(TIF)

Table S1 Paralog-specific primers used for qRT-PCR (5′-3′ order).
(DOCX)

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Author Contributions

Conceived and designed the experiments: MC RL LK. Performed the experiments: MC RL YW ORG RAS. Analyzed the data: MC RL YW RAS. Contributed reagents/materials/analysis tools: LK. Wrote the paper: MC RL YW LK RAS.

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Appendix 4

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

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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

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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

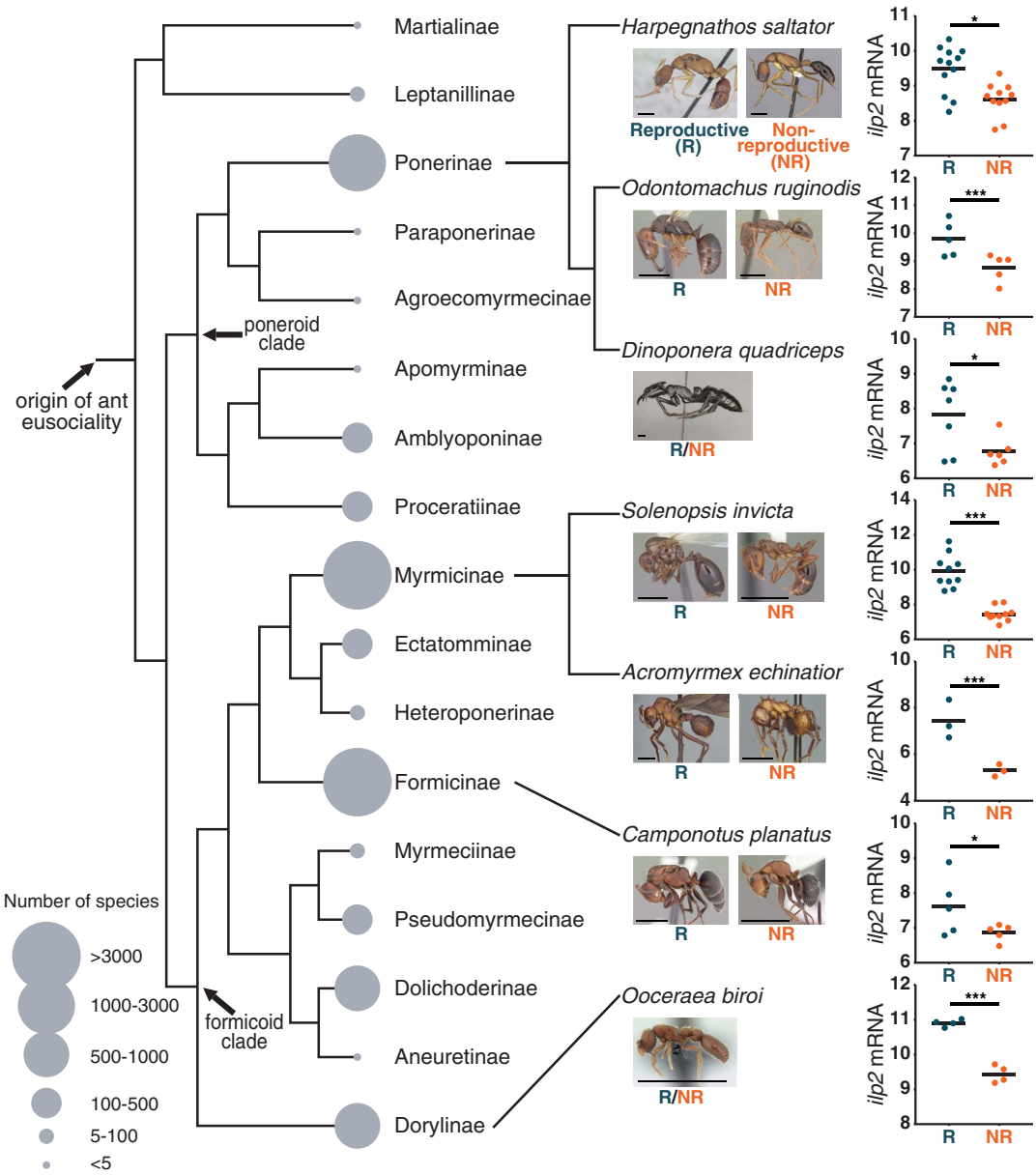

Fig. 1. Brain gene expression in seven ant species identifies one conserved differentially expressed gene. The figure shows the summary cladogram of the seven ant species used in this study in the context of the entire ant phylogeny with all subfamilies labeled. Five of the focal species have queens, 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*).

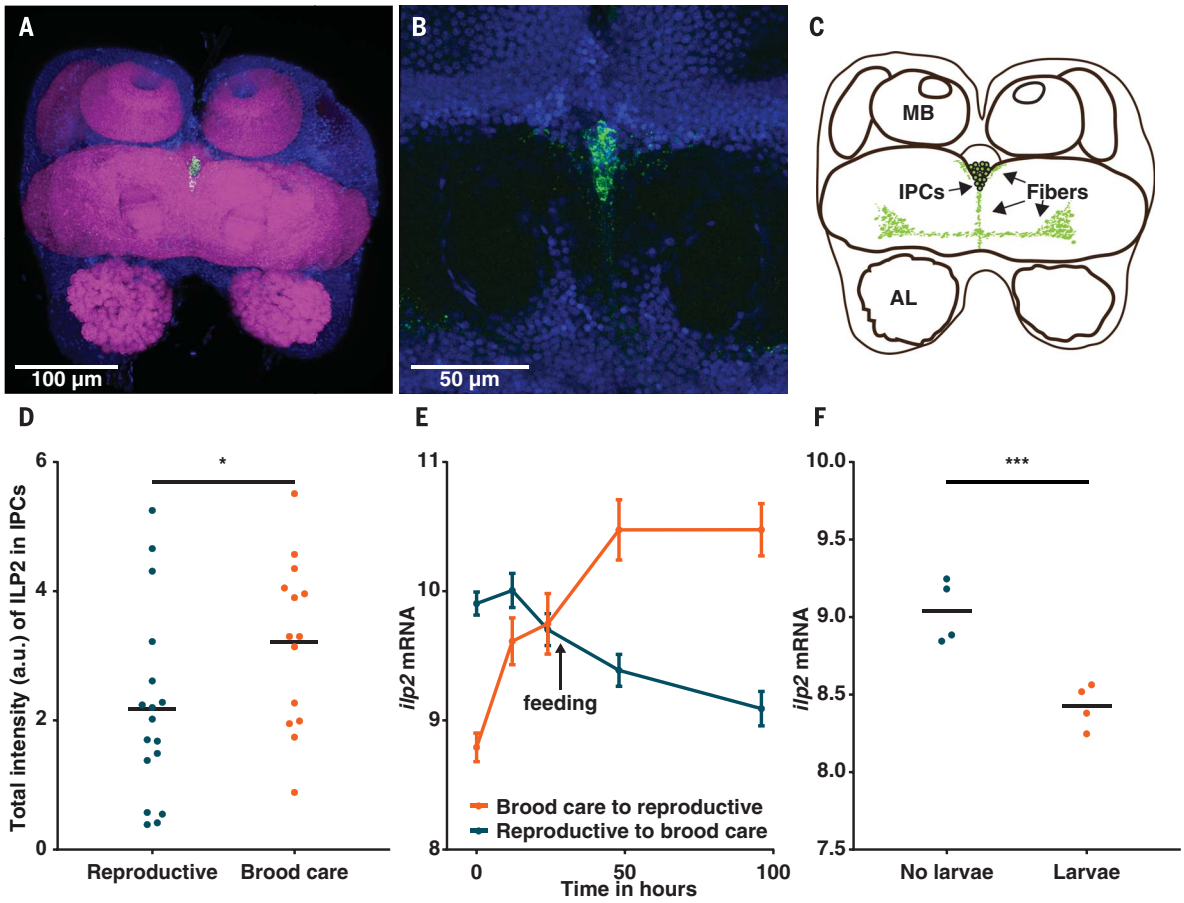

Fig. 2. Larvae regulate *ilp2* in adults. (**A** to **C**) Immunohistochemistry with antibody against ILP2 (anti-ILP2) on an *O. biroi* brain localizes ILP2 peptide to a single cluster of insulin-producing cells (IPCs) in the pars intercerebralis (body-axis dorsal view). Green, anti-ILP2; blue, DAPI (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

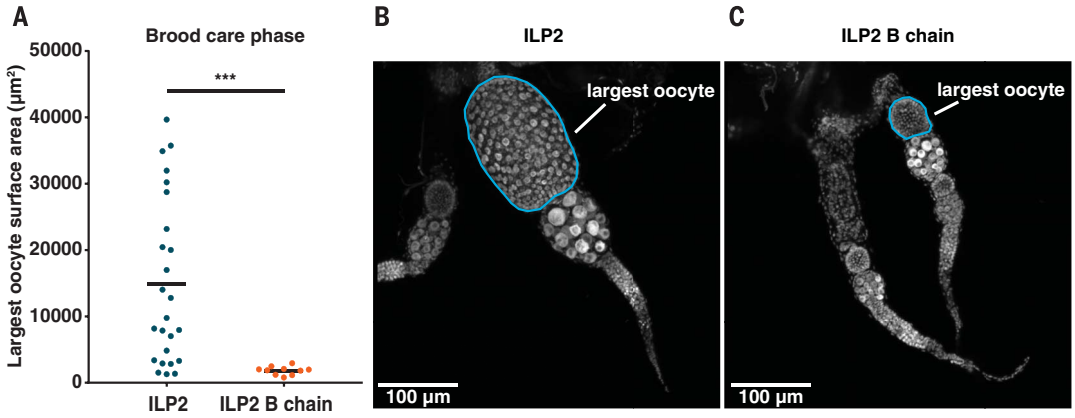

Fig. 3. ILP2 supplementation overrides larval suppression of adult reproduction. (**A**) Workers injected with 100 μM ILP2 in the brood care phase activate their ovaries relative to controls injected with 100 μM ILP2 B chain, despite being in contact with larvae [$n \geq 10$, Welch's t test with Bonferroni 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.

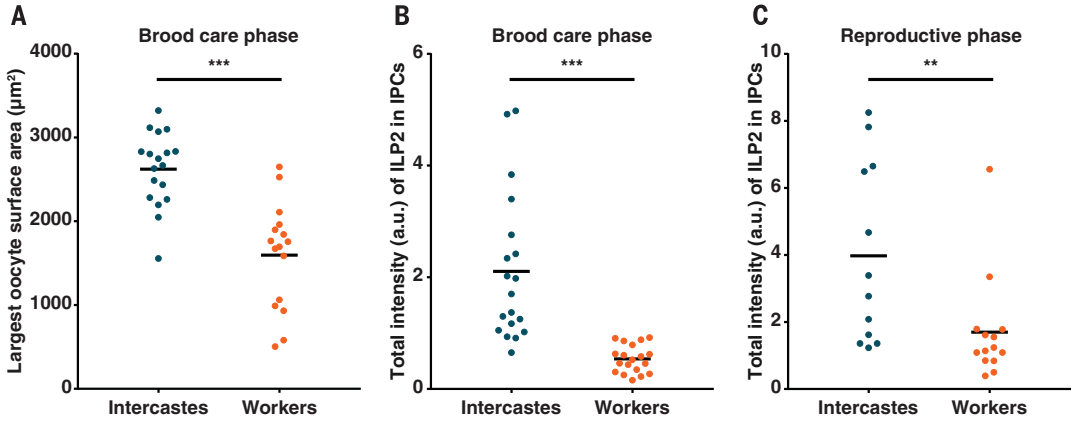

Fig. 4. Intercastes respond less to larvae and have more ILP2 than regular workers. (**A**) Intercastes have more active ovaries than age-matched regular workers in the brood care phase, despite both groups being in contact with larvae ($n \geq 16$, Welch's t test; $^{***}P < 0.0001$). (**B**) In the 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*).

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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*)

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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.

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Appendix 5

Clonal raider ant brain transcriptomics identifies candidate molecular mechanisms for reproductive division of labor

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Clonal raider ant brain transcriptomics identifies candidate molecular mechanisms for reproductive division of labor

Romain Libbrecht[1,2*†], Peter R. Oxley[1,3†] and Daniel J. C. Kronauer[1*]

Abstract

Background: Division of labor between reproductive queens and workers that perform brood care is a hallmark of insect societies. However, studies of the molecular basis of this fundamental dichotomy are limited by the fact that the caste of an individual cannot typically be experimentally manipulated at the adult stage. Here we take advantage of the unique biology of the clonal raider ant, *Ooceraea biroi*, to study brain gene expression dynamics during experimentally induced transitions between reproductive and brood care behavior.

Results: Introducing larvae that inhibit reproduction and induce brood care behavior causes much faster changes in adult gene expression than removing larvae. In addition, the general patterns of gene expression differ depending on whether ants transition from reproduction to brood care or vice versa, indicating that gene expression changes between phases are cyclic rather than pendular. Finally, we identify genes that could play upstream roles in regulating reproduction and behavior because they show large and early expression changes in one or both transitions.

Conclusions: Our analyses reveal that the nature and timing of gene expression changes differ substantially depending on the direction of the transition, and identify a suite of promising candidate molecular regulators of reproductive division of labor that can now be characterized further in both social and solitary animal models. This study contributes to understanding the molecular regulation of reproduction and behavior, as well as the organization and evolution of insect societies.

Keywords: Eusociality, Social behavior, Social insects, Gene expression, Gene regulation, Time course, Brood care, Reproduction

Background

The evolution of social life from solitary organisms, one of the major transitions in evolution [1], is best exemplified by eusocial hymenopterans (ants, some bees, and some wasps). At the core of hymenopteran societies lies reproductive division of labor, whereby one or several queens monopolize reproduction while workers perform all the non-reproductive tasks necessary to maintain the colony [2]. To better understand the evolution of eusociality requires investigating the mechanisms that plastically regulate reproductive and non-reproductive tasks in social insects.

Studies of reproductive division of labor have primarily focused on comparing the queen and worker castes, both at the adult stage and during larval development when caste differentiation occurs [3–13]. Such studies have provided valuable insights into the mechanisms regulating the alternative developmental trajectories of queens and workers, and contributed greatly to the elaboration of theories regarding the evolution of eusociality [14–19].

However, there are three major limitations associated with the comparison of morphologically distinct queens and workers. First, at the adult stage, the two castes not only differ in reproductive status and behavior, but also in morphology, baseline physiology, immunity, and lifespan [2, 20, 21]. Thus it is difficult to disentangle differences between queens and workers that are actually associated with plastic variation in reproduction and behavior from those associated with other traits. Second, the caste is fixed when

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females reach adulthood and thus cannot be experimentally manipulated in adults, making it challenging to establish causality between molecular and phenotypic differences. Third, morphologically distinct queen and worker castes represent the derived state: comparing them does not necessarily provide accurate information on the mechanisms under selection during the evolutionary transition to eusociality from a totipotent ancestor. These limitations do not apply to eusocial insect species with flexible queen and worker castes, and studying the molecular basis of reproductive division of labor in such species has the potential to provide complementary insights to studies of species with fixed morphological castes [22–24].

Eusocial hymenopterans are derived from subsocial wasp-like ancestors that alternated between reproductive and brood care phases [15, 25–27]. The evolution of eusociality involved a decoupling of these phases in different individuals, the queens and the workers, respectively. To understand the evolution of such decoupling requires investigating the molecular mechanisms regulating the transitions between phases. Unfortunately, extant wasp species with a subsocial cycle and progressive provisioning of their larvae are rare tropical species (e.g., *Synagris* wasps in sub-Saharan Africa [28] or *Stenogaster* wasps in southeast Asia [29]) that have not been studied from a molecular perspective because they cannot be experimentally manipulated under controlled laboratory conditions.

The clonal raider ant *Ooceraea biroi* (formerly *Cerapachys biroi* [30]) is a promising model system to study the evolution of eusociality because it alternates between reproductive and brood care phases in a cycle that is reminiscent of the subsocial cycle of the ancestors of eusocial hymenopterans [31, 32]. This species has no queen caste, and colonies consist of morphologically uniform and genetically identical workers. Colonies alternate between reproductive phases of ca. 18 days during which workers reproduce asexually in synchrony and brood care phases of ca. 16 days during which workers have regressed ovaries, forage, and nurse larvae [31, 33]. Social cues derived from the larvae regulate the transitions between phases: when larvae hatch towards the end of the reproductive phase, they soon suppress ovarian activity and induce brood care behavior in the adults, and when larvae pupate towards the end of the brood care phase, the adults begin to activate their ovaries and foraging activity ceases [34, 35]. This allows precise experimental manipulation of the cycle by adding or removing larvae of a particular developmental stage at standardized time points during the cycle (Fig. 1).

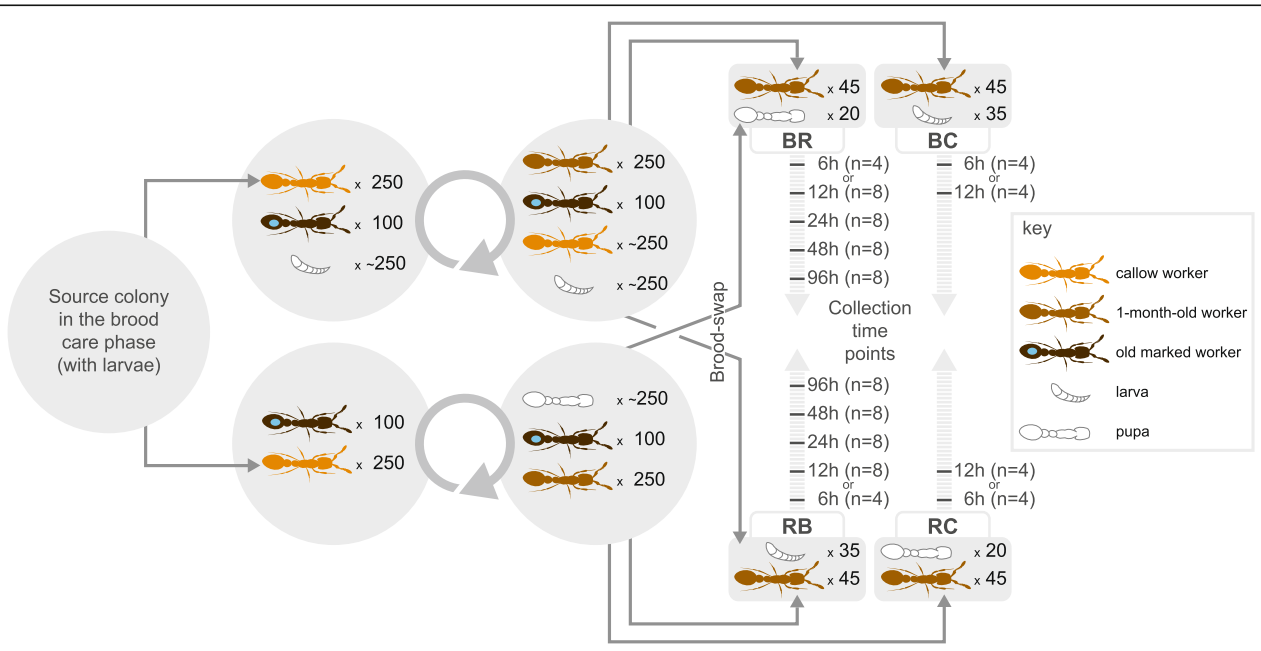

Fig. 1 Design of the brood-swap experiment. For each biological replicate, a large source colony in the brood care phase was used to establish two colonies of 250 1-month-old workers and 100 marked ≥ 3-month-old workers. One of these colonies received approximately 250 larvae. After a full colony cycle, each colony contained a complete cohort of brood and workers and was in either peak brood care phase (with larvae) or early reproductive phase (with eggs and pupae). On the day the first eggs were laid, the 1-month-old workers were subdivided in colonies of 45 workers each. One colony from each phase served as the control colony and was given brood from the mother colony. The remaining colonies received brood from the mother colony in the opposite phase of the cycle, triggering the transition toward the alternative phase. Colonies were subsequently collected 6, 12, 24, 48, or 96 h post treatment. BR workers transitioning from the brood care phase to the reproductive phase (after larvae were removed and pupae added), RB workers transitioning from the reproductive phase to the brood care phase (after pupae and eggs were removed and larvae added), BC workers from the brood care phase with larvae (brood care phase control), RC workers from the reproductive phase with pupae (reproductive phase control)

At the same time, *O. biroi* affords maximal control over the genetic composition and age structure of experimental colonies, arguably the two most important factors that affect division of labor in social insects [31, 35–37]. This study takes advantage of the unique biology of *O. biroi* to investigate the molecular underpinnings of behavioral transitions from reproduction to brood care and vice versa, and identify candidate genes potentially involved in the evolutionary transition from subsocial to eusocial living.

Results

We experimentally manipulated the presence of larvae in *O. biroi* colonies of age-matched, genetically identical individuals to induce transitions from the reproductive to the brood care phase (hereafter "RB transition") or from the brood care to the reproductive phase (hereafter "BR transition"). We then collected brain gene expression data from individuals sampled across five consecutive time points at 6, 12, 24, 48, and 96 h post manipulation (eight biological replicates per time point) to evaluate gene expression changes over time in response to changes in brood stimuli (Fig. 1). After checking for outliers, we judged the 6-h time points to mostly reflect a response to recent experimental disturbance and thus removed them from further analysis ("Methods"; Additional file 1).

Brain gene expression changes when ants transition between phases

We conducted two independent differential expression analyses (one for each transition) that revealed 2043 genes with significant changes in expression over time in the RB transition (hereafter "RB-DEGs") and 626 genes with significant changes in expression over time in the BR transition (hereafter "BR-DEGs") (adjusted p values < 0.05; "Methods"). These analyses also detected genes with similar changes in expression over time in both transitions, which likely stem from experimental manipulations. Thus we conducted a more conservative analysis that would not detect such genes by identifying genes that showed transition-specific expression changes over time ("Methods"). We detected 596 genes with different changes in expression over time between RB and BR transitions (hereafter "DEGs"; time-by-transition interaction with adjusted p values < 0.05; gene identifiers and annotations in Additional file 2).

PCA clustering of samples according to brain gene expression segregated samples according to ovary score (Fig. 2a). Samples that were early in the transition were most similar to their corresponding control samples. Samples that were late in the transition were most similar to the control samples for the opposite transition (i.e., closest to the phase opposite from where they started in the experiment). This shows that our experimental timeline appropriately spanned both transitions from beginning to end and that brain gene expression is an accurate corollary of the ovarian development of *O. biroi* individuals.

The timing of gene expression changes differs between transitions

The average distance between samples (Fig. 2a, b) indicated a more gradual change in gene expression when

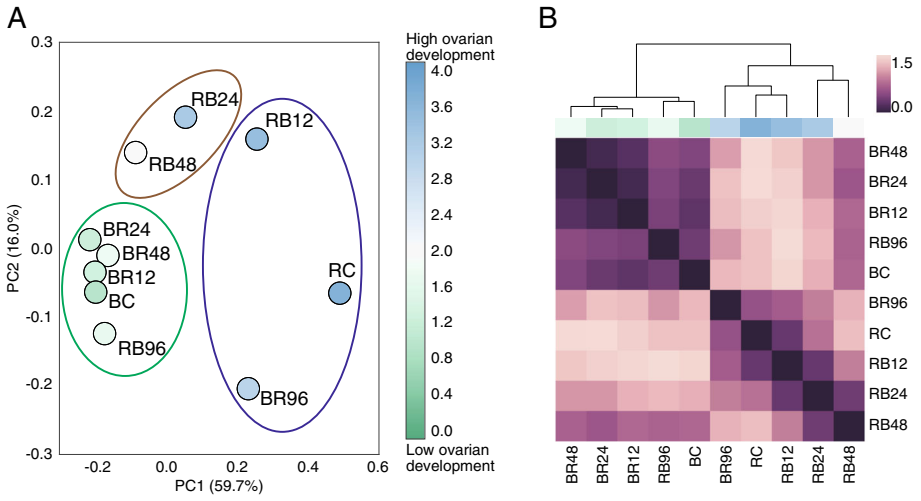

Fig. 2 Cluster analysis of samples based on the mean gene expression of each time point, for 596 differentially expressed genes (adjusted p value ≤ 0.05). **a** PCA plot of brood-swap and control samples. Percentages on each axis indicate the proportion of variance explained by the indicated principal component. The blue, brown, and green ellipses show the k-means cluster assignment. The color of each sample indicates the average ovary activation score as per [77]; 0 indicates no signs of ovary activation while 4 indicates fully developed eggs are present. Sample names are as per Fig. 1. **b** Heatmap showing Euclidean distances between all time points. The dendrogram was constructed using the average distances between time points. The blue and green color bar above the heatmap indicates average ovary activation score, as in **a**. Sample names are as per Fig. 1

transitioning to the brood care phase than when transitioning to the reproductive phase. The unbiased clustering of samples further suggested that changes in gene expression patterns occurred earlier after adding larvae to ants in the reproductive phase than after removing larvae from ants in the brood care phase (Fig. 2a). Only samples collected 12 h after addition of larvae clustered with the control samples for the reproductive phase, while later samples clustered either as an intermediary group (24 and 48 h) or with the brood care phase controls (96 h) (Fig. 2a). On the other hand, following the removal of larvae, all samples collected before 96 h clustered with the control for the brood care phase (Fig. 2a).

To further test whether gene expression dynamics differed between transitions, we used P-spline smoothing with mixed effects models [38] to fit the gene expression time course profiles into clusters (i.e., groups of co-expressed genes over time). This approach grouped all genes into 76 clusters for the BR transition and 96 clusters for the RB transition (Additional files 3 and 4). In order to compare clusters, we also identified their "maximum change vector" (MCV), which is the interval, magnitude, and direction of the largest average gene expression change between time intervals. For each transition, we used the MCV values to determine the number of genes showing their maximum change in expression for each time interval. If the timing of gene expression changes was similar in both transitions, we would expect a comparable distribution of such number of genes across time intervals for clusters showing significant changes over time (i.e., clusters enriched for DEGs). Contrary to this expectation, we found that among clusters enriched for DEGs, the distribution differed significantly between transitions ($\chi^2 = 1217.5$, $p < 0.00001$, Fig. 3, Additional file 5). Consistent with the PCA analysis, most gene expression changes in the BR transition occurred between 48 and 96 h, whereas changes in the RB transition were weighted towards earlier time intervals (Fig. 3).

The nature of gene expression changes differs between transitions

The independent analyses of the RB and BR transitions revealed a weak overlap between the lists of RB-DEGs and BR-DEGs: only 7.4% (185/2484) of the genes differentially expressed over time in one transition were differentially expressed over time in both transitions. In addition, 55.7% (103/185) of the overlapping DEGs had the same MCV in both transitions, suggesting that their expression changes were a result of experimental manipulation. This suggests that the genes and/or pathways associated with transitioning between phases are specific to each transition.

The gene co-expression clusters further corroborate this finding. Constructing a network from cluster membership in both transitions revealed a highly connected, homogenous network (Additional file 6), showing that most genes were co-expressed with different genes in each transition. This is similarly illustrated by cluster enrichment for Gene Ontology (GO) terms. We found 27 enriched clusters (including four clusters enriched for DEGs) for the BR transition and 35 (including seven clusters enriched for DEGs) for the RB transition (Additional file 3). Among clusters enriched for DEGs, only

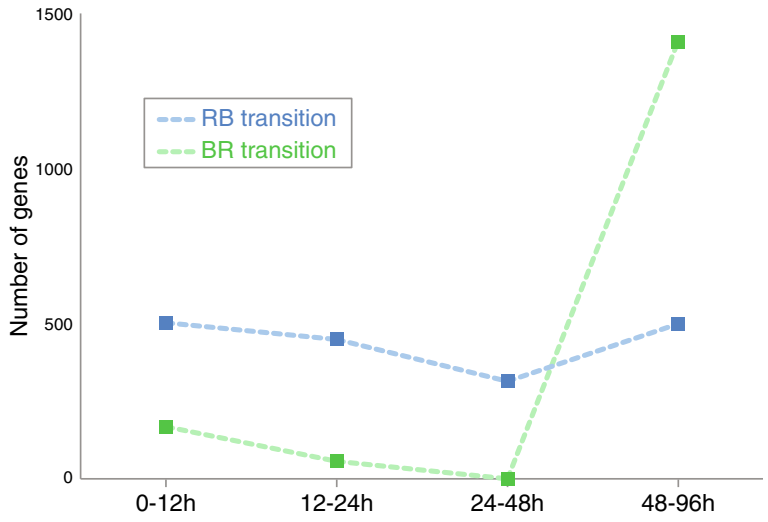

Fig. 3 Number of genes in clusters (enriched for DEGs) with maximal change in expression for each time interval. The distribution of such numbers across time intervals differed significantly between transitions ($\chi^2 = 1217.5$, $p < 0.00001$, Additional file 5). This suggests that the transition from reproduction to brood care (RB transition; blue) and the transition from brood care to reproduction (BR transition; green) are associated with distinct time dynamics of gene expression

6.9% (2/29) of the GO terms associated with one transition were also associated with the other transition (Additional file 7).

Furthermore, the expression patterns of genes that were co-expressed with the same genes in both transitions were inconsistent with a symmetrical molecular regulation. We identified all conserved co-expression clusters in the network (i.e., clusters whose members were more similar between transitions than expected by chance) ("Methods", Additional file 6). If the primary molecular mechanisms regulating phase transitions were reversible, then co-expressed genes would show expression changes in opposite directions in each transition. In that case, network edges that link clusters of genes regulated in opposite directions between transitions would represent a higher proportion of edges in the conserved network (with only non-random connections) compared to the complete network (which includes random connections). We found the reverse pattern: edges linking clusters of genes showing opposite changes of expression over time between transitions were less frequent in the conserved network (24.4%) than in the complete network (45.8%; $\chi^2 = 22.4$, $p < 0.00001$; Additional file 8).

Using the time course data to identify candidate genes

Ranking the 596 DEGs according to their change in expression between the control and the 96-h time point for each transition allowed us to identify genes most likely to be involved in the molecular regulation of one or both transitions (the lists of the top 40 DEGs when ranked according to log2 fold change are available in Additional file 9). This includes genes that encode proteins with neuroendocrine functions (*queen vitellogenin*), neuropeptides (*insulin-like peptide 2*, *neuroparsin-A*), and neuropeptide receptors (*leucine-rich repeat-containing G-protein-coupled receptor 4*) and enzymes involved in neuropeptide processing (*carboxypeptidase M*, *aminopeptidase N*), neurotransmitter receptors (*glycine receptor subunit alpha 2*) and proteins involved in neurotransmission (*synaptic vesicle glycoprotein 2C*, three *kinesin-like proteins*), neuronal function (*leucine-rich repeat neuronal protein 2*, *trypsin inhibitor*, *gliomedin*), hormone binding (*transferrin*), transcription (*hunchback*, *transcription termination factor 2*, *speckle-type POZ protein B*, *zinc finger BED domain-containing protein 1*, *lymphoid-specific helicase*), and protein synthesis and modification (*peptidyl-prolyl cis-trans isomerase D*, *hyaluronan-mediated motility receptor*, *alpha-(1,3)-fucosyltransferase 6*). The expression patterns for some of these candidate genes are shown in Fig. 4. In addition, we identified among these genes those with highest change in expression between the control and the 12-h time point (Additional file 9), i.e., genes that could function upstream in the molecular processes regulating the transitions. These include candidate genes with early changes in the RB transition (*hunchback*, *alpha-(1,3)-fucosyltransferase 6*), in the BR transition (*insulin-like peptide 2*, *glycine receptor subunit alpha 2*, *transcription termination factor 2*, *hyaluronan-mediated motility receptor*, *annulin*), or in both transitions (*leucine-rich repeat-containing G-protein-coupled receptor 4*, *leucine-rich repeat neuronal protein 2*, *transferrin*).

Both transitions are associated with overlapping sets of transcription factors

For each transition, we tested whether gene clusters were enriched for transcription factor binding sites. We used the JASPAR database to identify 27 clusters (including four clusters enriched for DEGs) in the BR transition and 12 clusters (including four clusters enriched for DEGs) in the RB transition that were significantly enriched for transcription factor binding sites (Additional file 3). A number of transcription factors were repeatedly associated with clusters enriched for DEGs and were present in both transitions (Additional file 10). Of particular note, in each transition, there was only one cluster enriched for a single transcription factor binding site, and in both cases, it was for the *forkhead* binding site. We identified all genes with at least one highly conserved binding site for *forkhead* ("Methods") to show that these genes cluster samples according to ovary activation and chronological distance (Additional file 11), which is consistent with *forkhead* being involved in the regulation of both transitions.

Discussion

Colonies of *O. biroi* alternate between brood care and reproductive phases, and our time course analyses of the brain transcriptome reveal that the transitions from brood care to reproduction and from reproduction to brood care involve distinct overall patterns of gene expression changes. The timing of brain gene expression changes after manipulating social cues differs between transitions. The addition of larvae leads to a rapid change in gene expression, whereas larval removal results in a much slower change. Inappropriately timed production of eggs incurs individual and colony-level fitness costs. At the individual level, eggs laid in the presence of larvae are eaten, wasting the resources taken to produce them. Furthermore, individuals with active ovaries are aggressed and eventually killed by nestmates [35]. Such policing behavior has been hypothesized to minimize colony-level costs because unsynchronized egg-laying would jeopardize the colony cycle [39]. Such fitness costs will exert selective pressure on the regulation of reproductive physiology [40]: in line with our findings, regulatory mechanisms should be slow to activate ovaries and quick to suppress or reverse egg production.

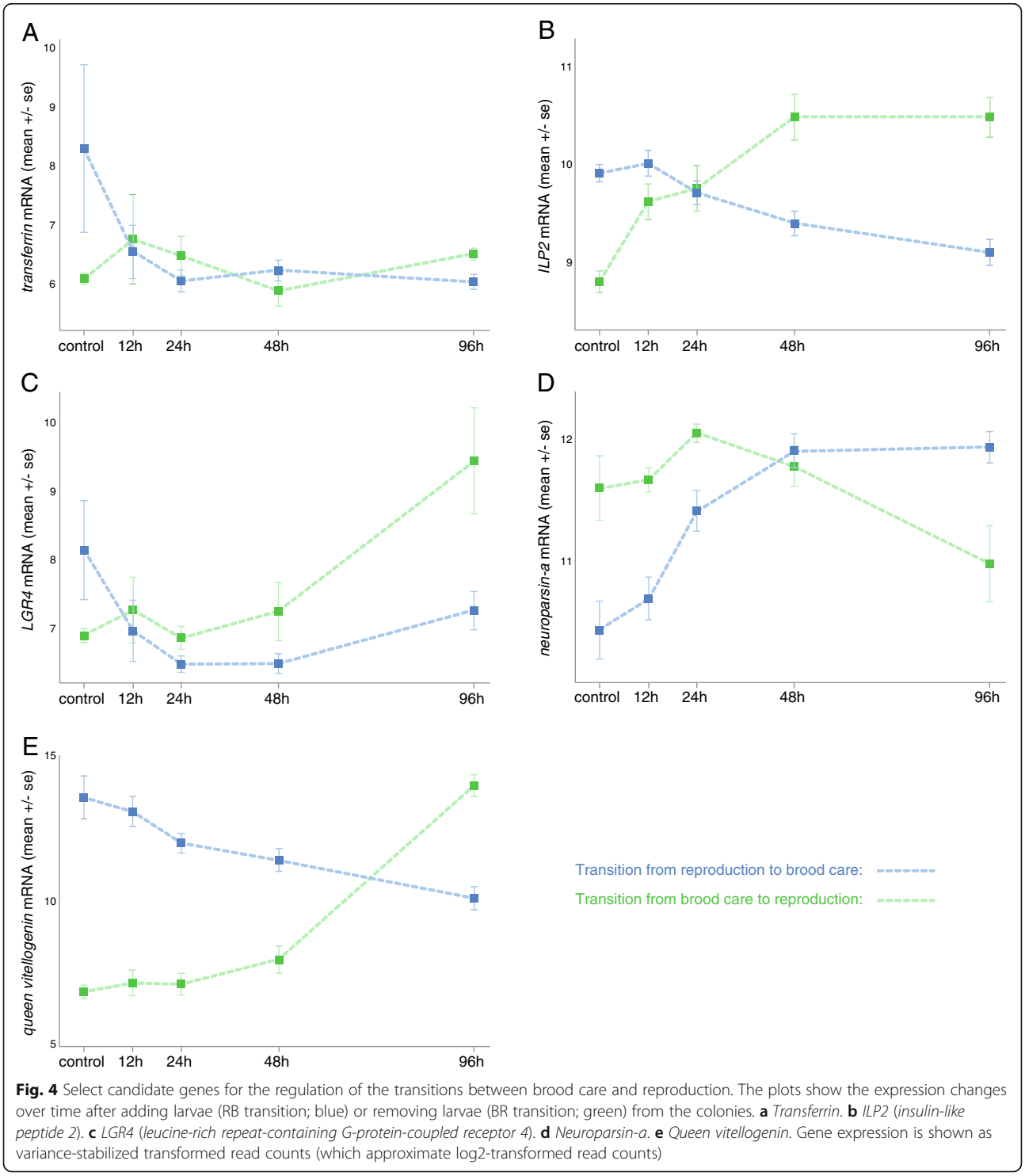

Fig. 4 Select candidate genes for the regulation of the transitions between brood care and reproduction. The plots show the expression changes over time after adding larvae (RB transition; blue) or removing larvae (BR transition; green) from the colonies. **a** *Transferrin*. **b** *ILP2* (*insulin-like peptide 2*). **c** *LGR4* (*leucine-rich repeat-containing G-protein-coupled receptor 4*). **d** *Neuroparsin-a*. **e** *Queen vitellogenin*. Gene expression is shown as variance-stabilized transformed read counts (which approximate log2-transformed read counts)

In addition, our results are consistent with larval cues acting as a reinforcement signal for brood care and for inhibition of reproduction, because the removal of the brood signal is accompanied by a delay in gene expression and physiological adjustments. Such a delay is necessary in *O. biroi* to prevent premature transitioning to reproduction, such as during foraging, when some individuals frequently exit the nest during the brood care phase and are thus only sporadically exposed to larval cues. Comparable resistance to change has been observed in other species and contexts. In behavioral sciences, the resistance to change in behavior after removal

of a stimulus has been compared to the inertial mass [41] and applied to behaviors as diverse as drug addiction in humans [41] or food-reinforced behaviors in birds [42]. Physiological regulations are also subject to resistance to change. For example, physiological changes that occur in rats in response to a stressful stimulus (e.g., cold temperature or low oxygen) take several days to return to baseline levels after stimulus removal [43, 44]. This pattern of rapid response to stimulus exposure but slow response to stimulus removal also parallels the adaptation and deadaptation rates seen in many molecular systems, such as the cAMP-mediated cGMP response inducing cell aggregation in the slime mold *Dictyostelium discoideum* [45].

Our findings are not consistent with the *O. biroi* colony cycle being regulated by discrete gene networks in which expression is coordinately and symmetrically up- or downregulated during transitions between phases. Indeed, neither the differential expression nor the network analyses found substantial overlap in gene membership between transitions. In other words, the sequence of gene expression changes that is associated with the transition to the reproductive phase is not the reverse sequence of gene expression changes associated with the transition to the brood care phase.

Finding transcriptome-wide differences in expression between transitions does not necessarily imply that individual genes or groups of genes cannot play a regulatory role in both transitions. In fact, some of the candidate genes identified here are involved in both the BR and the RB transition (see below). Our differential expression analysis revealed that genes with some of the highest expression changes over time have neuroendocrine, neuronal, and gene regulatory functions, and regulate neuropeptide signaling and neurotransmission. Among these genes, we have highlighted five candidates for the regulation of reproduction and brood care in *O. biroi* (Fig. 4) by identifying the DEGs with the largest changes in expression along one or both transitions (Additional file 9) that belong to molecular pathways with caste-biased activity in other social insects.

The gene *transferrin* (Fig. 4a) has large and early changes in expression in both transitions and shows caste-biased expression in multiple species of social insects. In the ant *Temnothorax longispinosus* and in the wasp *Polistes canadensis*, whole-body RNA sequencing revealed higher expression in queens compared to workers [5, 46]. While in insects the protein encoded by *transferrin* transports iron into the eggs, reduces oxidative stress, and interacts with the vitellogenin and juvenile hormone pathways [47], its role in the brain remains poorly understood.

Another candidate gene identified in our study is *insulin-like peptide 2* (*ILP2*; Fig. 4b), a neuropeptide that belongs to the insulin signaling pathway, which is a conserved pathway that regulates nutrition, fertility, and longevity in animals [48, 49]. Insulin signaling, together with the juvenile hormone and vitellogenin pathways [50–52], is involved in caste determination and division of labor in social insects [14, 50, 53–56]. Interestingly, *ILP2* shows one of the earliest responses to the removal of larvae (in the BR transition), and it has recently been shown that *ILP2* indeed regulates ant reproduction [57]. Another candidate gene with early expression changes in both transitions is *leucine-rich repeat-containing G-protein-coupled receptor 4* (*LGR4*; Fig. 4c). It encodes a G-protein-coupled receptor predicted to bind relaxin-like peptides [58], which belong to the insulin family, together with insulin-like peptides and insulin-like growth factors [59]. The expression of the neuropeptide *neuroparsin-a* increases gradually when transitioning to brood care (Fig. 4d), which is consistent with neuroparsins having anti-gonadotropic roles through interactions with the vitellogenin and insulin signaling pathways [60]. Together, these expression patterns support the hypothesis that insulin signaling plays an important role in linking changes in social cues to reproductive changes [23, 57, 61].

Queen vitellogenin (Fig. 4e) is differentially expressed between reproductive and non-reproductive castes in multiple species of ants, bees, wasps, and termites [5, 9, 17, 31, 53, 62–66]. This gene encodes the yolk protein precursor vitellogenin, which is instrumental to egg formation. In formicoid ants, the *vitellogenin* gene has been duplicated, and some gene copies have been co-opted to regulate non-reproductive functions such as behavior [17, 67]. The changes in *queen vitellogenin* expression mirror the ovarian development and overall alterations of the transcriptome: *queen vitellogenin* displays a gradual and early decrease during the RB transition but a sharp and delayed increase during the BR transition (Fig. 4e).

The protein vitellogenin is typically synthesized in the fat body, secreted into the hemolymph, and transported into the developing oocytes [68]. In addition, vitellogenin has been localized in the honeybee brain, suggesting that it also has a neuroendocrine function [69]. Here we show that *vitellogenin* gene expression in the brain is correlated with changes in reproductive physiology. This finding is consistent with *vitellogenin* changes in expression associated with adult caste differentiation and reproductive activation in queenless ants of the genus *Diacamma* [23] and with previous reports of caste-biased *vitellogenin* expression in the head [31, 53] or in the brain [64]. Such accumulation of evidence for caste-biased *vitellogenin* expression across the phylogeny of social insects, and in species with and without distinct morphological castes, identifies *vitellogenin* genes as key players in the evolution and regulation of reproductive division of labor. Our analyses of gene expression

changes over time reveal that, although *queen vitellogenin* shows one of the highest changes in expression in both transitions, such changes occur rather late after manipulating social cues. This supports the notion that the role of vitellogenin in the brain is likely to be downstream of earlier molecular changes (e.g., in the insulin signaling pathway) [57].

A recent study compared gene expression between reproductive and non-reproductive *Diacamma* ants, where caste is determined at the adult stage via social dominance and aggressive interactions [23]. Similar to *O. biroi*, this avoids the problem of morphological differences between castes and allows for the induction of changes in behavior and reproduction by experimentally manipulating social interactions. Interestingly, despite several differences in experimental design, the overlap between the genes differentially expressed in *Diacamma* [23] and *O. biroi* includes genes in the insulin signaling and vitellogenin pathways. Given that the two species are phylogenetically only distantly related, this opens the possibility that these genes are important in regulating reproductive division of labor across the ants and may have played a role during the evolutionary origin of ant eusociality [57].

Recent studies have proposed that changes in gene regulatory mechanisms were associated with the evolution of eusociality [70, 71]. In our study, many DEGs that showed early changes in gene expression have gene regulatory functions such as the onset (*hunchback*) and termination (*transcription termination factor* 2) of transcription, as well as the synthesis (*PPID*, *annulin*), glycosylation (*alpha-(1,3)-fucosyltransferase 6*), and phosphorylation (*hyaluronan-mediated motility receptor*) of proteins. In addition, gene clusters enriched for DEGs were also frequently found to be enriched for genes with certain transcription factor binding sites. This suggests complex transition-specific gene expression and regulation, affected by multiple transcription factors. Nevertheless, genes that are putatively regulated by a few transcription factors exhibit predictable patterns of regulation. For example, the expression of genes associated with *forkhead* transcription factor binding sites provided significant predictive power as to the physiological state of an individual. Interestingly, *forkhead* transcription factors regulate reproduction in other insect species. For example, knocking down *forkhead* transcription factors in the yellow fever mosquito *Aedes aegypti* and the brown planthopper *Nilaparvata lugens* reduced offspring production and the activity of the vitellogenin pathway [72, 73]. In addition, *forkhead* plays a role in the regulatory network of salivary glands in insects [74], which include the mandibular glands that produce caste-specific compounds in honeybees [75]. Interestingly, the promoter region of *forkhead* shows a depletion of transcription factor binding sites in ants compared to solitary insects, which may have facilitated *forkhead* pleiotropy and its implication in caste-specific regulatory networks [71]. The decoupling of brood care and reproductive phases in different female castes during the evolution of eusociality was associated with the co-option of gene function and regulation [15]. Our findings suggest that transcription factors such as *forkhead* may be among the regulatory elements responsible for the co-option of gene regulatory networks during this evolutionary transition.

Conclusion

Assuming that the colony cycle of *O. biroi* indeed represents a partial reversal to the life cycle of the subsocial ancestor of ants and possibly other eusocial hymenopterans, one parsimonious way to compartmentalize such a cycle would be to disrupt the transition to brood care in response to larval cues in a subset of individuals, which would then act as queens. Given that these queens would now lay eggs continuously, any additional females that emerge at the nest would immediately and permanently be exposed to larval cues and thus locked in the brood care phase of the ancestral cycle. This would then give rise to reproductive division of labor, which could be acted upon by natural selection, driving continued divergence in fertility, and ultimately leading to eusociality. In this study, we report that patterns of gene expression changes over time differ between the transition to brood care and the transition to reproduction in *O. biroi*. Our results are therefore not consistent with the transitions being regulated by mirrored sequences of gene expression changes in a pendular manner. On the contrary, patterns of gene expression appear to be circular, with the involvement of transition-specific sets of genes. This implies that, on a molecular level, the transition to brood care could have been disrupted in a variety of ways without affecting the reverse transition. However, especially given our finding that exposure to larval cues entails rapid and large-scale changes in brain gene expression, we would assume that this disruption happened early and upstream in the gene expression cascade. Our time-course data allowed us to identify molecular candidate pathways that respond rapidly to larval cues and could therefore be upstream of the longer-term behavioral and physiological responses. These constitute prime candidates, both for broad comparative analyses across social hymenopterans and for functional experiments in *O. biroi* and other species.

Methods

Biological samples

Source colonies (Fig. 1) were derived from two separate clonal lineages: MLL1 and MLL4 [76]. Clonal lineage and source colony identity are recorded for all RNA sequencing libraries, which are uploaded to the NCBI Bioproject PRJNA273874. Large source colonies in the

brood care phase were used to establish two experimental colonies each (250 1-month old workers and 100 ≥ 3-month old workers), one of which received approximately 250 larvae. After a full colony cycle, each colony contained a complete cohort of brood and workers and was in either peak brood care phase or early reproductive phase. On the day the first eggs were laid in the reproductive phase colony, the 1-month old workers were subdivided into colonies of 45 workers. One of these colonies from each phase served as the control colony and was given brood from the colony the workers were derived from (i.e., larvae for the brood care phase control and eggs and pupae for the reproductive phase control). The remaining colonies received brood from the colony at the opposite stage of the cycle (sub-colonies originally in the reproductive phase received larvae and vice versa), thereby inducing the transition toward the opposite phase. All colonies with larvae were fed every 24 h, immediately after samples for the respective time points had been collected. Colonies were collected 6, 12, 24, 48, or 96 h after experimental manipulation. This process was repeated eight times: four times with and four times without the 6-h time point. In each instance, the control sample was collected at the same time as the earliest time point. After looking for outliers, we removed all samples collected at the 6-h time point (see details below), thus resulting in four biological replicates for the controls and eight biological replicates per time point in both transitions (Fig. 1, Additional file 12). Source and experimental colonies were kept at 25 °C and 60% humidity, and when in the brood care phase were fed frozen *Solenopsis invicta* brood.

Sample collection and RNA sequencing

At the specified time for each colony, all ants were flash frozen and subsequently stored at − 80 °C. Ovaries and brains were dissected in 1× PBS at 4 °C. To estimate ovarian development, ovary activation was scored according to [77] for 200 ants (20 ants per time point) from two source colonies (Additional file 13). Brains of individuals with two ovarioles were transferred immediately to Trizol, and once ten brains from a colony were pooled, the sample was frozen on dry ice.

RNA was extracted with RNEasy column purification, as explained in Oxley et al. [31]. Clontech SMARTer low input kits were used for library preparation, and RNA sequencing was performed on a HiSeq 2000, with 100 bp single-end reads. Sequencing batches included all time points for both transitions of any given colony, for two source colonies at a time.

Identification of outlier samples

Nine hundred sixty-seven genes had more than twofold change in expression across all samples. Because these genes contribute the greatest variation between samples, they were used to observe the general pattern of sample clustering, in order to remove outlier samples prior to differential gene expression analysis (Additional file 1).

All 6-h samples (controls and treatments) clustered more closely with each other than with their respective (expected) transition groups. Looking at individual gene expression time courses, it was clear that the 6-h time points frequently deviated wildly from the other time points. This suggests that the majority of gene expression changes observed in the 6-h time points was induced by the experimental disturbance. However, removing the 6-h time points could prevent us from detecting genes that legitimately changed as a result of the actual brood-swap, instead of the experimental manipulation. We therefore looked at the change in sensitivity and specificity of the experiment after removing the 6-h samples from the analysis.

Removing the 6-h time points reduced the number of genes with greater than or equal to twofold difference by 335. Fifty-one percent of these 335 genes were differentially expressed between 6- and 12-h control samples of the same phase and were therefore a priori likely to be false positives. Seventy-three genes were expressed greater than or equal to twofold between 6-h control and treatment samples and were therefore potentially genes regulated by the change in brood stimuli. Of these 73 genes, only 5 were not present in the 632 genes still identified as having greater than or equal to twofold differences after removal of the 6-h time points (Additional file 1). If these genes were real target genes, we would only lose 6.8% of the early-responding genes. Removing the 6-h time points as outliers therefore increased the specificity of our differential expression analysis, with negligible loss of sensitivity.

Identification of differentially expressed genes

Fastq reads from all samples were aligned to the *Ooceraea biroi* genome (NCBI assembly CerBir1.0) using STAR (default parameters). HTSeq was then used to determine the number of reads aligned to each gene (NCBI *Cerapachys biroi* Annotation Release 100). DESeq2 was used for differential gene expression analysis.

To analyze each transition separately, we contrasted the following models in DESeq2:

Full model: colony + bs(time, df = 3)
Reduced model: colony

using the bs function from the splines library (v. 3.2.3) in R for evaluating the spline function of all time points (controls coded as time 0). This contrast identified genes with a significant change at any point in time, not just genes significantly different from the control samples. This analysis was run for both BR and RB transitions.

To account for the effects of experimental manipulation, the following models were contrasted:

Full model: colony + transition + bs(time, df = 3) + transition: bs(time, df = 3)
Reduced model: colony + transition + bs(time, df = 3)

This model contrast identified the genes that were differentially expressed over time, after accounting for the differences in gene expression between reproduction and brood care phases. Without using the spline function, we could simply be comparing gene expression at each time point to "time 0" (i.e., the control samples). However, this would not reveal genes whose expression changed temporarily, before returning to their baseline value.

We identified only those genes with a significant time by transition interaction. It has been shown that expression of certain genes can have opposing effects, depending on the context [78]. Genes that show significant change in expression over time, but no significant interaction with phase, may therefore still be important in regulating transitions between phases. However, such genes are confounded with, and cannot be disentangled from, genes that are expressed as a stress response resulting from the brood-swap experimental procedure, and we therefore decided to ignore them in our present analyses.

Clustering of gene expression time courses

We clustered the samples using P-spline smoothing and mixed effects models according to the algorithm by Coffey et al. [38]. To determine the optimal number of clusters for each transition, we calculated the BIC score for all even cluster sizes between 2 and 120 clusters (Additional file 4). We selected the smallest cluster size of the lower BIC values that did not precede a higher BIC value (Additional file 4).

Enrichment analyses for expression clusters

Transcription factor binding site enrichment of each cluster was determined with Pscan, using the available position weight matrices from the JASPAR database. Assessment of clusters for enrichment for DEGs and GO terms was determined using Fisher's exact test followed by Benjamini and Hochberg [79] false discovery rate adjustments. GO term enrichment was calculated using genomepy's genematch.py module (github.com/oxpeter/genomepy). To identify all *O. biroi* annotated genes with *forkhead* transcription factor binding sites, we used the R packages TFBSTools and biostrings, with the position weight matrix for *Drosophila* from the JASPAR database and a 95% minimum score for matching.

Network analysis of the identified clusters

We first constructed the complete network that consisted of all gene clusters from both transitions. Each node in this network represented a cluster of genes, and edges represented the genes that are shared between clusters. Since each gene is uniquely assigned to a single cluster in each transition, no two clusters from the same transition will ever be connected. Similarly, every gene is represented once, and only once, among all the edges.

The conserved network was constructed by looking at the Jaccard Index for each pair of clusters as a measure of similarity that does not rely on untested assumptions. We then conducted a permutation analysis by calculating 1000 random cluster networks (each cluster had the same number of genes as the original) and calculated the Jaccard Indices of all node pairs. Our conserved network was then created by choosing only those edges that represent a Jaccard Index greater than 95% of all scores from the random networks.

Additional files

Additional file 1: Outlier analysis. PCA and distance map of genes showing greater than twofold change in expression. A) PCA plot of brood-swap and control samples. Clustering was based on the mean gene expression of each group, for 967 genes with more than twofold change in expression between samples. Percentages on each axis indicate the proportion of variance explained by the indicated principal component. The color of each sample indicates the expected similarity to the control samples; dark blue indicates reproductive phase and dark green indicates brood care phase. Sample names are as per Fig. 1. B) Heatmap showing Euclidean distance between all samples, based on all genes with more than twofold change in expression, and clustered according to average distances between samples. Blue and green color bar above the heatmap indicates similarity to control samples, as in A. Sample names are as per Fig. 1. C) Venn diagram showing outcome of eliminating the 6-h time points. Numbers in the small circles indicate genes with greater than twofold change in expression between 6-h control and treatment samples in the reproduction to brood care transition (blue) and brood care to reproduction transition (green) (a priori true positives). Red numbers indicate genes that show greater than twofold change in expression after removal of the 6-h time points. Thus, elimination of the 6-h samples does not substantially reduce the number of DEGs identified with large expression changes. (PDF 79 kb)

Additional file 2: All 596 DEGs ranked according to *p* value (smaller to larger). (PDF 101 kb)

Additional file 3: All gene clusters identified, and their corresponding enrichment for differentially expressed genes, gene ontology terms, and transcription factor binding sites. (PDF 124 kb)

Additional file 4: Evaluation of BIC scores for selection of optimal number of clusters. Genes were clustered into all even numbered cluster sizes between 2 and 120 (brood care to reproduction) or 2–110 (reproduction to brood care). The optimal cluster size was determined to be the cluster with the lowest BIC score after stabilization to the plateau seen on the right of each graph. Arrows show the cluster selected for each transition. (PDF 53 kb)

Additional file 5: Summary of clusters enriched for DEGs. (PDF 29 kb)

Additional file 6: Basic network statistics of gene clusters for time course gene expression between both reproductive and brood care phase transitions. (PDF 51 kb)

Additional file 7: GO terms significantly enriched in clusters enriched for DEGs. Only two of these GO terms were common to both transitions (RB: transition from reproduction to brood care; BR: transition from brood care to reproduction). The diameter of the circles is proportional to the number of enriched GO terms. (PDF 97 kb)

Additional file 8: The conserved network (with only non-random connections) shows a lower proportion of edges linking clusters of genes regulated in opposite direction compared to the complete network (which includes random connections) ($\chi^2 = 22.4$, $p < 0.00001$). This finding is inconsistent with the same genes regulating both transitions. (PDF 47 kb)

Additional file 9: Top 40 DEGs (ranked according to log2 fold change in expression for control vs 12-h time point and control vs 96-h time point for each transition). (PDF 50 kb)

Additional file 10: Summary of clusters enriched for differentially expressed genes (DEGs) and transcription factor binding sites. (PDF 53 kb)

Additional file 11: Genes associated with *forkhead* also segregate with position in the colony cycle. Heatmap showing Euclidean distance between all samples for the 438 genes that contained at least one transcription factor binding site for *forkhead* with a minimum score of 95%. The dendrogram was constructed using the average distances between samples. The blue and green color bar above the heatmap indicates average ovary activation score, as in Fig. 2a. Sample names are as per Fig. 1. (PDF 49 kb)

Additional file 12: Number of replicates in the analyses (after outlier removal). (PDF 28 kb)

Additional file 13: Ovary activation scores. (XLSX 11 kb)

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Availability of data and materials
The sequencing data generated and analyzed in this study are available in the NCBI Bioproject PRJNA273874 and the scripts used for the analyses at https://doi.org/10.5281/zenodo.1318306. The ovary activation scores are available in Additional file 13.

Authors' contributions
PRO and DJCK designed the study. RL and PRO conducted the experiments and analyzed the data. RL wrote the manuscript with input from PRO and DJCK. DJCK supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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Appendix 6

Robust DNA methylation in the clonal raider ant brain

Libbrecht R, Oxley PR, Keller L & Kronauer DJC

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Report

Current Biology

Robust DNA Methylation in the Clonal Raider Ant Brain

Highlights

- A large proportion of brain DNA methylation in the clonal raider ant is robust
- Genes with robust methylation show high and stable expression
- DNA methylation is not associated with different reproductive and behavioral states
- Evidence for caste-specific DNA methylation in social insects is weak

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In Brief

Libbrecht et al. show that in the clonal raider ant, brain DNA methylation is robust, particularly in genes with high and stable expression, and is not associated with different reproductive and behavioral states. They also report that currently there is little evidence of differential DNA methylation between the female castes of social insects.

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Current Biology
Report

Robust DNA Methylation in the Clonal Raider Ant Brain

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SUMMARY

Social insects are promising model systems for epigenetics due to their immense morphological and behavioral plasticity. Reports that DNA methylation differs between the queen and worker castes in social insects [1–4] have implied a role for DNA methylation in regulating division of labor. To better understand the function of DNA methylation in social insects, we performed whole-genome bisulfite sequencing on brains of the clonal raider ant *Cerapachys biroi*, whose colonies alternate between reproductive (queen-like) and brood care (worker-like) phases [5]. Many cytosines were methylated in all replicates (on average 29.5% of the methylated cytosines in a given replicate), indicating that a large proportion of the *C. biroi* brain methylome is robust. Robust DNA methylation occurred preferentially in exonic CpGs of highly and stably expressed genes involved in core functions. Our analyses did not detect any differences in DNA methylation between the queen-like and worker-like phases, suggesting that DNA methylation is not associated with changes in reproduction and behavior in *C. biroi*. Finally, many cytosines were methylated in one sample only, due to either biological or experimental variation. By applying the statistical methods used in previous studies [1–4, 6] to our data, we show that such sample-specific DNA methylation may underlie the previous findings of queen- and worker-specific methylation. We argue that there is currently no evidence that genome-wide variation in DNA methylation is associated with the queen and worker castes in social insects, and we call for a more careful interpretation of the available data.

RESULTS AND DISCUSSION

The clonal raider ant *Cerapachys biroi* provides a good system to investigate insect DNA methylation, because age-matched individuals that are genetically identical can be collected easily [7]. *C. biroi* has no distinct queen and worker castes. Instead, all ants in a colony produce female offspring by parthenogenesis [8], and colonies undergo stereotypical cycles alternating between queen-like reproductive phases (ants lay eggs inside the nest) and worker-like brood care phases (ants do not lay eggs but nurse the brood and forage for food) [5]. To characterize the brain methylome of *C. biroi*, we sequenced eight samples of bisulfite-treated DNA extracted from pools of 20 brains dissected from age-matched ants collected in the reproductive phase (four samples) and in the brood care phase (four samples) from four source colonies belonging to two different clonal lineages (Experimental Procedures).

The average proportion of methylated cytosines across the eight samples was 2.1% ± 0.1% (mean ± SD), which is substantially higher than what has been reported for the honeybee (0.1%) [1] and other ant species (0.3% in *Camponotus floridanus* and 0.2% in *Harpegnathos saltator*) [2]. Methylation-sensitive AFLP on additional samples confirmed higher levels of methylation in *C. biroi* than in other social insects (Table S1; Supplemental Experimental Procedures). DNA methylation was found primarily in CpG dinucleotides (66.3% ± 1% of the methylated cytosines) and within genes (82.5% ± 0.6%), especially in exons (57% ± 0.9%). Such exonic CpG methylation has been reported in other insect species and in mammals, and it may affect gene function through histone modifications [9], nucleosome stability [10], and/or alternative splicing [1, 2, 11]. As previously shown in other ant species [2], levels of DNA methylation in *C. biroi* were associated with patterns of alternative splicing (Figure S1; Supplemental Experimental Procedures), and transposable elements were hypomethylated compared to the genome baseline (Wilcoxon rank-sum test, W = 64, p = 0.0002; Table S2; Supplemental Experimental Procedures).

Robust DNA Methylation Is Associated with Highly Expressed Genes Involved in Core Functions

On average, 29.5% ± 1.7% of the methylated cytosines in a given sample showed robust methylation, as they were methylated in all eight samples, despite behavioral, reproductive, and genotypic differences among samples. Additionally, the percentage of sequencing reads indicating methylation was higher for the cytosines that were methylated in all samples (58.2% ± 0.4%) than for those that were methylated in only a subset of samples (17.4% ± 1.9%). Strikingly, 99.3% ± 0.1% of the cytosines with more than 60% reads indicating methylation were methylated in all samples (Figure S2). This suggests that DNA methylation is not only robust across samples but also within samples, hence across individual brains. However, to more definitively assess variation in DNA methylation across

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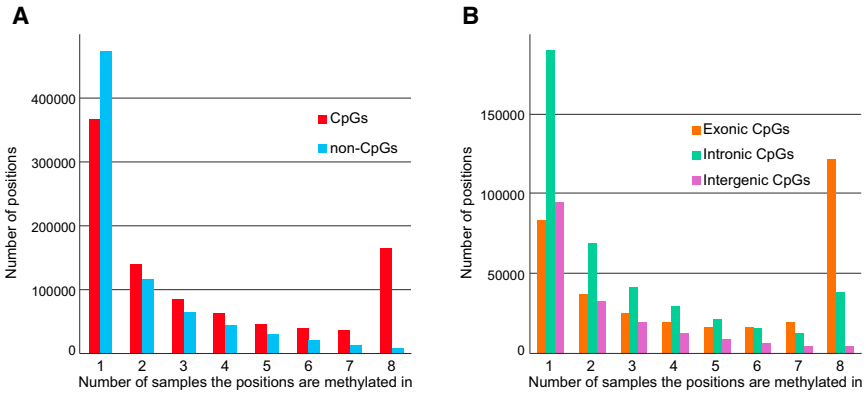

Figure 1. Robust Methylation Is Context and Location Dependent

The graphs show the number of methylated cytosines that are methylated in different numbers of samples (from one to eight) for CpG and non-CpG contexts (A) and for exonic, intronic, and intergenic CpGs (B). Most methylated cytosines are methylated in one sample only (random or sample-specific methylation) or in all eight samples (robust methylation). See also Tables S1 and S3 and Figure S2.

(A) Levels of robust methylation differ between CpG and non-CpG contexts, as illustrated by the sharp increase observed between seven and eight samples for CpGs, but not for non-CpGs.

(B) Levels of robust methylation differ across genomic locations: DNA methylation is more robust in exons compared to introns (sharper increase between seven and eight samples for exonic CpGs than intronic CpGs) and in introns compared to intergenic regions (increase between seven and eight samples for intronic CpGs, but not intergenic CpGs).

individuals would require very deep sequencing coverage from single brains.

The degree of robust DNA methylation differed between CpG and non-CpG contexts and across genomic locations. While 164,258 CpG positions (41.3% ± 2.2% of the methylated CpGs) were methylated in all eight samples, only 9,047 non-CpG positions (4.8% ± 0.4% of the methylated non-CpGs) were methylated in all samples, revealing that CpG methylation is more robust than non-CpG methylation (Figure 1A). Similarly, while 121,858 exonic CpGs (60.9% ± 3.8% of the methylated exonic CpGs) were methylated in all eight samples, only 38,036 intronic CpGs (26.2% ± 1.5% of the methylated intronic CpGs) and 4,364 intergenic CpGs (8.3% ± 0.5% of the methylated intergenic CpGs) were methylated in all samples, revealing that DNA methylation is more robust in exons compared to introns and in genic (exons and introns) compared to intergenic regions (Figure 1B).

The comparison between genes with and without robust methylation revealed that genes with robust methylation (i.e., with at least one cytosine methylated in all eight samples) were significantly enriched for gene ontology (GO) terms related to core processes, such as DNA repair; RNA binding and processing; and protein translation, folding, transport, and binding (Table S3). Genes with robust methylation also were more expressed than genes without robust methylation (Wilcoxon rank-sum test, $W = 5{,}216{,}694$, $p < 0.0001$). More generally, there was a positive relationship between the level of expression and the level of methylation (Spearman rank-correlation test, $rho = 0.59$, $p < 0.0001$; Figure 2A). DNA methylation may preferentially target highly expressed genes and/or DNA methylation may enhance gene expression.

DNA Methylation Is Not Associated with Reproduction and Behavior

To determine whether parts of the *C. biroi* methylome are associated with reproduction and behavior, we performed two analyses to investigate whether DNA methylation differs between brains of age-matched ants in the reproductive phase and in the brood care phase. First, we compared the proportion of methylated reads between the two phases for each CpG. There was no CpG for which the proportion of methylated reads significantly differed between phases after correcting for multiple testing (all p values > 0.22). Second, we used the methylation status of each CpG (methylated or not methylated) to calculate the number of CpGs that were methylated in all four samples from one phase and not methylated in all four samples from the other phase. Then we determined whether such a number of differentially methylated CpGs could be expected by chance by repeating the analysis for all possible sample randomizations. We found 1,560 differentially methylated CpGs between the reproductive phase and the brood care phase, while random comparisons returned an average of 1,727 ± 222 differentially methylated CpGs (median = 1,705; ranging from 1,418 to 2,115; Figure S3). This suggests that the 1,560 apparently differentially methylated CpGs were false positives. Therefore, our analyses did not detect any significant differences in DNA methylation between brains of ants in the reproductive phase and brains of ants in the brood care phase.

In line with the finding that DNA methylation is not associated with reproduction and behavior in the context of colony cycles in *C. biroi*, there was a strong negative relationship between the level of DNA methylation and the level of differential gene expression. Genes that were differentially expressed between the reproductive phase and the brood care phase had fewer methylated sites, while genes with a stable expression between phases tended to be more methylated (Spearman rank-correlation test, $rho = -0.32$, $p < 0.0001$; Figure 2B). Because our analyses did not detect differentially methylated CpGs and DNA methylation is less likely to be found in genes that are differentially expressed between phases, it is unlikely that DNA methylation is involved in the regulation of the clonal raider ant colony cycles.

Re-evaluating the Evidence for Caste-Specific DNA Methylation

Our finding that DNA methylation is robust and not associated with changes in reproduction and behavior in *C. biroi* seems to contradict previous studies that reported DNA methylation differences between the queen and worker castes in four social insect species. Although the findings of caste-specific DNA methylation have been reviewed extensively in the

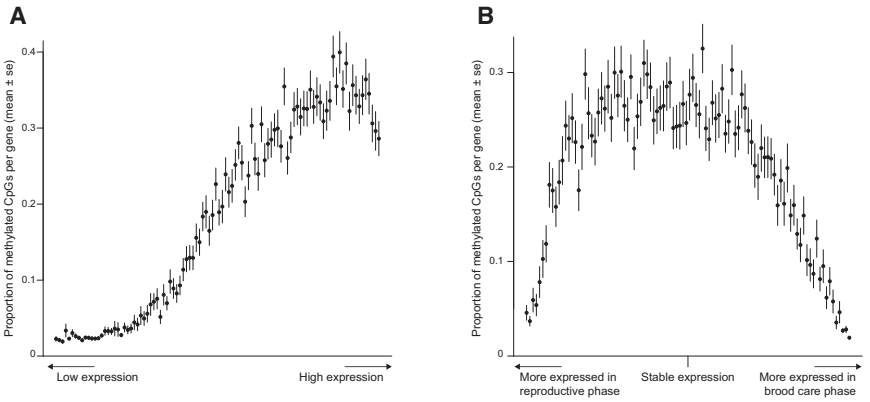

Figure 2. Relationship between DNA Methylation and Gene Expression and between DNA Methylation and Proportional Change in Gene Expression between the Phases of the Colony Cycle

(A) There is a positive relationship between the proportion of methylated CpGs per gene and gene expression. Genes were ranked according to their mean expression across the eight samples before being divided into 100 bins. For each bin, we plotted the mean ± SE proportion of methylated CpGs per gene.

(B) Genes with stable expression between phases tend to be more methylated than genes with differential expression. Genes were ranked depending on how differential their expression was before being divided into 100 bins: in the center are genes with stable expression, on the left those that are more expressed in the reproductive phase compared to the brood care phase, and on the right those that are more expressed in the brood care phase compared to the reproductive phase. For each bin, we plotted the mean ± SE proportion of methylated CpGs per gene. See also Table S2 and Figure S1.

literature [12–27], there are only four empirical studies that used whole-genome bisulfite sequencing to report such differences in ants and bees [1–4]. All those studies investigated differential methylation using the same statistical method, which does not require biological replicates but is prone to producing false positives stemming from sample-specific DNA methylation.

We used the *C. biroi* methylome to assess the validity of the statistical method used in previous studies. First, we investigated whether sample-specific DNA methylation occurred in *C. biroi* by comparing DNA methylation across the eight samples. We found that, on average, 105,321 ± 18,935 cytosines (17.8% ± 2.7% of the methylated cytosines) and 46,027 ± 6,453 CpGs (11.5% ± 1.3% of the methylated CpGs) showed sample-specific DNA methylation. Second, we applied the statistical method used in previous studies to our own data (Supplemental Experimental Procedures). Instead of performing one analysis with four replicates, we performed four separate analyses, each comparing the reproductive phase and the brood care phase of one source colony. We found several hundred differentially methylated exons between the phases for all four source colonies (Figure 3), which is in striking contrast to our combined analysis of the four replicates. However, overlapping the results from the four separate comparisons revealed no exon that was consistently significantly differentially methylated between the two phases in all four analyses (Figure 3). This shows that the lists of differentially methylated exons generated by the statistical method used in previous studies are random or colony specific, and they likely stem from sample-specific DNA methylation.

To our knowledge there are only two empirical genome-wide studies of DNA methylation in social insects that used a replicated experimental design to test whether methylation differs between queens and workers in honeybees [28], *Dinoponera* ants, and *Polistes* wasps [29]. Neither of the two studies detected significant differences in DNA methylation between queen and worker brains (Supplemental Experimental Procedures), which is consistent with our finding that brain DNA methylation does not differ between the reproductive and brood care phases in the clonal raider ant.

Conclusions

The use of biological replicates allowed us to conduct a proper study of the brain methylome of the clonal raider ant *C. biroi*. Our analysis reveals that a large proportion of methylation is robust both across and within samples, especially in exonic CpGs of highly expressed genes involved in general processes. We also report that DNA methylation is unlikely to be involved in regulating the reproductive and behavioral dynamics of the *C. biroi* colony cycle. Finally, evaluating the statistical method used in previous studies with our data indicates that there currently is no empirical evidence for genome-wide variation in DNA methylation associated with the queen and worker castes in other social insect species. Such a lack of well-supported evidence does not necessarily imply that caste-specific methylation does not exist, but rather calls for more controlled and carefully replicated studies of DNA methylation in insect societies.

EXPERIMENTAL PROCEDURES

Sample Preparation

In *C. biroi*, the presence or absence of larvae triggers the switch between the phases of the colony cycle [30]. We used this effect of the larvae to prepare the samples for our study. We first collected 500 callow (recently eclosed) workers, which are light-colored age-matched ants, from a source colony in the brood care phase. We split those callows into two subcolonies, from one of which we removed all the larvae. The subcolony with the larvae remained in the brood care phase, while the other entered a new reproductive phase. We then waited a complete cycle (circa 34 days) until the two subcolonies were again at opposite ends of the cycle. The subcolony in the brood care phase was flash frozen 6 days after the ants started foraging, while the subcolony in the reproductive phase was flash frozen when the first eggs were laid. Thus, the ants collected in the brood care phase and in the reproductive phase were the same age, and they were morphologically and genetically identical (all came from the same source colony, i.e., the same clonal genotype).

For each subcolony, we dissected the brains of 30 individuals with two ovarioles [8], pooled 20 brains to extract DNA for whole-genome bisulfite sequencing, and pooled ten brains to extract RNA for RNA sequencing (see the Supplemental Experimental Procedures for DNA and RNA extraction protocols). We repeated this entire process four times using four different source colonies spanning two clonal lineages: source colonies A1 and A2 (C1 and C16 from clonal lineage A or MLL1 in [31]), and B1 and B2 (STC1 and STC6 from clonal lineage B or MLL4 in [31]). This resulted in eight DNA samples and eight

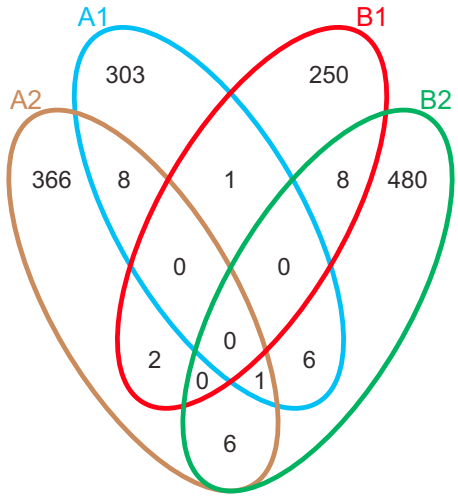

Figure 3. The Lists of Differentially Methylated Exons Returned by the Statistical Method Used in Previous Studies without Biological Replicates Are Random or Colony-Specific Lists of Exons

This graph shows the number of differentially methylated exons between the reproductive phase and the brood care phase for each source colony: 319 in colony A1, 383 in colony A2, 261 in colony B1, and 501 in colony B2 (see details in the Supplemental Experimental Procedures). There was no exon that was consistently differentially methylated between phases in all four source colonies. This shows that the statistical method used in previous studies, especially when used without biological replicates [1–4, 6], is prone to return random or colony-specific lists of exons. See also Figure S3.

RNA samples (four in the reproductive phase and four in the brood care phase for both DNA and RNA).

Library Preparation and Sequencing

Library preparation for whole-genome bisulfite sequencing and RNA sequencing, sequencing, and post-processing of the raw data were performed at the Epigenomics Core at Weill Cornell Medical College (see the Supplemental Experimental Procedures for details). Each phase and each clonal lineage was equally represented in each of the two batches of library preparation and sequencing.

Methylated Cytosines

For each position with coverage $\geq$ 10 in each sample (on average 63.6% ± 4.6% of the cytosines had a coverage $\geq$ 10), the methylation status (methylated or not methylated) was determined by comparing the proportion of sequencing reads indicating methylation (methylated reads) to a binomial distribution, where the number of trials is the number of reads (coverage), the number of successes is the number of methylated reads, and the probability of success is the conversion rate of the bisulfite sequencing treatments. If the proportion of methylated reads could not be explained by chance ($p < 0.05$ after correcting for multiple testing [32]), the position was considered methylated. If it could, the position was considered unmethylated.

Differentially Methylated CpGs

Quantitative Method

For each CpG with coverage $\geq$ 10 in all samples, we performed a paired t test to compare the proportion of methylated reads between the reproductive phase (four replicates) and the brood care phase (four replicates), and then we corrected the p values for multiple testing [32].

Permutation Method

We counted the number of CpGs with coverage $\geq$ 10 in all samples that were methylated in the four samples of one phase but unmethylated in the four samples of the other phase. We then compared this number to the numbers for all possible combinations of four and four samples to assess the number of differentially methylated CpGs that could be expected by chance.

ACCESSION NUMBERS

The accession number for the sequencing data reported in this paper is NCBI BioProject: PRJNA304722.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.12.040.

AUTHOR CONTRIBUTIONS

R.L., P.R.O., L.K., and D.J.C.K. designed the experiments. R.L. conducted the experiments. R.L. and P.R.O. analyzed the data. R.L., P.R.O., L.K., and D.J.C.K. wrote the manuscript.

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Appendix 7

Experimental increase of worker diversity benefits brood production in ants

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Experimental increase of worker diversity benefits brood production in ants

Marina N. Psalti[†], Dustin Gohlke[†] and Romain Libbrecht[*]

Abstract

Background: The reproductive division of labor of eusocial insects, whereby one or several queens monopolize reproduction, evolved in a context of high genetic relatedness. However, many extant eusocial species have developed strategies that decrease genetic relatedness in their colonies, suggesting some benefits of the increased diversity. Multiple studies support this hypothesis by showing positive correlations between genetic diversity and colony fitness, as well as finding effects of experimental manipulations of diversity on colony performance. However, alternative explanations could account for most of these reports, and the benefits of diversity on performance in eusocial insects still await validation. In this study, we experimentally increased worker diversity in small colonies of the ant *Lasius niger* while controlling for typical confounding factors.

Results: We found that experimental colonies composed of workers coming from three different source colonies produced more larvae and showed more variation in size compared to groups of workers coming from a single colony.

Conclusions: We propose that the benefits of increased diversity stemmed from an improved division of labor. Our study confirms that worker diversity enhances colony performance, thus providing a possible explanation for the evolution of multiply mated queens and multiple-queen colonies in many species of eusocial insects.

Keywords: Social evolution, Social insects, Division of labor, Behavior, Genetic diversity

Introduction

Genetic relatedness plays an important role in the evolution of altruistic behaviors in animals [1]. Extreme altruism is found in colonies of eusocial Hymenoptera (ants, bees and wasps), where the workers forgo their own reproduction to help the queens produce offspring [2]. Such reproductive division of labor evolved in a context of high genetic relatedness, with a single female reproductive mated with a single male [3, 4]. Most extant eusocial Hymenoptera species are still characterized by high genetic relatedness [3].

Other species evolved colonies with lower relatedness among individuals, and thus higher genetic diversity [5]. In these species, colonies have one multiply mated queen and/or multiple queens. Prominent examples include the honeybee *Apis mellifera*, where queens can mate with up to 20 males [6–9], and the Argentine ant *Linepithema humile*, where nests may contain up to 60 queens [10]. However, there are costs associated with strategies that increase genetic diversity. Multiple mating increases risk of disease or predation, and requires more energy to locate the sexual partners and copulate [11, 12]. Having multiple queens per nest lowers relatedness in the worker force and may favor the emergence of conflicts among workers [13–17].

The evolution of such strategies to increase genetic diversity in some eusocial insect species shows that they must have benefits in certain ecological conditions [18].

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The potential benefits of increased genetic diversity include increased resistance to diseases and parasites via improved social immunity [19–27] and a more efficient behavioral division of labor among workers [28–33].

Behavioral division of labor is the repartition of tasks in the worker force. For example, some workers tend to stay inside the nest to nurse the brood while others forage for food. These tendencies likely stem from workers differing in their internal response threshold to perform specific tasks [34]. This response threshold is determined by a combination of extrinsic and intrinsic factors, such as the social environment, location in the nest, morphology, age, individual experience and genetic background [35–42]. The increasing evidence for genetic effects on worker behavior and division of labor [29–33, 43–45] is consistent with the hypothesis that increased genetic diversity in the worker force would result in a larger variation in threshold responses, and thus a more efficient division of labor.

Several lines of evidence suggest that intracolonial genetic diversity increases fitness. The reports of such findings fall in one of three categories. First, there are theoretical studies that supported a link between diversity and performance [17, 46–48]. Second, there are reports of correlations between genetic diversity and one or several fitness correlates in several species of bees, wasps and ants [28, 49–57]. Third, there are reports of experimental manipulations of genetic diversity that affected colony performance, mostly in bees [19, 20, 23, 24, 38, 51, 58–61].

However, there is still debate over whether increased genetic diversity directly benefits colony performance [62]. First, finding correlations between genetic diversity and fitness components does not imply causation, and other correlative studies did not detect such an association [49, 53, 63–65]. Then, the strategy of many studies that experimentally manipulated genetic diversity was to decrease it in species with naturally high diversity. For example, in the highly polyandrous honey bee, the artificial insemination of queens with the sperm from a single male reduced the performance of their colonies compared to queens inseminated with the sperm from multiple males [38, 51, 61]. In these studies, the decrease in colony performance associated with the low diversity treatment could be confounded by potential stress associated with not being in the natural state. Two studies in *Bombus terrestris* showed some benefits of artificially increased genetic diversity in a species with naturally lower diversity, but mostly in terms of resistance to pathogens [59, 60]. Finally, experiments based on artificial insemination cannot disentangle between direct effects of genetic diversity among workers produced by the artificially inseminated queen and indirect maternal effects via the queen (e.g., on the number and quality of eggs produced) in response to the insemination with variable sperm diversity.

One way to get around the confounding maternal effects is to directly manipulate the diversity in the worker force. This experimental approach has so far been restricted to the study of the effect of worker diversity on pathogen resistance in bumble bees [20] and ants [23]. Here, we experimentally increased worker diversity in small colonies of the black garden ant *Lasius niger*, while controlling for potential maternal effects. We produced colonies composed of workers from either one (low diversity) or three (high diversity) source colonies. These experimental colonies were then provided with a single, unrelated queen, and brood production was monitored over time. We found an increased brood production in experimental colonies with a more diverse worker force, thus showing that worker diversity enhances colony performance.

Results

The experimental increase in worker diversity enhanced the production of larvae, but not eggs

To measure a potential effect of worker diversity on offspring production we monitored the number of eggs and larvae in experimental colonies with low (control) and high (treatment) worker diversity. The change over time in the number of eggs recorded in the experimental colonies did not differ between control ($n = 23$) and treatment ($n = 18$) colonies ($p \geq 0.1$ for all parameters; Table 1; Fig. 1). Consistently, we could not detect any effect of treatment on the maximum number of eggs recorded in the colonies (ANOVA: $\chi^2 = 1.03$, $p = 0.31$; Additional file 1: Figure S1A).

The change over time in the number of larvae recorded in the experimental colonies differed significantly between control and treatment colonies (Table 2; Fig. 2). Specifically, we found that colonies with higher worker diversity reached a higher horizontal asymptote by the end of the experiment (*Asym*, $t = 2.56$, $p = 0.011$; Table 2; Fig. 2), and showed a higher logistic growth rate (*scal*, $t = 2$, $p = 0.046$; Table 2; Fig. 2). We did not detect any effect of worker diversity on the timing of the logistic growth (*xmid*, $t = 0.94$, $p = 0.35$; Table 2; Fig. 2).

In one control colony, no larvae were produced throughout the experiment (Fig. 2). To ensure that the effect of worker diversity on larva production did not stem from this colony only, we repeated the analysis after excluding this colony and found qualitatively similar results, with worker diversity still influencing the level of the asymptote reached at the end of the experiment (*Asym*, $t = 2.36$, $p = 0.018$, *xmid*, $t = 0.77$, $p = 0.44$, *scal*, $t = 1.87$, $p = 0.061$). We also found that the

Table 1 Parameters of the models for egg production over time in the control (n = 23) and treatment (n = 18) colonies

Parameter	Estimate control (± se)	Estimate treatment (± se)	t-value	p-value
a	3.23 ± 0.78	2.95 ± 1.17	−0.24	0.81
b	0.40 ± 0.07	0.12 ± 0.11	1.10	0.27
c	−0.03 ± 0	−0.006 ± 0	−1.52	0.13
d	0.0006 ± 0	0.0001 ± 0	1.63	0.10
e	−0.000004 ± 0	−0.000001 ± 0	−1.56	0.12

The models are based on a quintic function $y = a * x + b * x^2 + c * x^3 + d * x^4 + e * x^5$. y stands for the number of eggs, x is the number of days after setup, and a, b, c, d and e are the parameters estimated by the models

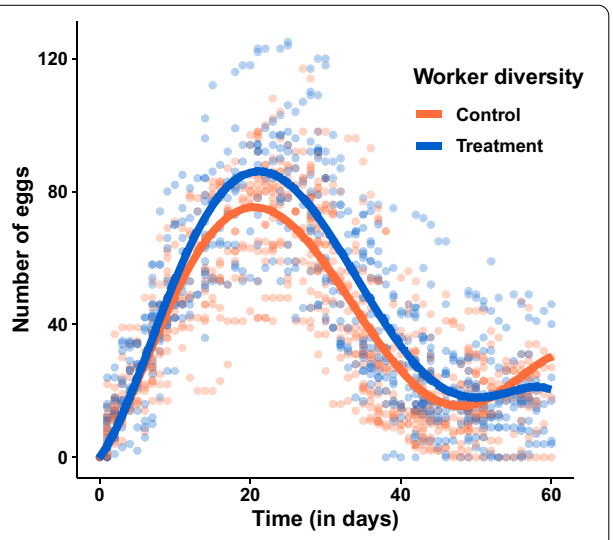

Fig. 1 Number of eggs in control (n = 23) and treatment (n = 18) colonies over time. The dots show the raw data for all colonies and time points. The curves depict the output of the models for control (orange) and treatment (blue) colonies. None of the parameters of the quintic function differed significantly between control and treatment colonies (all $p \geq 0.1$; Table 1)

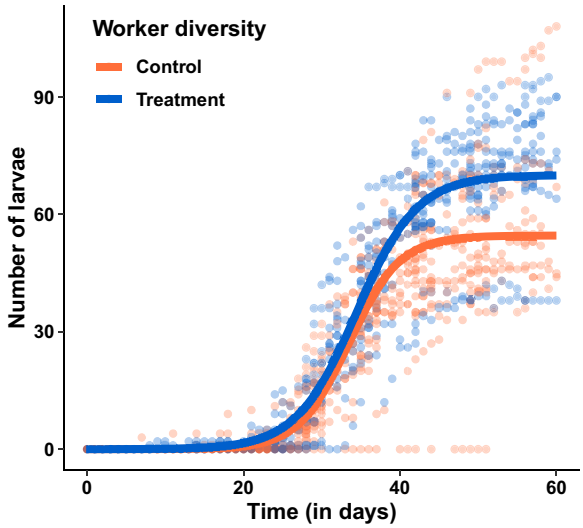

Fig. 2 Number of larvae in control (n = 23) and treatment (n = 18) colonies over time. The dots show the raw data for all colonies and time points. The curves depict the output of the models for control (orange) and treatment (blue) colonies. Two parameters of the logistic growth function differed significantly between control and treatment colonies (*Asym*, t = 2.56, p = 0.011; *scal*, t = 2, p = 0.046; Table 2)

Table 2 Parameters of the models for larva production over time in the control (n = 23) and treatment (n = 18) colonies

Parameter	Estimate control (± se)	Estimate treatment (± se)	t-value	p-value
Asym	54.6 ± 4.0	70.0 ± 6.0	2.56	0.011
xmid	33.5 ± 0.7	34.5 ± 1.0	0.94	0.349
scal	3.24 ± 0.2	3.9 ± 0.3	2.00	0.046

The models are based on a logistic growth function $y = \frac{Asym}{1+e^{\frac{xmid-x}{scal}}}$. y stands for the number of larvae, x is the number of days after setup, and the parameters estimated by the models are the asymptote (Asym), the timing of the growth (xmid) and the rate of the growth (scal)

maximum number of larvae recorded in each experimental colony was higher in treatment than in control colonies (ANOVA: $\chi^2 = 4.87$, p = 0.027, Additional file 1: Figure S1B).

Experimental increase in worker diversity did not affect foraging

To investigate whether the beneficial effect of worker diversity on the production of larvae stemmed from improved foraging efficiency, we submitted the experimental colonies to a foraging test. We could not show that control and treatment colonies differed in the maximum number of workers at the food (ANOVA: $\chi^2 = 2.05$, p = 0.15, Fig. 3A), the proportion of maximum workers at the food (ANOVA: $\chi^2 = 1.03$, p = 0.31), or the time for the first worker to reach the food (ANOVA: $\chi^2 = 0.11$, p = 0.74, Fig. 3B).

Experimental increase in worker diversity enhanced variation in body size

To better understand the positive influence of worker diversity on larva production, we conducted further

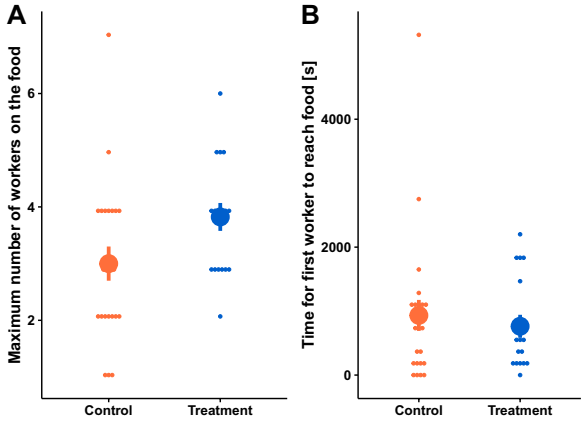

Fig. 3 Foraging did not differ between control (n = 23) and treatment colonies (n = 17). The large dots depict the mean ± standard error. The small dots represent the raw data points. We did not detect any significant difference between control and treatment colonies in (**A**) the maximum number of workers (ANOVA: $\chi^2 = 2.05$, $p = 0.15$) and (**B**) the time for the first worker to reach the food (ANOVA: $\chi^2 = 0.11$, $p = 0.74$)

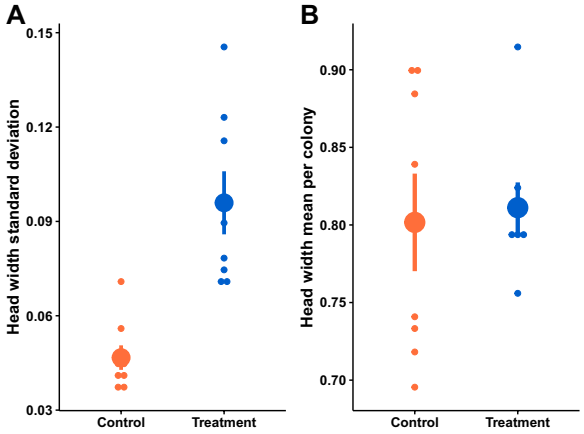

Fig. 4 Control (n = 8) and treatment (n = 8) colonies differed in the standard deviation, but not the average, in head width (measured in mm). The large dots depict the mean ± standard error. The small dots represent the raw data points. The experimental increase in worker diversity resulted in (**A**) a higher within-colony variation in head width (ANOVA: $F_{1,14} = 26.42$, $p < 0.001$) but (**B**) no difference in the average head width per colony (ANOVA: $F_{1,14} = 0.099$, $p = 0.76$)

analyses on the workers that emerged from the pupae that were used to set up the experimental colonies.

We extracted the standard deviation in head width for each experimental colony to find that this measure of body size variation differed between control and treatment colonies, as experimental colonies with higher worker diversity showed higher variation (ANOVA: $F_{1,14} = 26.42$, $p < 0.001$, Fig. 4A). However, we did not find such an effect of worker diversity on the average mean head width per colony (ANOVA: $F_{1,14} = 0.099$, $p = 0.76$, Fig. 4B).

The number of workers that emerged from the pupae used to set up the experimental colonies varied among colonies (20.1 ± 5.4, mean ± sd), but did not differ between control and treatment colonies (ANOVA: $\chi^2 = 1.83$, $p = 0.18$). Furthermore, we found that the standard deviation in the time needed for the workers to emerge from the pupae was larger in colonies with higher worker diversity (ANOVA: $\chi^2 = 8.7$, $p = 0.003$). We could not detect such an effect of worker diversity on the average time until worker emergence (ANOVA: $\chi^2 = 0.003$, $p = 0.96$).

Discussion

The aim of this study was to test the effect of worker diversity on colony performance in eusocial insects. To do so, we experimentally increased worker diversity of small *Lasius niger* colonies by combining workers from three different source colonies and compared them to colonies composed of workers from a single source colony. In addition, we provided the experimental colonies with unrelated queens to disentangle any effects of worker diversity from maternal effects. We found that increased worker diversity enhanced the production of larvae but not of eggs.

Our finding that worker diversity enhanced larva production in small, experimentally produced laboratory colonies is consistent with previous reports of benefits provided by higher genetic diversity in other species of eusocial Hymenoptera [19, 24, 38, 51, 58–61], a phenomenon termed 'social heterosis' [66]. In the honey bee *Apis mellifera*, decreased genetic diversity by artificial insemination, or restricted natural mating was shown to result in lower productivity and fitness [24, 51, 61]. Similarly, in bumble bees (*Bombus terrrestris*) higher genetic variance was shown to decrease parasite load and enhance reproductive success [20, 59, 60]. Our study adds to previous reports because we found benefits of experimentally increasing worker diversity in a species with lower natural levels of diversity, while controlling for any maternal effects caused by the experimental manipulation. Our study validates the hypothesis that worker diversity positively affects larva production, and possibly colony performance.

Worker diversity may improve colony performance via a more efficient division of labor [44]. Behavioral division of labor among workers likely stems from workers differing in their response thresholds to perform specific tasks [34]. In our study, the experimental colonies produced with workers from three source colonies were more diverse than the control colonies in terms of genetic

background and size variation. Genetic effects on worker size and morphology have been reported in multiple species of ants [67–69] and worker size differs among colonies in *L. niger* [70]. Worker size and genetic background influence the response threshold of individual workers [30, 67, 71–73], and worker size polymorphism is generally associated with improved division of labor [74–76] (but there were contradictory results [77, 78]). In our study, worker emergence in high diversity colonies was more spread over time, possibly improving division of labor via a broader age distribution [79]. Overall, a more diverse worker force would have resulted in a more heterogeneous mix of response thresholds, possibly enhancing the efficiency of division of labor among workers [38–41, 80–82].

The beneficial effect of worker diversity on the production of larvae likely stemmed from more efficient brood care. We detected an increase in the number of larvae, but not in the number of eggs, and we could not detect any effect on foraging. This suggests that increased worker diversity improved the survival and/or development of larvae, but probably not via better food provisioning to the colony. It may be that the actual distribution of food to the brood was improved by increased worker diversity or that our experiment failed to detect a difference in foraging because of the low number of foragers. However, if more diverse colonies were better at foraging for food, we would have expected the better nourished queens in those colonies to produce more eggs. Our results do not support this expectation. Another explanation that could explain our inability to detect a difference in egg number is egg cannibalism by the larvae, which is common in eusocial Hymenoptera [2, 83, 84]. More diverse colonies had more larvae, which in turn may have eaten more eggs compared to low diversity colonies. According to this scenario, the number of eggs would differ between more and less diverse colonies before the first larva emerged from the eggs. This was not the case, as the maximum number of eggs—which was reached before larvae appeared—was not affected by worker diversity. Our findings are more consistent with worker diversity improving brood care, although we do not have direct evidence for such an effect.

Another, non-mutually exclusive explanation for the enhanced brood production is that more diverse experimental colonies were better at resisting diseases and parasites. This is supported by studies in wasps, bees and ants that reported a positive association between genetic diversity and pathogen resistance [19–27].

A minor proportion of previous studies did not detect an association between diversity and colony performance [51, 61, 62, 64, 65, 81, 82, 85] or had ambiguous findings [58], including in *L. niger* field colonies [49, 65]. Discrepancies among studies suggest that the effect of diversity on division of labor and colony fitness is context- and/or species-dependent. In our study, we used an experimental approach to control for confounding factors and study the benefits of worker diversity in small experimental colonies with young queens, thus in conditions that resemble the early stage of a colony life. Newly founded colonies are very vulnerable and subject to strong competition, and only a small proportion of founding queens manage to establish colonies [2, 86, 87]. Our findings indicate that in this context, increasing worker diversity enhances brood production, which may provide a competitive advantage and increase the chances of a successful colony foundation [87].

The experimental colonies in the high diversity treatment were composed of workers produced by different queens. This situation resembles founding colonies established by several cooperating, unrelated founding queens. This process, called pleometrosis, has been described in multiple ant species, including *L. niger* [88–93]. Pleometrosis increases and accelerates brood production [88, 90, 91, 93–96], which is consistent with our finding that higher worker diversity enhances the production of larvae. Furthermore, workers of young *L. niger* colonies may raid and steal the brood from other colonies in the founding stage [88–91, 93]. Such social parasitism could increase worker diversity in a similar manner to the experimental manipulations conducted in our study, and could similarly benefit colony growth and performance.

We did not manipulate genetic diversity directly, but combined workers from multiple source colonies to produce diversity in the worker force. We confirmed that our experimental manipulation increased size variation in the more diverse colonies. Such size diversity could stem from genetic differences across colonies, but could also be explained by environmental and maternal effects [67, 68, 70, 97]. The genetic background affects size and morphology in eusocial insects [67–69], thus one strategy to increase worker size diversity is to increase genetic diversity. Additionally, we found that the high diversity colonies showed a higher variance in the time of worker emergence from the pupae used to set up the experimental colonies. This result is consistent with the higher variation in worker size, as size and developmental time are correlated [98, 99]. The variation in size and developmental time could also be explained by genetic or source colony effects, as well as other indirect effects of the social context experienced as larvae [100].

So far, evidence for benefits of worker diversity in eusocial insects came from correlative studies, experimental studies where low diversity was also the unnatural situation and/or where other confounding factors such as maternal effects could have played a role. In this study,

we experimentally increased worker diversity and controlled for maternal effects. We found that increased worker diversity improved larva production, possibly via enhanced division of labor. Our findings confirm that increased diversity can benefit colony performance in some situations, which could have led to the evolution in some eusocial insects of multiply mated queens and multiple-queen colonies [5, 11, 56, 101].

Methods

Lasius niger as a study system

To manipulate genetic diversity in the worker force, we used the black garden ant *L. niger* to experimentally combine workers from one or three source colonies and provided them with an unrelated queen. *L. niger* colonies have a single queen, which in Northwestern Europe is usually mated with a single male, leading to highly relatedness among workers [102]. Queens in this species can also be mated twice or more, but mostly in other geographic regions [18, 65]. Established colonies in the field are large, with as much as 10,000 workers [103], which makes it easy to collect large quantities of brood. After their nuptial flights in summer, hundreds of young mated queens can easily be collected as they roam on the ground looking for a nest site [103].

Collection and housing

We collected 44 *L. niger* queens after their nuptial flight on July 10th 2019 on the campus of Johannes Gutenberg University of Mainz, Germany. One day after collection, we transferred each queen to a glass tube half filled with water blocked by cotton and closed with another piece of cotton. Then, we kept the queens in darkness at 21 °C and approximately 80% humidity and without food, as *L. niger* founding queens do not feed [104]. These queens had produced a first cohort of at least five workers by the time the experiments began.

We collected workers and brood from nine different *L. niger* colonies in the area around the Opel Arena stadium in Mainz, Germany between October and December 2019. The species was identified according to Seifert [105]. In the laboratory, we relocated workers and brood from the soil into glass tubes with water blocked by cotton and covered with aluminum foil. Workers and brood from the same colony were stored in closed boxes ($31 \times 22 \times 5$ cm) coated with fluon in a climate cabinet at 28 °C and approximately 100% humidity, and fed five times a week with frozen crickets and a mixture of honey, eggs and vitamins [106]. At the time of collection, these colonies (hereafter referred to as "source colonies") contained 682 ± 414 (mean $\pm$ sd) larvae. We regularly checked the source colonies for pupae to be used for the setup of experimental colonies. All source colonies contributed to both types of experimental colonies (Additional file 1: Table S1).

Setup of experimental colonies

To manipulate worker diversity, we grouped workers produced by either one or multiple source colonies. Because *L. niger* workers are aggressive towards workers from other colonies, we combined pupae, rather than adult workers, from one or multiple source colonies. The workers that later emerged from those pupae produced the experimental colonies used in this study.

The low diversity experimental colonies were produced by combining 30 pupae from a single source colony and are thus referred to as "control" colonies. We produced the high diversity experimental colonies (hereafter referred to as "treatment" colonies) by combining 30 pupae from three different source colonies (10 pupae per colony). For each experimental colony, we combined the 30 pupae with one unrelated, founding queen and five of its workers (hereafter referred to as "chaperones") to care for the pupae. For each experimental colony, we removed those chaperones, as well as all the eggs present at the time, once three workers had emerged from the pupae. This day was considered as day 0 in the analysis. Most workers that composed the experimental colonies survived until the end of the monitoring ($94.8\% \pm 8\%$, mean $\pm$ sd), and survival did not differ between control and treatment colonies (Wilcoxon test: $W = 214$, $p = 0.85$).

We kept the experimental colonies in closed plastic boxes ($11 \times 15 \times 3$ cm) coated with fluon, which contained a glass tube filled with water and cotton as a nest and water source, and a small petri dish for food. We fed the experimental colonies twice a week with a mixture of honey, eggs and vitamins [106]. From day 0 to day 2, we kept the experimental colonies in a dark climate cabinet at approximately 28 °C and 100% humidity. On day 3, we moved the experimental colonies to a climate chamber at 21 °C and approximately 80% humidity and in dark conditions.

In total, we set up 43 colonies. We excluded two treatment colonies from our monitoring because no workers emerged or survived the experimental setup. This resulted in 23 control and 18 treatment colonies in the analysis.

Brood production monitoring

In each experimental colony, we monitored brood production by counting the number of eggs, larvae and pupae five times a week for 70 days after colony setup. Because the experimental colonies varied in the time to reach day 0 (9 ± 6.6, median $\pm$ sd), we only kept the time points between day 0 and day 60 in the analysis to ensure

that at any given time point, more than half the experimental colonies were monitored. By the end of the monitoring, only 14 out of 41 colonies had pupae, and even those had a low number of pupae (1.9 ± 2, mean $\pm$ sd), and no workers had emerged from the eggs produced in the experimental colonies. Thus, we restricted our analysis of brood production to the production of eggs and larvae.

Foraging assays

We performed the foraging assays 28 days after the last pupa was observed in the experimental colonies to limit age differences across colonies. One treatment colony was not tested because it still contained two pupae at the end of the experiment. Five days prior to the foraging assays, we removed the food to increase the motivation of workers to forage. We performed the foraging assays inside the box of the experimental colonies by placing a small petri dish with a small cotton roll soaked with a honey solution (0.5 ml honey in 1 ml water). Then we observed the colonies for two hours to score the maximum number of workers observed at the food source at any given time point and to record the time when the first worker arrived at the food.

Body size measurements

At the end of the experiments, all workers from the experimental colonies were frozen at −18 °C for later morphological measurements. To estimate body size, we measured the width of the worker heads as the distance between the outer points of the eyes [78, 85, 107, 108]. The frozen workers were placed flatly on modeling clay, photographed with a Leica S9i microscope, and measured with LAS V4.12 Leica computer software. We measured all 326 workers that survived the experiment in eight control (151 workers) and eight treatment (175 workers) colonies, while making sure that we only used one experimental colony per source colony.

Statistical analysis

To test whether control and treatment colonies differed in brood production over time, we built non-linear mixed effect models with the R package *nlme* [109].

To model the egg production, we used the quintic function

$$y = a*x + b*x^2 + c*x^3 + d*x^4 + e*x^5$$

where y is the number of eggs, x is the number of days, and the parameters a, b, c, d and e are estimated by the model to provide the best fit to the empirical data.

To model the larva production, we used a logistic growth equation with the SSlogis() function

$$y = \frac{Asym}{1 + e^{\frac{xmid - x}{scal}}}$$

where y is the number of larvae, x is the number of days, and the parameters *Asym*, *xmid* and *scal* are estimated by the model to provide the best fit to the empirical data. *Asym* represents the horizontal asymptote of the logistic growth function, *xmid* the x value of the sigmoid's midpoint, and *scal* the rate of the logistic growth. The starting values for the parameters were obtained by fitting non-linear models without random effects using the nls() function. For both eggs and larvae, the non-linear mixed effects models were fitted using the function nlme(), and included the worker diversity (control or treatment) as fixed effect and the experimental colony as random effect. The summary() function was used to extract the estimate for each parameter and each treatment, as well as to test whether estimates differed between treatments. In addition to the non-linear modeling of the change in brood number over time, we used the simpler approach of extracting the maximum number of eggs and larvae recorded in each colony during the experiment. This allowed us to confirm that any effect that would be detected by the non-linear models would not merely stem from the source colony not being included as random effect in the models. We then tested the effect of worker diversity on the maximum numbers of brood using the lmer() function with the package *lme4* [110] to fit a linear mixed effects model with worker diversity as fixed effect and source colony as random effect.

We tested the effect of worker diversity on the maximum number of foragers at the food source, as well as the square root transformed data of the time for the first worker to reach the food by building a linear mixed effect model using the lmer() function, with worker diversity as fixed effect and source colony as random effect.

To test whether there was a difference in the size of workers used to set up the control and treatment colonies, we calculated the square root of the average head width per colony. To investigate the effect of worker diversity on size variation, we extracted the standard deviation of head size in each colony. Then we built linear models with the lm() function to explain both measurements by worker diversity.

We tested the effect of worker diversity on the number of workers that emerged from the pupae used to set up the experimental colonies, and the standard deviation and mean of the time needed for the workers to emerge with linear mixed effect models using the

lmer() function, with worker diversity as fixed effect and source colony as random effect. We checked all linear models for normal distribution of the residuals and used the Anova() function of the package *car* [111] to test the effect of the explanatory variables. We produced all plots with the packages *ggplot2* [112] and *ggpubr* [113], and used the package *dplyr* [114] for data handling. We ran all analyses in R [115] version 3.6.1. The R script is provided in Additional file 2, and all data in Additional file 3.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12862-021-01890-x.

Additional file 1. Figure S1. Maximum number of brood items in the different treatments. **Table S1**. Number of control and treatment colonies that have been created by the respective source colonies.

Additional file 2. The R script for all statistical analyses.

Additional file 3. All data generated and analyzed in this study.

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Authors' contributions
Conceptualization: RL; methodology and investigation: DG and RL; analysis: MP and RL; writing: MP, DG and RL; resources and supervision: RL. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in Additional file 3.

Declarations

Ethics approval and consent to participate
The research project was conducted on invertebrate species that are not subjected to any specific ethical issue and legislation.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

Appendix 8

Fecundity determines the outcome of founding queen associations in ants

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Fecundity determines the outcome of founding queen associations in ants

Eva-Maria Teggers, Falk Deegener & Romain Libbrecht✉

Animal cooperation evolved because of its benefits to the cooperators. Pleometrosis in ants—the cooperation of queens to found a colony—benefits colony growth, but also incurs costs for some of the cooperators because only one queen usually survives the association. While several traits in queens influence queen survival, they tend to be confounded and it is unclear which factor specifically determines the outcome of pleometrosis. In this study, we used the ant *Lasius niger* to monitor offspring production in colonies founded by one or two queens. Then, we experimentally paired queens that differed in fecundity but not in size, and vice versa, to disentangle the effect of these factors on queen survival. Finally, we investigated how fecundity and size differed between queens depending on whether they were chosen as pleometrotic partners. Our results indicate that pleometrosis increased and accelerated worker production via a nutritional boost to the larvae. The most fecund queens more frequently survived the associations, even when controlling for size and worker parentage, and queens selected as pleometrotic partners were less fecund. Our results are consistent with fecundity being central to the onset and outcome of pleometrosis, a classic example of cooperation among unrelated animals.

While animal cooperation typically occurs among related individuals[1], there are cases of unrelated cooperators in some mammal, bird and insect species[2]. These have raised interesting questions on the origin, maintenance and benefits of cooperation among unrelated animals. Such questions include how the internal (e.g., physiology) and external (e.g., environment) conditions affect the decision to behave cooperatively, the benefits of cooperation, and its outcome. While social insects are typical model systems for kin-based cooperation, the early stage of the colony life of some ant species provides a classic example of cooperation among unrelated partners[2].

Pleometrosis in ants is the foundation of colonies by two or more cooperating newly-mated queens that settle in the same nest site after a nuptial flight[3–6]. In some cases, pleometrosis results in mature multiple-queen colonies, as in pleometrotic populations of the harvester ant *Pogonomyrmex californicus*[7,8]. However, in most species, pleometrotic associations are only transitory: the queens stay together to produce eggs, but upon worker emergence, queens engage in fights (sometimes initiated and/or joined by the workers) until a single queen survives[4]. Pleometrosis results in an earlier production of larger groups of workers compared to solitary foundation, which may be beneficial in dense populations where young colonies compete for food and brood raiding can be common[3,7,9–12]. Whether pleometrosis merely increases egg production or also provides additional benefits in term of brood development or immunity remains unclear[13,14].

The queens that do not survive the pleometrotic associations have no fitness benefits, as they die before producing sexuals (the fertile individuals that are only produced in mature colonies). Thus, understanding the factors that determine which queens survive the associations would allow researchers to draw and test hypotheses on whether or not queens with given characteristics should decide to cooperate with other queens, as well as interpret previous reports of such queen decisions[3,15].

Several determinants of queen survival in pleometrotic associations have been identified. Surviving queens tend to be larger[16–19], heavier[15,18], to spend more time on the brood pile[20,21], and to lose less weight during colony foundation[17,20]. However, these reports may have been subject to confounding effects because the factors tested are usually interconnected. For example, body size and mass may correlate with fecundity[16], which in turn influences the number of workers produced and therefore the distribution of parentages in the offspring (although there is no evidence that workers favour their own mother[16,17,20]). One strategy to control for such confounded factors is to experimentally pair queens that differ in one factor but not in others, thus allowing the identification of factors that affect queen survival irrespective of others.

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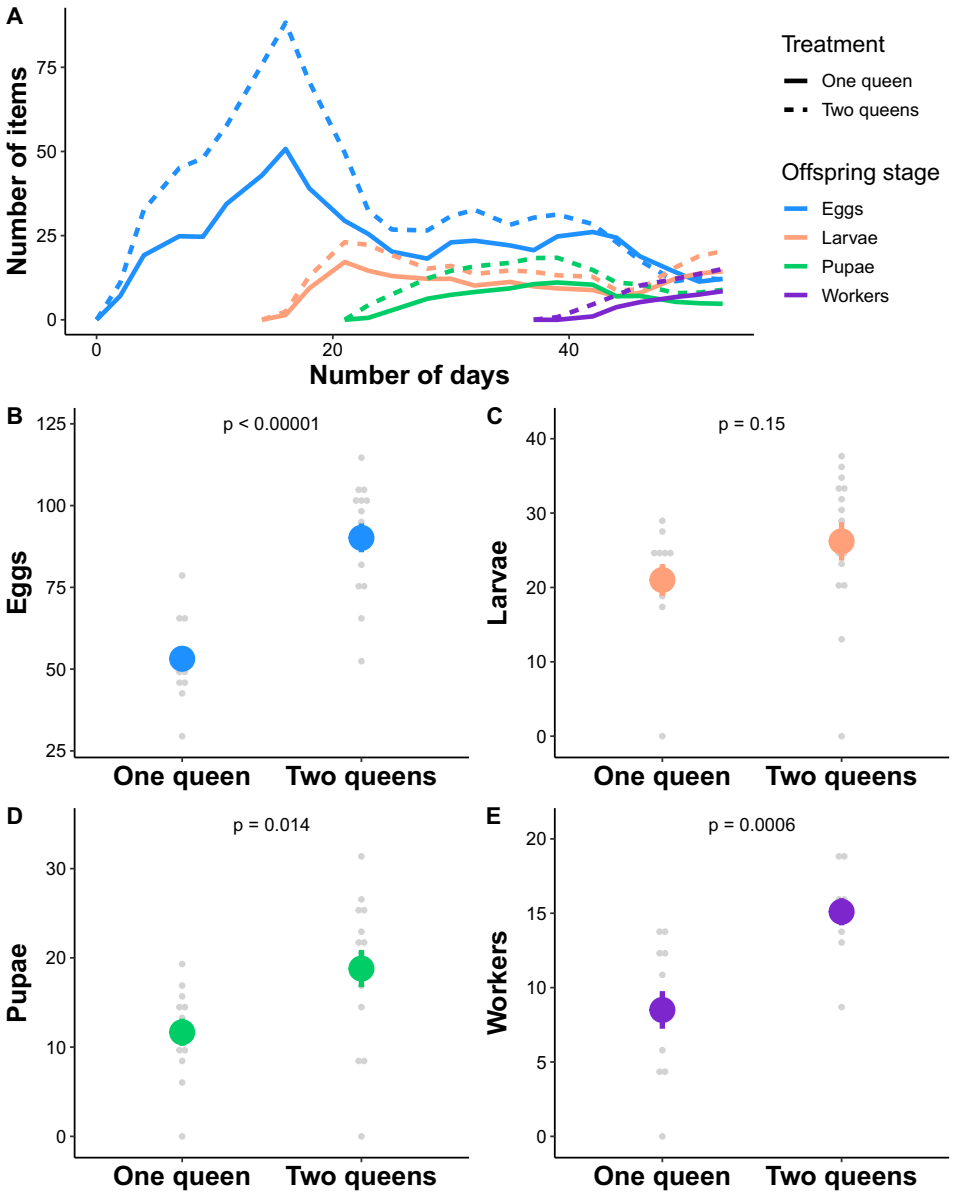

Figure 1. Effect of queen number on (**A**) mean offspring number over time since colony foundation (colors denote the brood type, while line type indicates treatment), maximum number of (**B**) eggs, (**C**) larvae, (**D**) pupae recorded in the colonies, and (**E**) number of workers at the end of the experiment (mean ± standard error).

In this study, we used the black garden ant *Lasius niger* to study cooperation among unrelated individuals in pleometrotic associations. We first quantified the benefits of pleometrosis in terms of brood production and development. Then we tested whether queen fecundity and body size, two factors that may be associated with queen survival[16–18,20] were correlated. We investigated the effect of fecundity and size on queen survival in experimental pleometrotic associations, in which paired queens differed in one trait but not the other, while controlling for worker parentage. Finally, we compared fecundity and size between queens that were selected as pleometrotic partners and queens that were not.

Results

Effect of pleometrosis on offspring production. To investigate the effect of pleometrosis on offspring production, we set up experimental colonies with one or two *L. niger* founding queens. Our comparisons of the number of eggs, larvae, pupae and workers produced in these experimental colonies revealed benefits of pleometrosis (Fig. 1A). We found that pleometrosis increased the maximum number of eggs (ANOVA: $F_{1,25} = 39.18$, $P < 0.00001$; Fig. 1B) and pupae (ANOVA: $F_{1,25} = 6.93$, $P = 0.014$; Fig. 1D) recorded in the colonies, as well as the number of workers produced (ANOVA: $F_{1,20} = 16.62$, $P = 0.0006$; Fig. 1E). However, our analysis did not detect such an effect for the maximum number of larvae (ANOVA: $F_{1,25} = 2.25$, $P = 0.15$; Fig. 1C).

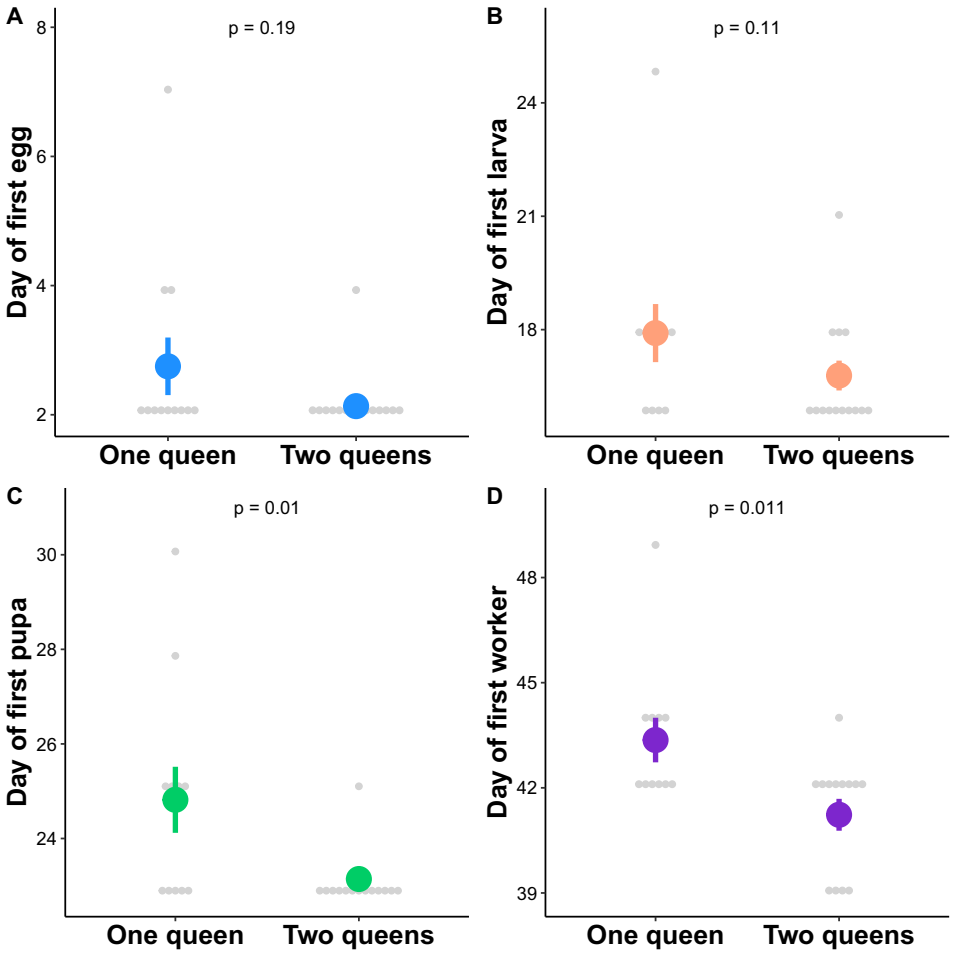

Figure 2. Effect of queen number on the day (mean ± s.e.) when the first (**A**) eggs, (**B**) larvae, (**C**) pupae, and (**D**) workers were recorded in the colonies.

The benefits of pleometrosis disappeared when correcting for the number of queens present in the colonies. The *per capita* numbers of eggs and larvae were actually reduced in colonies with two queens (ANOVA for eggs: $F_{1,25} = 3.97$, $P = 0.057$; ANOVA for larvae: $F_{1,25} = 10.94$, $P = 0.0029$), and we found no effect of pleometrosis on the numbers of pupae and workers per queen (ANOVA for pupae: $F_{1,25} = 1.60$, $P = 0.22$; ANOVA for workers: $F_{1,20} = 0.43$, $P = 0.52$).

We did not detect any effect of queen number on the time to produce the first egg (Wilcoxon test: $W = 107$, $P = 0.19$; Fig. 2A) and larva (Wilcoxon test: $W = 103$, $P = 0.11$; Fig. 2B), but colonies with two queens were significantly faster than those with one queen in producing their first pupa (Wilcoxon test: $W = 114.5$, $P = 0.01$; Fig. 2C) and worker (Wilcoxon test: $W = 111$, $P = 0.011$; Fig. 2D).

Finally, once the first workers emerged, colonies with two queens showed a faster increase in worker number than colonies with a single queen (ANOVA: $F_{1,171.4} = 44.6$, $P < 0.00001$; Fig. 3).

Association between fecundity and size. To identify the factors that influence queen survival in pleometrotic associations requires the decoupling of candidate variables, such as fecundity and size. We found a weak but significant, positive correlation between thorax length and brood number in *L. niger* founding queens (ANOVA: adjusted $R^2 = 0.034$, $F_{1,229} = 9.13$, $P = 0.0028$). This result confirms that the effects of size and fecundity can be confounded, but suggests that it is possible to disentangle them experimentally.

Effect of fecundity and size on queen survival. To investigate the effect of fecundity and size on queen survival, we produced experimental pairs of *L. niger* queens that differed in one factor, but not the other. Among the 35 pairs of queens that differed in fecundity (but not size), seven pairs still had two queens at the end of the experiment. Out of the remaining 28 pairs that lost a queen, the most fecund queen survived in 75% (21 out of 28) of the cases, which represented a significant departure from 50% (exact binomial test, $P = 0.012$; Fig. 4A). Among the 33 pairs of queens that differed in size (but not fecundity), five pairs still had two queens at the end of the experiment, and in one additional case, the two queens died on the same day. Out of the remaining 27 pairs

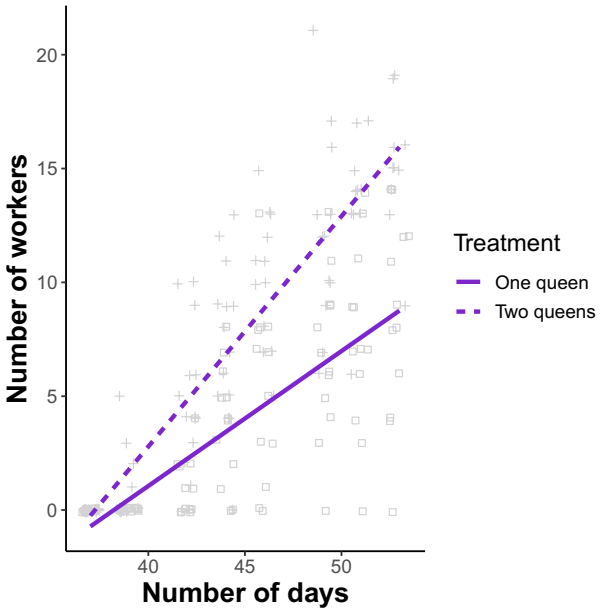

Figure 3. The number of workers increased faster in colonies with two queens (crosses, dotted regression line, slope = 1.01, $R^2 = 0.77$) compared to those with one queen (squares, solid regression line, slope = 0.59, $R^2 = 0.53$) (ANOVA: $F_{1,171.4} = 44.6$, $P < 0.00001$).

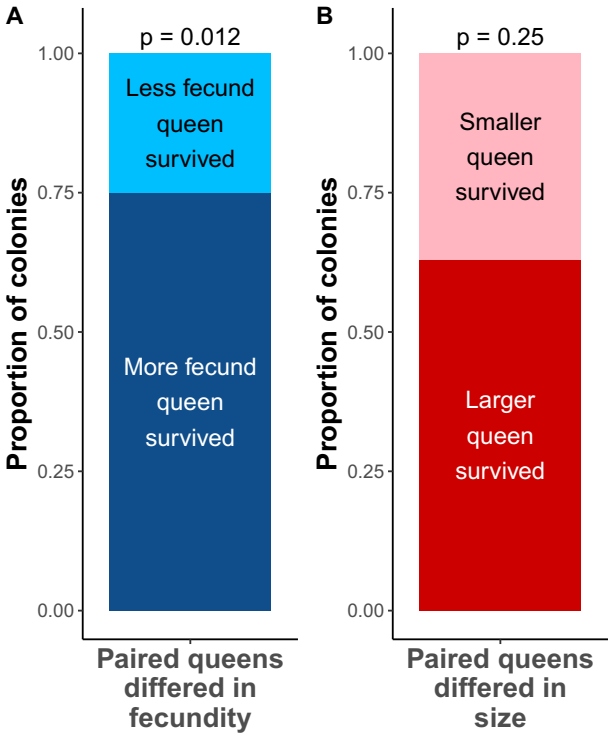

Figure 4. Likelihood of surviving the pleometrotic association depending on queen (**A**) fecundity (n = 28) and (**B**) size (n = 27). *P* values come from binomial tests.

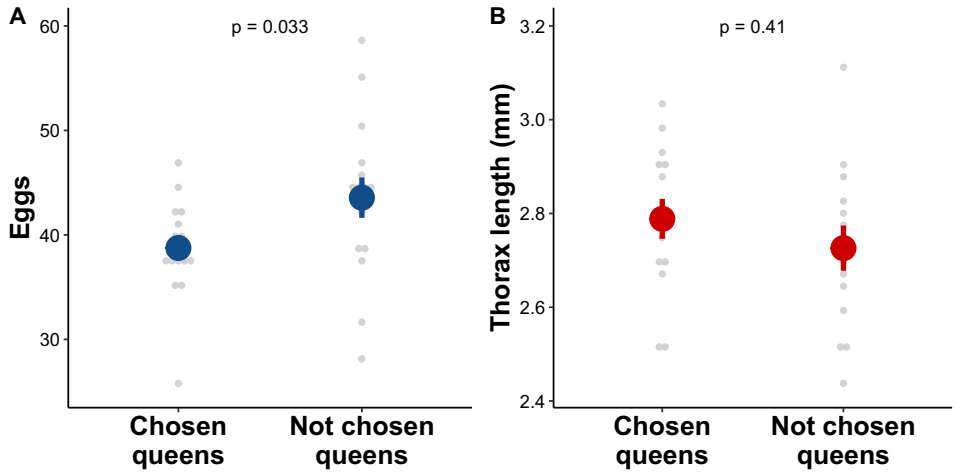

Figure 5. Difference between queens that were chosen and not chosen in the choice tests in (**A**) maximum number of eggs recorded and (**B**) thorax length (mean ± s.e.).

that lost a queen, the largest queen survived in 63% (17 out of 27) of the cases, which did not differ significantly from 50% (exact binomial test, $P = 0.25$; Fig. 4B).

Difference between chosen and not chosen queens in fecundity and size. To study the traits associated with partner selection in pleometrotic *L. niger* queens, we provided founding queens with the choice between two, randomly selected queens. Then we compared the brood production and size of chosen and not chosen queens. We found that the maximum number of eggs recorded was lower for queens that were previously chosen as pleometrotic partners compared to queens that were not chosen (ANOVA: $\chi^2 = 4.56$, $P = 0.033$; Fig. 5A). We could not find such difference for the maximum number of larvae (ANOVA: $\chi^2 = 0.41$, $P = 0.52$) and pupae (ANOVA: $\chi^2 = 0$, $P = 1$). In addition, we could not detect any difference in thorax length between chosen and not chosen queens (ANOVA: $\chi^2 = 0.66$, $P = 0.41$; Fig. 5B).

Discussion

In this study, we used the black garden ant *Lasius niger* to investigate the benefits and factors of pleometrosis, the transitory association between founding queens. The monitoring of colonies founded by one or two queens showed that pleometrosis increased and accelerated offspring production. Then, the experimental pairing of *L. niger* founding queens revealed that in pairs of queens of different fecundity but similar size, the most fecund queen was more likely to survive. Our experiment could not detect a similar effect of size when controlling for fecundity. Finally, we found that queens associated preferentially with less fecund queens.

Our findings of pleometrosis benefiting offspring production are in line with the literature for this, and other ant species[3,7,9,10,12,22,23]. Interestingly, we only detected these benefits at the colony level, as pleometrosis had either no effect or a negative influence on the *per capita* offspring production[9,12,22]. However, colony-level measurements are more relevant in the case of pleometrosis, as the queen that survives the association inherits all the offspring produced during colony foundation. In the field, colonies with a faster, more efficient worker production would have a competitive advantage over neighbouring founding colonies[3,4]. This is especially true for *L. niger*, which shows high density of founding colonies that compete for limiting resources and raid the brood of other colonies[10]. Thus, the competitive advantage provided by pleometrosis likely enhances colony growth and survival.

The increased and faster production of workers in colonies with two queens may stem from a nutritional boost for the larvae. *L. niger* founding queens do not forage, and produce the first cohort of workers from their own metabolic reserves. Larvae have been observed to cannibalize both viable and non-viable (trophic) eggs[24]. We found that colonies with two queens produced more eggs, but that this did not translate in them having more larvae. However, more of these larvae became pupae—and ultimately workers. In addition, while the time to produce the first egg and larva did not differ between colonies with one and two queens, the first pupa and worker were produced faster when two queens were present, consistent with a shorter larval stage. We propose that larvae in pleometrotic colonies developed faster and were more likely to reach pupation because they had more eggs that provided nutrients, boosting the development rate of the first workers.

These benefits of pleometrosis are only inherited by the queens that survive, it is thus important to understand the factors that determine queen survival in pleometrotic associations. Although this question has been relatively well studied[3,16–21], it has remained challenging to disentangle the effects of correlated factors. For example, we found that size, which has been reported to predict queen survival[16,19], correlated with fecundity, which would itself be confounded with the parentage of workers in the first cohort produced. To address this issue, we disentangled size and fecundity experimentally, and used foreign workers that developed from pupae collected in field colonies to prevent any potential nepotistic behaviour.

We found that fecundity, but not size, determined queen survival. The finding that, despite being of similar size, more fecund queens are more likely to survive indicates that the outcome of pleometrosis is not the mere consequence of physical dominance. The higher fecundity could reflect a better health condition, which may give the advantage to the more fecund queen in direct fights[3,15], or if workers initiate the fights. Natural selection may have favoured workers that skew aggression toward the less fecund queen, both because this queen would be less efficient at building a colony, and because the workers would be more likely to be the offspring of the more fecund queen. The latter would not necessarily involve direct nepotistic behaviours (the workers would not behave according to parentage, but to fecundity), which have remained elusive in social insects in general[25–27], and in pleometrotic associations in particular[16,17,20]. Despite regular behavioural observations, we did not observe who initiated aggression in our experiments, and it remains unclear whether the queens and/or the workers are responsible for the onset of fights. Consistently with previous studies[16,23], we found that a certain proportion of queen death occurred before worker emergence, suggesting that worker presence is not required for queen execution. Finally, we cannot rule out that the least fecund queens were more likely to die because of a weaker health status, possibly combined with the stress of being associated with another, healthier queen.

Although it has not been directly reported before, our finding that fecundity determines queen survival is consistent with previous reports of weight being associated with queen survival[17], more fecund queens being more aggressive[28], cuticular hydrocarbon profiles differing between surviving and culled queens[21], and between more and less fecund queens[28]. We could not directly support previous reports of size correlating with survival[16,19]. This could be because in those studies, size could have been confounded with fecundity, and/or because we lacked the statistical power to detect such effect in our experiment.

Pleometrosis provides clear benefits, but these benefits are only inherited by the surviving queens, and the losing queens pay the great cost of dying without contributing to the next generation. Natural selection should thus favour queens that decide whether or not to join a pleometrotic association based on the relative benefits compared to individual foundation—these may differ across ecological contexts[29]—and the likelihood of surviving the association. As fecundity appears to determine queen survival in *L. niger*, queens may have evolved the ability to choose among potential partners according to their fecundity. Our results are consistent with this hypothesis, as queens preferentially associated with partners that would later produce fewer eggs, possibly because they were less fecund, and therefore less healthy and easier to eliminate. This suggests that founding queens may assess the fecundity of potential partners, possibly via their cuticular hydrocarbon profile[28]. This result further supports our finding that fecundity plays an important role in pleometrotic associations. It is important to note that this difference in egg production could have alternative explanations. First, it could stem from more fecund queens having no interest in forming an association because they are able to start a competitive colony alone. Second, it could be a consequence, rather than a cause, of the outcome of the choice experiment. We cannot rule out that entering an association with another queen and/or leaving this association prematurely at the end of the choice experiment may have been stressful for the chosen queens, and affected their later production of eggs. We could not detect any difference between chosen and not chosen queens in the number of larvae and pupae produced, which are likely influenced by factors other than fecundity (e.g., brood care behaviour). Interestingly, we did not find that queens chose according to size, consistent with our finding that size may not affect which queen survives the pleometrotic association.

Our study informs on the benefits and factors of pleometrosis, and highlights the role of fecundity in the decision to associate with another queen, and in determining which queen survives the association. As such, it contributes to a better understanding of the onset and outcome of pleometrosis, a classic case of cooperation between unrelated animals.

Methods

Sample collection. We used *L. niger* as study organism because queens in this species commonly found new colonies in pleometrotic associations that consist of typically unrelated individuals[3]. We collected 411 *L. niger* founding queens in Mainz, Germany right after nuptial flights on June 26th 2017 (cohort 1, 138 queens), July 9th 2017 (cohort 2, 138 queens) and July 4th 2020 (cohort 3, 135 queens). All founding queens had shed their wings at the time of collection. The queens were housed individually in glass test tubes (10 × 1.2 cm) with water blocked by a cotton ball, and then kept in the dark at 21 °C.

Experimental designs. We randomly selected 45 queens from cohort 2 to investigate the benefits of pleometrosis. Half the test tubes contained a single queen (n = 15), and the other half contained two queens (n = 15). Then we recorded the number of eggs, larvae, pupae and workers three times a week for 53 days. Upon emergence of the first workers, we placed the tubes in larger plastic boxes (11 × 15 × 3 cm) and fed the colonies once a week with a mixture of honey, eggs, agar and water[30]. Eight queens died over the course of the experiment: three queens in the single-queen treatment died four, 11 and 16 days after set-up, and were removed from the analyses. Five queens in the two-queen treatment died 37, 42 (two associations) and 49 (two associations) days after set-up. In each of those five cases, one queen of the two queens survived. These tubes were kept in the analyses because queen execution is expected in pleometrotic associations.

We used 231 queens (138 from cohort 1, 93 from cohort 2) to investigate the effect of size and fecundity on queen survival. On day 17 after the nuptial flights, we recorded the number of brood items (eggs and larvae) in each tube, and used this measure as a proxy for fecundity, thus assuming that egg production changes similarly over time across queens. The number of brood items equalled the number of eggs for most of the queens (152/231), as they had only produced eggs at this point. On days 19 and 20 after the nuptial flights, we used a Leica stereomicroscope to measure the thorax length of the founding queens, and used it as a proxy for size in all subsequent analyses.

To test how difference in fecundity between queens affects the outcome of the pleometrotic association, we experimentally paired 70 paint-marked queens (35 pairs) that differed in the number of brood items (high fecundity: 65.5 ± 7.8, mean ± sd; low fecundity: 30.5 ± 11.6; one sample t-test against zero, t = − 15.1, df = 34, $P < 0.0001$), but not in thorax length (high fecundity: 3.2 mm ± 0.4; low fecundity: 3.2 mm ± 0.4; one sample t-test against zero, t = 0.59, df = 34, $P = 0.56$). To do so, we paired queens that had a high number of brood items (top 25% of the distribution) with queens that had a low number of brood items (bottom 25% of the distribution, excluding the queens that did not produce any eggs), while making sure that they had a medium size (both in the middle 50% of the distribution). We used a similar strategy to investigate how size difference between queens affects their survival, and paired 66 paint-marked queens (33 pairs) that differed in size (large: 3.2 mm ± 0.4; small: 2.8 mm ± 0.3; one sample t-test against zero, $t = -18.15$, $df = 32$, $P < 0.0001$) but not in fecundity (large: 49.4 ± 8.9; small: 49.2 ± 9.7; one sample t-test against zero, $t = -0.36$, $df = 32$, $P = 0.72$). Before being paired, each queen was marked with a color dot on the thorax using a toothpick dipped into Edding marker paint. Although there is no evidence that workers favour their own mother in pleometrotic associations[16,17,20], we wanted to prevent worker parentage from being confounded with queen fecundity. Thus, we did not provide the queen pairs with their own brood, but with 25 pupae from a pool of pupae collected in field colonies around Mainz, Germany.

We used 135 queens (cohort 3) to investigate how fecundity and size correlate with the likelihood of being chosen as a pleometrotic partner. To do so, we first performed choice test experiments on the three days following the nuptial flight. We used test arenas that consisted of two plastic tubes (length: 4 cm) covered with red foil, and connected them on opposite sides to a plastic petri dish (diameter: 5 cm). We tethered a queen to each tube by attaching one end of a metal wire (length: ca. 1 cm; diameter: 0.02 mm) to its petiole and the other end to the bottom of the tube. We then introduced a choosing queen in the central petri dish, which could move freely into both tubes, and interact with the queens attached to the bottom of each tube. This set-up provided the choosing queen with two possible pleometrotic partners. We used Sony FDR-AX33 video cameras to record the arenas for 12 h after introduction of the choosing queen. We tested 45 groups of three queens. For each test, we randomly selected one of the three queens to be the choosing queen. We used a set of three criteria to consider that a choosing queen had made a choice: (1) it should be observed inside one of the two tubes at the end of the video (12 h after its introduction to the arena), (2) it should not have left the tube for at least one hour before the end of the video, and (3) it should be observed in the same tube the next day (24 h after its introduction to the arena). In 27 choice tests (out of 45), no decision was made because the choosing queen did not settle in one of the two tubes, or because one of the choice queens escaped their metal wire. Thus in the remaining 18 tests, the choosing queen made a decision, which provided us with 18 chosen queens and 18 not chosen queens. These queens were then kept individually at 21 °C, and monitored once a week to count the number of eggs, larvae and pupae present in their tube. The monitoring stopped when the first worker emerged or 80 days after the choice test (12 queens did not produce workers by that time). We used a Leica stereomicroscope to measure the thorax length of the queens at the end of the experiment. Two queens died during monitoring, and were removed from the analysis, as well as the other queens from the same test arena, resulting in 16 chosen and 16 not chosen queens in the analysis.

Statistics. To investigate the effect of the number of queens on absolute and per capita offspring production, we have extracted for each colony the highest number of brood items recorded for each brood type, as well as the number of workers at the end of the experiment, and compared those values between single-queen and two-queen colonies using type II ANOVAs on linear models. To investigate the effect of queen number on the timing of offspring production and development, we compared the days when the first egg, larva, pupa and worker were recorded between colonies with one and two queens using non-parametric Wilcoxon tests. To further investigate the effect of queen number on worker production, we focused on the days after the first workers were produced, and conducted a type II ANOVA on a mixed-effect linear model with the number of workers as response variable, time and number of queens as fixed response variable, and queen identity as a random effect. To investigate the association between size and fecundity in *L. niger* founding queens, we conducted a type II ANOVA on a linear model that explained the number of brood items with thorax length (values were standardized within queen collection cohorts for both measurements). To test whether size and fecundity determined queen survival, we compared proportion of surviving queens to 50% using exact binomial tests. To investigate whether chosen and not chosen queens differed in brood production, we extracted for each queen the maximum number of brood items recorded for each brood type, and conducted a type II ANOVA on a mixed-effect linear model with the maximum number of brood items as response variable, queen category as a fixed response variable, and test arena as a random effect. We performed the same analysis to test the effect of queen category on thorax length. All linear models built in this study were checked for normal distribution of the residuals.

Data availability

All data is accessible as supplementary material.

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Appendix 9

Ontogeny of superorganisms: Social control of queen specialization in ants

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RESEARCH ARTICLE

Ontogeny of superorganisms: Social control of queen specialization in ants

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Abstract

1. The functioning of biological systems relies on the cooperation of specialized components and understanding the processes that produce such specialization is a major challenge in biology. Here, we study the ontogeny of biological systems at a new phenotypic level: the superorganisms (i.e. insect societies with specialized individuals).
2. We investigate how founding queens, the earliest developmental stage of ant colonies, transition from expressing behavioural pluripotency to becoming strictly specialized in egg production.
3. We demonstrate that the presence of workers both initiates and maintains this queen specialization, and propose that such a social control of queen behaviour is common in ants and regulated by ancestral mechanisms.
4. These findings contradict the traditional view of social insect queens as being intrinsically specialized in egg production and may reshape our understanding of the division of labour in insect societies.

KEYWORDS
behavioural plasticity, brood care, colony foundation, division of labour, juvenile hormone, major evolutionary transitions, social behaviour, social insects

1 | INTRODUCTION

A central question in life sciences is to understand the evolution and functioning of biological systems that are constituted by specialized components. Typical examples of such biological systems include multicellular organisms that are composed of specialized cells and insect societies that are composed of specialized individuals (Szathmáry & Smith, 1995). Social insect colonies (also called superorganisms) are analogous to multicellular organisms in that they have queens that monopolize reproduction (similar to germ cells), and functionally sterile workers that perform all non-reproductive tasks and thus act as somatic cells (Boomsma & Gawne, 2018; Wheeler, 1911). Both types of biological systems evolved from solitary and non-specialized ancestors in major evolutionary transitions (Szathmáry & Smith, 1995): multicellular organisms from unicellular organisms and insect societies from solitary insects. Interestingly, in both cases, the specialization also needs to be established in every generation during the ontogeny of these biological systems (i.e. the

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developmental process that produces the self-assembly and specialization of their components).

In this context, it is important to understand the factors and mechanisms underlying the emergence and maintenance of specialization and reproductive division of labour in social insect colonies. The many investigations that tackled this question can be separated in three broad categories. First, studies of caste determination and differentiation uncovered critical information on the regulation of alternative developmental trajectories that lead to the production of queens and workers (Ashby et al., 2016; Cameron et al., 2013; Collins et al., 2020; Corona et al., 2016; Genzoni et al., 2023; Libbrecht et al., 2011; Libbrecht, Corona, et al., 2013; Libbrecht, Oxley, et al., 2013; Montagna et al., 2015; Mutti et al., 2011; Psalti & Libbrecht, 2020; Schultner et al., 2023; Schwander et al., 2008; Schwander & Keller, 2008; Wheeler et al., 2006). Second, studies comparing adult queens and workers in mature colonies revealed a suite of caste-specific phenotypic and molecular differences (Bonasio et al., 2012; Chandra et al., 2018; Corona et al., 2007, 2013; Feldmeyer et al., 2014; Grozinger et al., 2007; Kronauer & Libbrecht, 2018; Libbrecht, Oxley, et al., 2013; Patalano et al., 2015). Third, there have been investigations of condition-dependent plasticity in the reproductive activity of workers (Amarasinghe et al., 2014; Holman, 2014; Holman et al., 2016; Libbrecht et al., 2016, 2018; Macedo et al., 2016; Negroni et al., 2021; Ronai et al., 2016; Ulrich et al., 2016). Overall, studies of reproductive division of labour between queens and workers primarily aimed to understand the development and functioning of queens as egg-laying machines, the worker-specific expression of a variety of non-reproductive behaviours, and the plasticity of worker reproduction. In contrast, there is limited research on the regulation of non-reproductive behaviours in queens (Chouvenc, 2022; Woodard et al., 2013).

By reducing queens to their reproductive activity, studies of division of labour amplified the notion, also predominant in popular science, that queens are intrinsically specialized in egg production once they reach the adult stage and that this robust specialization does not depend on environmental conditions. However, the maturation process of social insect queens is not completed by the time they emerge as adults. This is best exemplified by the behaviour of queens in the process of colony foundation, especially in the many species of social insects where mated queens found their colony independently (Peeters, 2020). These pluripotent founding queens are not specialized in egg production yet, as they express a broad repertoire of both reproductive and non-reproductive behaviours to produce the first workers (Augustin et al., 2011; Brossette et al., 2019; Cassill, 2002; Haskins & Haskins, 1955; Helms Cahan & Fewell, 2004; Jeanson & Fewell, 2008; Norman et al., 2016; Rissing & Pollock, 1986; Rüppell et al., 2002; Walsh et al., 2018; Wheeler, 1932, 1933; Woodard et al., 2013). For example, while workers provide brood care in mature colonies, founding queens need to groom and feed the larvae during the founding stage (Schultner et al., 2017). Founding queens may also dig the first chamber of the nest (Haskins & Haskins, 1955; Helms Cahan & Fewell, 2004; Rissing & Pollock, 1986) or cultivate the mutualistic fungus in leaf-cutting ant species (Augustin et al., 2011). It is only once the colonies are established (i.e. they contain workers) that the queens stop expressing non-reproductive behaviours and become strictly specialized in egg production (Augustin et al., 2011; Chouvenc, 2022; Hölldobler & Wilson, 1990; Wilson, 1971; Woodard et al., 2013).

While the process of queen specialization is central to the ontogeny of superorganisms, the factors and mechanisms that control the specialization of pluripotent founding queens remain poorly understood. It is also unclear whether queen specialization is condition-dependent, and whether queens become permanently specialized once the colonies are established (i.e. they lose the ability to express non-reproductive behaviours). In this study, we aimed to address these questions by investigating queen specialization in ants. We showed that the presence of workers was necessary and sufficient to inhibit brood care behaviour in founding queens—and thus initiate their specialization in egg production—and we identified potential mechanisms underlying these behavioural modifications. We found the queen specialization to be reversible and dependent on the social environment, as queens reverted to expressing brood care upon the experimental removal of their workers in two ant species. This continuous social regulation of queen specialization contradicts the prevailing notion of ant queens as intrinsically specialized egg-laying machines.

2 | METHODS

2.1 | General procedures

To investigate the factors and mechanisms that control the queen specialization in egg production, we conducted a series of experiments using the black garden ant *Lasius niger* as our main study system. In this section, we describe the general procedures that apply to all experiments regarding the collection and keeping conditions of queens, the use of workers for experimental manipulation of worker presence, the experimental setup and behavioural observations, as well as the statistical analyses.

2.1.1 | Founding queen collection and keeping conditions

All *Lasius niger* founding queens used in the experiments were collected after their nuptial flights in 2017, 2018, 2019 and 2020 around Mainz and Ingelheim, Germany (Table S1 in Supporting Information). After collecting them in fluon-coated boxes containing humid paper towel, we transferred the founding queens individually into glass tubes (10 cm length × 1 cm diameter) half filled with water blocked by cotton and plugged with another piece of cotton. The tubes were placed in boxes (18 cm × 12 cm × 7 cm) kept in darkness. Most queens were kept in a climate chamber at

21°C and 80% humidity. Founding queens were not provided with food, since *L. niger* queens found their colonies without foraging (claustral colony founding; Janet, 1907; Keller & Passera, 1989; Peeters, 2020). Once the first workers emerged, each tube containing the queen, brood and workers was transferred into a small plastic box (15 cm × 11 cm × 12 cm) with walls coated with fluon to prevent the ants from escaping. These colonies were also kept in a climate chamber at 21°C and 80% humidity, but fed every second week with a drop of honey, one frozen cricket and a piece of artificial food with a 2:1 (carbohydrate: protein) ratio (Dussutour & Simpson, 2008). Every winter, the established colonies were hibernated for 3–4 months at 5°C. They entered and left hibernation via a 2-week gradual decrease and increase in temperature, respectively. Table S1 provides information on queen collection, keeping conditions and sample sizes in all experiments. This study did not require ethics approval.

2.1.2 | Experimental manipulations of worker presence

To experimentally provide workers to founding queens, we used 'callow' workers (Errard, 1984; Julian & Fewell, 2004; O'donnell, 1998; Psalti et al., 2021; Stuart, 1988; Stuart & Page, 1991; Teggers et al., 2021; Woodard et al., 2013). We sampled brood from *L. niger* field colonies and kept it in laboratory colonies that we monitored regularly to collect workers that recently emerged from the pupae (<12 h). These callow workers did not elicit aggression from foreign individuals and were readily accepted by founding queens, possibly because they did not possess the signature chemical profile of their own colony yet (Dahbi et al., 1998; Isingrini et al., 1985; Signorotti et al., 2014). Callow workers were easily recognizable due to their light grey colour. The laboratory colonies used for callow worker production were kept between 21°C and 28°C, and between 80% and 100% humidity, depending on the timing and availability of climate cabinets. In cases where callow workers were kept at a different temperature than the temperature of the experiment, they were moved to the room of the experiment at least 1 h before it started.

2.1.3 | Experimental setup

All behavioural analyses were conducted based on observations of videos that were recorded for 1 h with cameras standing ca. 50 cm over the observation arenas. Two white LED light bars illuminated the arenas approximately 70 cm above the observation arenas. One camera recorded one tray, which could contain up to 12 observation arenas simultaneously (Figure S1). Whenever multiple cameras were used simultaneously, the recordings belonged to the same batch. All experiments were filmed in a climate chamber at 21°C and 80% humidity, with 12 h/12 h light/dark cycle. The observation arenas consisted of airtight petri dishes (50 mm diameter, 9 mm height; Falcon) half filled with moistened, and blue plaster for better contrast and visibility (Figure S2). We used soft forceps and brushes to manipulate the ants. We ensured that all treatments of an experiment were represented and equally distributed on each tray. We assigned random numbers to the observation arenas to ensure that the experimenters were blind to the treatment during the experiments and video analyses.

2.1.4 | Behavioural analyses

To assess the behavioural specialization of queens, we observed their brood care behaviour towards larvae. We defined a brood care event as an active manipulation of the brood, with frequent contacts with the antennae and touches with the front legs. We also counted as a brood care event the placement of a brood item into a different position. However, merely carrying the brood item in the mouth parts was not scored as brood care. In the experiments 'Worker emergence' and 'Worker presence and feeding status', we counted the total number of brood care events and divided it by the duration of the videos. In all other experiments, we scored the brood care behaviour via scan sampling. We watched the first 10 s of every minute of each video and recorded the presence or absence of brood care behaviour. We performed 51 such scans per 1 h video, as we did not analyse the first and last 5 min of the videos to avoid potential disturbances due to the experimental manipulations. The videos were analysed using the software BORIS (version 8.0.5) or Pot player (version 1.7.21212).

2.1.5 | Statistical analyses

We performed all statistical analyses (Appendix S1) using R v. 4.0.4 and RStudio v. 1.4.1106. Table S2 provides the input data, syntax and outputs of all statistical models. We used the lm() command from R base to build linear models, the lmer() command from the *lme4* package (Bates et al., 2015) for linear mixed-effect models, the nls() command from R base for non-linear regressions, and the glmer() command from the *lme4* package to build generalized linear mixed-effect models. We used the lsmeans() command from the *emmeans* package (Lenth et al., 2019) for post hoc pairwise comparisons. To test the effect of the response variables, we either used the summary() command from R base or an ANOVA with the Anova() function of the *car* package (Fox & Weisberg, 2019). Whenever needed, we used a square root transformation to ensure that the residuals of the models followed a normal distribution. Table S2 provides for each experiment the fixed and random variables that were included in the models. We included time as a fixed variable in the analyses of all the experiments that involved the collection of data at multiple time points. Whenever the analyses could not detect an interaction between time and the treatment of interest, we pooled the numbers of scans with brood care over the multiple time points. Whenever the analyses could detect

an interaction between time and the treatment of interest, we investigated the effect of time separately for the different levels of the treatment variable (Table S2).

2.2 | Detailed experimental descriptions

In this section, we provide context for each experiment, as well as experiment-specific information that deviates from the general procedures. Table S1 provides an overview of the collection and keeping conditions of all queens.

2.2.1 | Brood production of founding queens

To verify that *L. niger* founding queens raise their first cohort of workers independently, we collected newly mated queens right after their nuptial flight and transferred them to closed, individual glass tubes. We used 24 founding queens collected in July 2019 on the campus of the Johannes Gutenberg University of Mainz, Germany (from now on referred to as JGU Mainz). We used a stereomicroscope to count for each queen the number of eggs, larvae, pupae and workers three times per week for 93 days after the nuptial flight.

2.2.2 | Effect of worker emergence

To confirm that *L. niger* founding queens express brood care behaviour, we used 12 founding queens collected in July 2017 on the campus of the JGU Mainz. We kept the queens in their collection boxes with humid paper towel for 7 days before transferring them into individual glass tubes. The queens were filmed inside their tubes with all their brood for 15 min before worker emergence (35 days after the nuptial flight), and after worker emergence (56 days after the nuptial flight).

2.2.3 | Effect of experimental manipulations of worker presence and feeding status

To experimentally manipulate worker presence and feeding status, we used 54 founding queens collected in June 2018 near the campus of the JGU Mainz. The queens were kept in glass tubes at 25°C and approximately 80% humidity with a 12 h/12 h light/dark cycle. Just before the start of the experiment, all pupae were removed to prevent the emergence of workers during the experiment (all eggs and larvae were left in the tubes). The experiment started 30 days after collection. The queens were divided into four treatments: (i) queens were fed and were given five callow workers ($n = 14$), (ii) queens were fed and received no workers ($n = 11$), (iii) queens were not fed and were given five callow workers ($n = 15$) and (iv) queens were not fed and received no workers ($n = 14$). For the feeding treatment, queens were hand-fed for 5–10 min with artificial food with a 2:1 (carbohydrate: protein) ratio (Dussutour & Simpson, 2008). We filmed the queens in their tubes for at least 30 min twice a day (morning and afternoon) over seven consecutive days (14 time points per queen).

2.2.4 | Effect of worker removal on established queens that had workers for 3 days

To test whether the presence of workers maintains queen specialization, we first used 48 founding queens collected in July 2019 in three locations in and around Mainz, Germany (Mainz-Bretzenheim, Mainz-Marienborn and campus of the JGU Mainz). The experiment started 55 days after collection, and all pupae were removed once 37 days after collection to ensure that no workers emerged before the start of the experiment. We assigned the queens to three different treatments: (i) queens with five larvae and five added callow workers ($n = 16$), (ii) queens with five larvae and without workers ($n = 16$) and (iii) queens with five larvae that formerly had five callow workers (workers removed; $n = 15$). In the 'workers removed' treatment, queens had been given five callow workers in their glass tubes 3 days prior to the experimental setup when the workers were removed, and all tubes were filmed just before the experimental setup. Then, the queens were filmed in the observation arenas 24 and 48 h after the experimental setup.

2.2.5 | Effect of worker removal on established queens that had workers for 2 years and 6 months

To further investigate the role of worker presence in maintaining queen specialization, we used 12 colonies that were established in the laboratory by founding queens collected in July 2017 on the campus of the JGU Mainz. The experiment started 932 days after collection. All colonies were treated the same way. First, we filmed each queen with five of its larvae and five of its workers to allow direct observations of the queen behaviour and standardize worker numbers across colonies. Then, we removed the last five workers, and filmed the queens 24 and 48 h after the experimental setup.

2.2.6 | Effect of worker removal on established queens that had workers for 3 years and 2 months

To complete our investigations of whether the presence of workers maintains queen specialization, we used 24 colonies that were established in the laboratory by founding queens collected in July 2017 on the campus of the JGU Mainz. The experiment started 1162 days after collection. The experimental setup consisted of one queen, five of its larvae and five of its workers in observation arenas. Then, we gradually removed one worker per day over the course of 6 days, until reaching zero workers. We filmed the observation arenas every day and removed the workers after each video recording.

2.2.7 | Effect of workers in unmated queens

To test whether mating is necessary for queens to become specialized in the presence of workers, we collected 27 unmated queens from field colonies in June and July 2021 near the campus of the JGU Mainz, and kept them at 24°C and 80% humidity in darkness. The experiment started right after collection. Each unmated queen was observed with five foreign larvae and either five ($n = 14$) or zero ($n = 13$) callow workers. We filmed the unmated queens six and 24 h after the experimental setup.

2.2.8 | Effect of worker number

To investigate whether the effect of workers on queen specialization is dose-dependent, we used 76 founding queens collected in July 2019 on the JGU campus. The experiment started 35 days after collection. We set up one queen with five larvae and either zero ($n = 14$), one ($n = 18$), two ($n = 15$), three ($n = 13$) or five ($n = 16$) callow workers. We recorded the queens 24, 48 and 72 h after the start of the experiment. A similar experiment was conducted using 80 founding queens collected in July 2020 in several locations in Mainz, Germany (Marienborn, Mainz-Hechtsheim and Mainz-Oberstadt). This second experiment started 47 days after collection. For the last experimental setups of this second experiment, all pupae were removed to ensure that no workers naturally emerged before the experiment was completed. The queens were provided with 15 larvae and either zero ($n = 16$), one ($n = 16$), two ($n = 16$), three ($n = 16$) or five ($n = 16$) callow workers, and were also filmed 24, 48 and 72 h after the experimental setup.

2.2.9 | Effect of worker cuticular hydrocarbons (CHC)

To test the effect of worker CHC on the brood care behaviour of founding queens, we used 36 queens collected in July 2019 on the campus of the JGU Mainz. The experiment started 40 days after collection. We produced five CHC extracts, each from 100 workers collected in a field colony around Mainz, Germany. The workers were sedated with CO_2, transferred into glass vials and immersed in *n*-hexane 10 min while occasionally swaying the vials. The liquid was transferred to a micro insert and completely exhausted under a gentle nitrogen stream. The CHC were then re-dissolved in 50 μL of hexane and stored at 4°C until used in the experiments. We applied either 10 μL of the worker CHC extracts (treatment) or 10 μL of pure *n*-hexane (control) on glass beads (~1.5 mm, Roth GmbH). Five glass beads were placed on a bowl made of aluminium foil (5 mm diameter) to ensure that the CHC extracts would not soak into the plaster below. One queen and five larvae were placed in each observation arena, together with either treatment ($n = 18$) or control ($n = 18$) beads. We filmed the observation arenas 3, 6 and 24 h after the experimental setup.

2.2.10 | Effect of former presence of workers

To investigate the effect of the presence of worker cues on queen specialization, we first used 36 founding queens collected in July 2019 on the campus of the JGU Mainz. The experiment started 42 days after collection. Each queen was placed together with five larvae in an observation arena that used to contain 20 field-collected workers for 48 h (workers removed just before the start of the experiment, $n = 18$) or in a clean observation arena ($n = 18$). We filmed the observation arenas six and 24 h after the experimental setup.

2.2.11 | Effect of the presence of dead workers

To further test whether cues of worker presence affect queen specialization, we then used 54 founding queens collected in July 2020 in Ingelheim and Mainz, Germany. The experiment started 65 days after collection. The tubes containing the queens were regularly checked prior to the experiment to remove any pupae; thus, ensuring that no workers had emerged before the experimental setup. We assigned the queens to four treatments: one queen with five larvae and (i) five dead workers ($n = 18$), (ii) five living workers ($n = 18$) and (iii) without any workers ($n = 18$). Dead workers were obtained by placing 90 workers at −80°C for 2 h, and then at −20°C for 24 h before the experimental setup. Freshly killed, frozen ants show a similar CHC profile as living ants (Wilson et al., 1958). We recorded the observation arenas 2, 6 and 24 h after the experimental setup.

2.2.12 | Effect of workers separated by a wire mesh

To investigate whether the queen brood care behaviour changed in response to workers separated from the queen and brood by a wire mesh, we used 43 founding queens collected in July 2020 in Ingelheim and Mainz, Germany. The experiment started 52 days after collection. The tubes containing the queens were regularly checked prior to the experiment to remove any pupae; thus, ensuring that no workers had emerged before the experimental setup. In this experimental setup, we modified the observation arenas by adding a wire mesh cylinder (0.2 mm thick, 0.2 mm mesh size and 2 cm cylinder diameter) in the middle of the arena (Figure S3). The wire mesh enabled antennation between the queen and the separated workers, but the workers had no close physical interactions with either the queen or the larvae. We assigned the queens to the following treatments: one queen and five larvae were placed outside the wire circle and the three callow workers were placed (i) on the inside of the circle, thus separated from the queen and brood (workers separated; $n = 13$), or (ii) on the outside of the circle together with the queen and brood (workers present; $n = 14$) or (iii) without any workers (workers absent; $n = 16$). We filmed the observation arenas 2, 6 and 24 h after the experimental setup.

2.2.13 | Effect of methoprene and precocene I and II

To investigate the potential implication of the juvenile hormone (JH) pathway in the regulation of brood care in queens, we subjected founding queens to treatments with a JH analogue (methoprene) or with chemical inhibitors of JH production (precocene I and II). We used 160 queens collected in July 2020 in Ingelheim, Mainz-Bretzenheim and the city centre of Mainz, Germany. The experiment started 52 days after collection for the methoprene experiment and 66 days after collection for the precocene I and II experiment. For the methoprene experiment, queens were treated with (i) methoprene at 1.5 µg/µL (PESTANAL®, a commonly used JH analogue (Henrick, 2007); $n = 35$) or (ii) acetone (solvent; $n = 35$). For the precocene I and II experiment, queens were treated with (i) precocene I at 1.5 µg/µL (Sigma; JH inhibitor; $n = 30$), (ii) precocene II at 1.5 µg/µL (Cayman; JH inhibitor; $n = 30$) or (iii) acetone (solvent; $n = 30$). To treat the queens, we attached them to an eraser with a fishing line and applied 1 µL of the treatment on their thorax with a glass pipette. Each queen was treated every morning for four consecutive days. In the first 2 days, we recorded the observation arenas without workers. On the third day, before the application of the treatments, we added five callow workers to half of the queens and kept them with these workers for another 2 days. The observation arenas were recorded between 2 and 6 h after each treatment.

2.2.14 | Effect of larvae on egg production in founding and established queens

To test the influence of brood presence on egg production in founding and established queens, we used 66 *L. niger* queens collected in July 2019 on the campus of the JGU Mainz. The queens were distributed into the following treatments: (i) founding queens with larvae ($n = 18$), (ii) founding queens without larvae ($n = 18$), (iii) established queens with larvae ($n = 15$) and (iv) established queens without larvae ($n = 15$). Founding queens were queens that had not yet produced pupae or workers and were tested 8 days after the nuptial flight. Established queens were queens that had produced at least five workers and were tested 82 days after the nuptial flight. To test the effect of larvae, we used five larvae collected in field colonies near the campus of the JGU Mainz. We recorded the number of eggs once a day for five consecutive days.

2.2.15 | Effect of worker removal on established *Temnothorax nylanderi* queens

To investigate whether the presence of workers induces the queen specialization in another species of ants, we collected 21 *T. nylanderi* colonies in April 2021 in the Lenneberg forest of Mainz, Germany. In the laboratory, each colony was transferred into plastered nest boxes containing an artificial nest consisting of a Plexiglas perimeter (3 mm high) with an entrance, sandwiched between two microscope slides (7.5 cm × 2.5 cm × 0.5 cm). The colonies were kept according to the general procedures and were fed twice a week with a drop of honey, half a cricket and water. The experiment started 2 weeks after collection. The treatments consisted of queens with five larvae and (i) five of their own workers ($n = 11$) and (ii) no workers ($n = 10$). We used CO_2 to anaesthetise and transferred the ants into the observation arenas. We filmed the observation arenas every 24 h for five consecutive days.

3 | RESULTS

3.1 | Queens become specialized after the emergence of the first workers

To verify that *L. niger* founding queens produce workers independently, we collected newly mated queens right after their nuptial flight and monitored brood production and development over the next 93 days (Figure S4). 91.67% of the queens (22/24) survived the experiment, and 77.28% of the surviving queens (17/22) produced workers within the observation time (5.26 ± 3.95 workers; mean ± sd; Figure S4). This indicates that founding queens provide care to the brood, as ant larvae cannot develop independently (Schultner et al., 2017). We confirmed this by direct observations of brood care behaviour (defined as active manipulation of the brood) in founding queens before and after worker emergence. We found that founding queens expressed brood care before they produced the first workers, but that the emergence of workers was associated with a sharp decrease in queen brood care behaviour ($\chi^2 = 35.63$, $p < 0.0001$, Figure 1a). Therefore, the emergence of workers correlates with the behavioural specialization of queens in *L. niger*.

3.2 | The presence of workers triggers the specialization of queens

The emergence of workers is confounded with the age and nutrition of queens, as established queens—defined as queens with workers—are older and fed by their workers. To disentangle the effects of age, nutrition and worker presence, we manipulated worker presence and feeding status of same-age founding queens that had not produced workers yet, and quantified their brood care behaviour for 7 days. We detected an interaction between time and worker presence ($\chi^2 = 17.24$, $p < 0.0001$), as queens with workers showed a stronger decrease in brood care over time ($\chi^2 = 49.69$, $p < 0.0001$, Figure 1b) than queens without workers ($\chi^2 = 8.99$, $p = 0.0027$, Figure 1b). In addition, we found very strong evidence that queens with workers performed less brood care overall than queens without workers ($\chi^2 = 596.33$, $p < 0.0001$, Figure 1b). This inhibition of brood care by workers was already detected 20 h

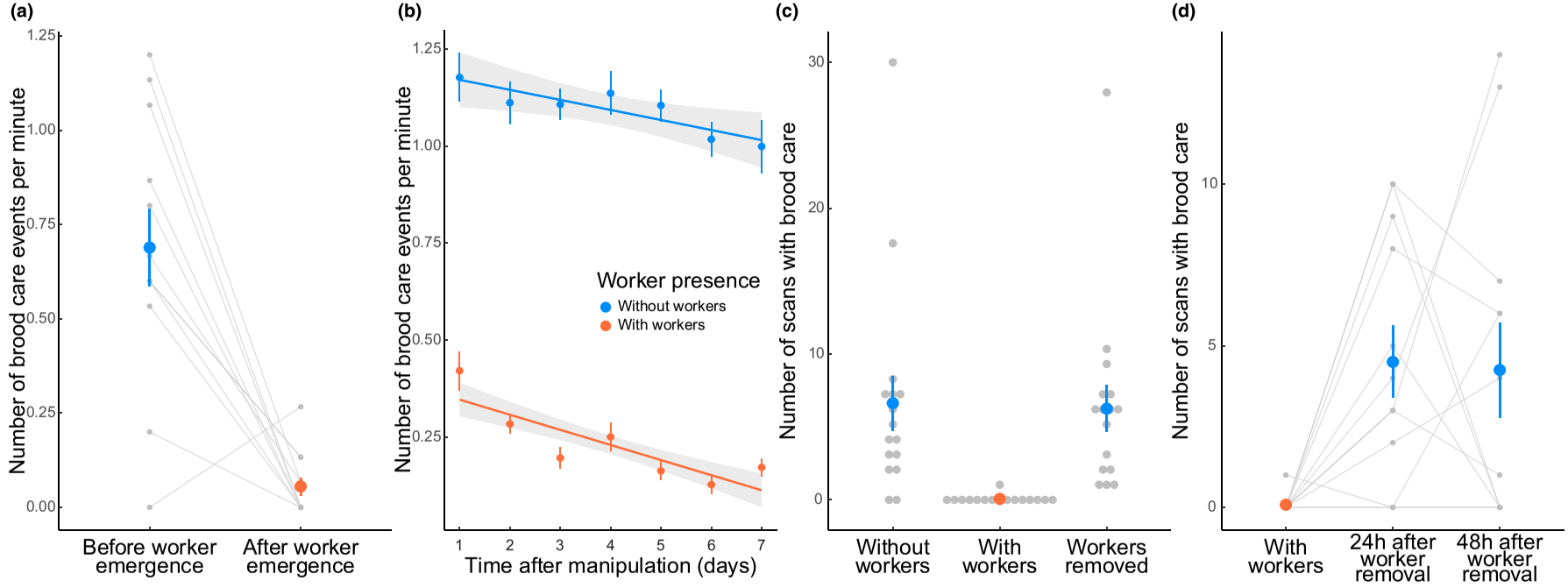

FIGURE 1 Presence of workers both initiates and maintains the queen behavioural specialization. (a) The emergence of workers correlates with the behavioural specialization of queens. Founding queens ($n = 12$) reduced brood care after producing the first workers ($\chi^2 = 35.63$, $p < 0.0001$). (b) The experimental addition of workers drives the queen specialization. Founding queens provided with workers ($n = 29$) showed lower brood care levels than founding queens kept without workers ($n = 25$, $\chi^2 = 596.33$, $p < 0.0001$). The effect of worker presence was already detected 20 h after the addition of workers ($\chi^2 = 221.28$, $p < 0.0001$). (c) The queen specialization is reversible after 3 days. Founding queens that had their workers removed ($n = 16$) performed more brood care than queens that just received workers ($n = 16$, $t = 6.19$, $p < 0.0001$), but similar brood care as queens that never had any workers ($n = 16$, $t = 0.15$, $p = 0.99$). (d) The queen specialization is reversible after 2 years and 6 months. Established queens ($n = 12$) showed increased brood care both 24 h ($t = 3.69$, $p = 0.0035$) and 48 h ($t = 3.07$, $p = 0.015$) after their workers were removed. Coloured dots and error bars represent means and standard errors, respectively, and grey dots show individual data points.

after the addition of workers ($\chi^2 = 221.28$, $p < 0.0001$, Figure S5). We could not detect any effect of the feeding status, neither as a main effect ($\chi^2 = 1.10$, $p = 0.29$) nor as an interaction with time ($\chi^2 = 1.90$, $p = 0.17$). This experiment demonstrates that the presence of workers is necessary and sufficient to initiate the behavioural specialization of *L. niger* queens.

3.3 | The presence of workers maintains the specialization of queens in established colonies

Then, we investigated whether the presence of workers is necessary to maintain the queen specialization in established colonies. First, we studied this question in *L. niger* queens that were provided with workers for 3 days. As expected, those queens expressed lower levels of brood care than same-age queens that never had any workers ($\chi^2 = 12.78$, $p = 0.00035$). We then removed the workers and compared the queen brood care behaviour to queens that either never had any workers or were just provided with workers. We found that queens that had their workers removed performed more brood care than queens that just received workers ($t = 6.04$, $p < 0.0001$, Figure 1c), but similar levels of brood care as queens that never had any workers ($t = 0.15$, $p = 0.99$, Figure 1c).

We report that the worker-driven inhibition of queen brood care behaviour is reversible after 3 days, but it may be that more time is needed for the queen specialization to be permanently established. Therefore, we investigated whether the queen behavioural specialization is reversible after several years. To do so, we used *L. niger* colonies that were founded in the laboratory between 2 years and 6 months before starting the experiment. We first recorded the queen brood care behaviour after removing all but five workers. Then, we removed the last five workers, and quantified queen brood care behaviour on the next 2 days. We found that queens expressed elevated brood care levels both 24 h ($t = 3.69$, $p = 0.0035$, Figure 1d) and 48 h ($t = 3.073$, $p = 0.015$, Figure 1d) after worker removal. We did not detect any difference between the two time points ($t = 0.61$, $p = 0.81$, Figure 1d), indicating that the queens did not show further behavioural changes after 24 h. To complete these findings, we quantified changes in queen brood care behaviour in response to the sequential removal of workers in laboratory colonies that were established between 3 years and 2 months before the experiment. We found that these established queens went back to expressing brood care upon the experimental removal of their last worker (Figure S6). These experiments show that the presence of workers not only initiates the specialization of *L. niger* queens during colony foundation, but also constantly maintains it in established colonies.

3.4 | Mating is not required for queens to become specialized in response to workers

Then, we investigated whether mating and reproductive activity are prerequisites for queens to express brood care behaviour, and whether it depends on worker presence. To do so, we manipulated the presence of workers and quantified the brood care behaviour of winged unmated queens collected from their colonies of origin (thus before their nuptial flight). We found that unmated queens performed brood care behaviour, but at very low levels. We did not observe brood care behaviour for 37.04% of the unmated queens (10/27), and those that did express brood care did so in only 1.76 ± 1.52 scans (out of 102). Expression of brood care by unmated queens has been reported in other ant species (Brown, 1999; Fletcher & Blum, 1983; Ito et al., 2017; Murakami, 2020; Nehring et al., 2012; Pyenson et al., 2022; Vieira et al., 2011). In addition, we found moderate evidence that the likelihood of expressing brood care was lower for unmated queens kept with workers compared with unmated queens kept without workers ($\chi^2 = 4.50$, $p = 0.034$, Figure S7). These results indicate that the presence of workers triggers the specialization of queens independent of their mating status and reproductive activity.

3.5 | Workers induce queen specialization in a dose-dependent manner

After demonstrating that the presence of workers controls the queen specialization, we set out to characterize the inhibitory effect of workers on brood care. First, we investigated whether the effect of workers is dose-dependent, as in other instances of social control of phenotypic variation in social insects (Ulrich et al., 2016; Winston et al., 1990). We provided same-age founding queens with five larvae, and either zero, one, two, three or five workers, and found a negative, non-linear correlation between queen brood care and the number of workers ($t = 8.45$, $p < 0.0001$, Figure 2a). While the presence of a single worker was sufficient to drive a reduction in brood care behaviour, additional workers inhibited brood care further, as the correlation remained after removing the queens that did not receive any workers from the analysis ($t = 3.35$, $p = 0.0014$). We repeated the same experiment with 15 larvae instead of five—thus increasing the larval need for care—and also found a negative correlation between the number of workers and the level of brood care expressed by queens, both when queens without workers were included ($t = 4.19$, $p < 0.0001$, Figure S8) and excluded from the analysis ($t = 2.37$, $p = 0.021$). These results indicate that workers have a dose-dependent negative effect on the brood care behaviour of queens.

3.6 | Mere worker cues do not induce the specialization of queens

Dose-dependent effects of social partners on phenotypic variation are often driven by variation in the quantity of social cues. Because social insects detect social partners via the blend of hydrocarbons on their cuticle (Richard & Hunt, 2013; Sprenger & Menzel, 2020), we extracted cuticular hydrocarbons (CHC) from pools of *L. niger* workers and applied the CHC to glass beads that we provided to same-age founding queens. We did not detect any effect of the CHC treatment on the

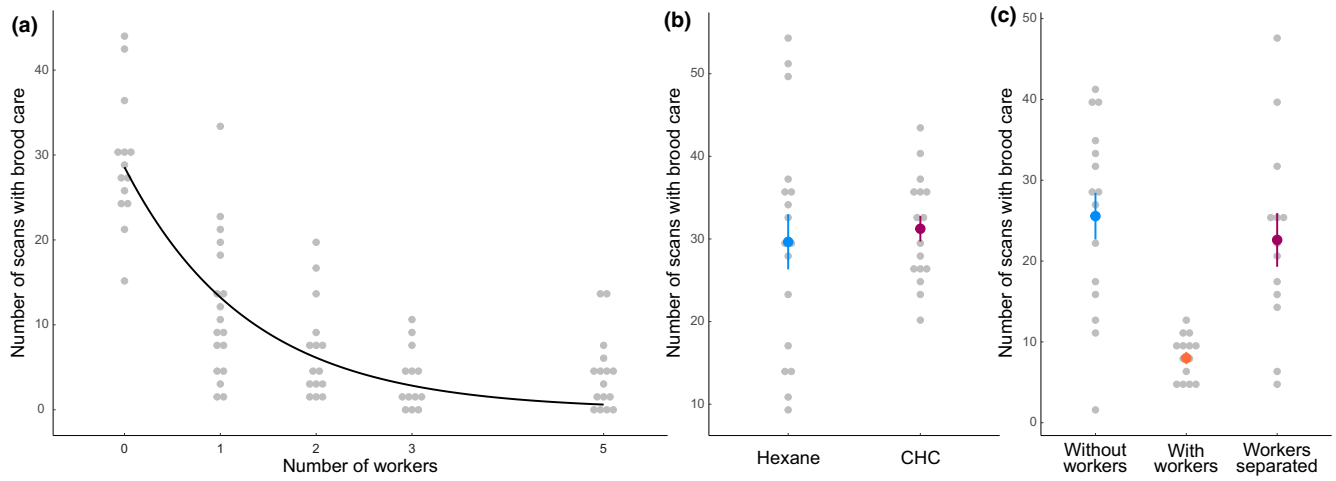

FIGURE 2 Characterization of the effect of workers on the queen behavioural specialization. (a) Workers show a dose-dependent inhibition of queen brood care. Founding queens were provided with zero ($n=14$), one ($n=18$), two ($n=15$), three ($n=13$) or five ($n=16$) workers. There was a negative, non-linear correlation between the number of workers and the queen brood care level ($t=8.5$, $p<0.0001$). The black line shows the non-linear regression curve of the model, and grey dots show individual data points. (b) Worker cuticular hydrocarbons (CHC) do not drive the queen specialization. No behavioural difference was detected depending on whether founding queens received glass beads covered in worker CHC ($n=18$) or hexane (control, $n=18$, $\chi^2=0.60$, $p=0.44$). (c) Workers separated by a wire mesh do not drive the queen specialization. The brood care behaviour of founding queens kept with workers separated by a wire mesh ($n=21$) differed from founding queens kept with workers ($n=14$, $t=3.95$, $p=0.001$), but not from founding queens kept without workers ($n=20$, $t=0.66$, $p=0.79$). Coloured dots and error bars represent means and standard errors, respectively, and grey dots show individual data points.

expression of brood care by queens ($\chi^2=0.60$, $p=0.44$, Figure 2b), indicating that queens do not modify their behaviour in response to the mere detection of worker CHC. This result was confirmed by three additional experiments. First, we did not detect any difference in brood care behaviour between founding queens depending on whether they were kept in boxes that used to contain many workers, or in boxes that never contained any ($\chi^2=0.48$, $p=0.49$, Figure S9). Second, we found that the presence of frozen workers, and thus of worker CHC (Modlmeier & Foitzik, 2011; Pamminger et al., 2011; Wilson et al., 1958), had no detectable effect on queen brood care ($t=0.57$, $p=0.84$, Figure S10). Finally, we investigated how the queen behaviour was affected by workers separated from the queen and brood by a wire mesh. This setup enabled workers to make antennal contacts with the queen and brood, but prevented closer interactions such as fluid exchange via trophallaxis (LeBoeuf, 2021). We found that queens kept with workers separated by a wire mesh expressed more brood care than queens kept together with workers ($t=3.95$, $p=0.001$, Figure 2c), but as much brood care as queens kept without workers ($t=0.66$, $p=0.79$, Figure 2c). This series of experiments shows that worker cues are not sufficient to drive the queen specialization and suggests that workers require close interactions with queens and/or larvae to inhibit the brood care behaviour of queens.

3.7 | Juvenile hormone may regulate the specialization of queens

The JH pathway is a good candidate for the regulation of brood care in queens because its function in regulating multiple worker behaviours (including brood care) has been demonstrated in many social insect species (Ferreira et al., 2023; Robinson, 1987b). To investigate the role of JH, we observed the behavioural response of same-age founding queens (kept with or without workers) to treatments with a JH analogue (methoprene) or with chemical inhibitors of JH production (precocene I and II). We found that the methoprene treatment decreased queen brood care in the first 2 days of the experiment, when brood care levels were relatively high ($\chi^2=4.43$, $p=0.035$, Figure 3a), consistent with previous reports of methoprene inhibiting brood care in *L. niger* queens (Pamminger et al., 2016). On the contrary, we found that queens treated with the JH inhibitor precocene I expressed elevated levels of brood care, although this was only detectable on the third and fourth day of the experiment, when brood care levels were overall low ($\chi^2=3.91$, $p=0.048$, Figure 3b; see Figure S11 and Table S2 for details on the effect of precocene II). These effects of methoprene and precocene I were independent of worker presence (Table S2). Finding that treatments with a JH analogue reduced brood care, while treatments with a JH inhibitor increased brood care, suggests a possible role of the JH pathway in regulating the brood care behaviour of *L. niger* queens (Ortiz-Alvarado & Rivera-Marchand, 2020).

3.8 | Founding queens show an ancestral physiological response to the presence of larvae

The JH pathway interacts closely with the insulin-signalling pathway (Al Baki et al., 2019; Libbrecht, Corona, et al., 2013; Perez-Hedo et al., 2014; Tatar et al., 2001), and both show variation between

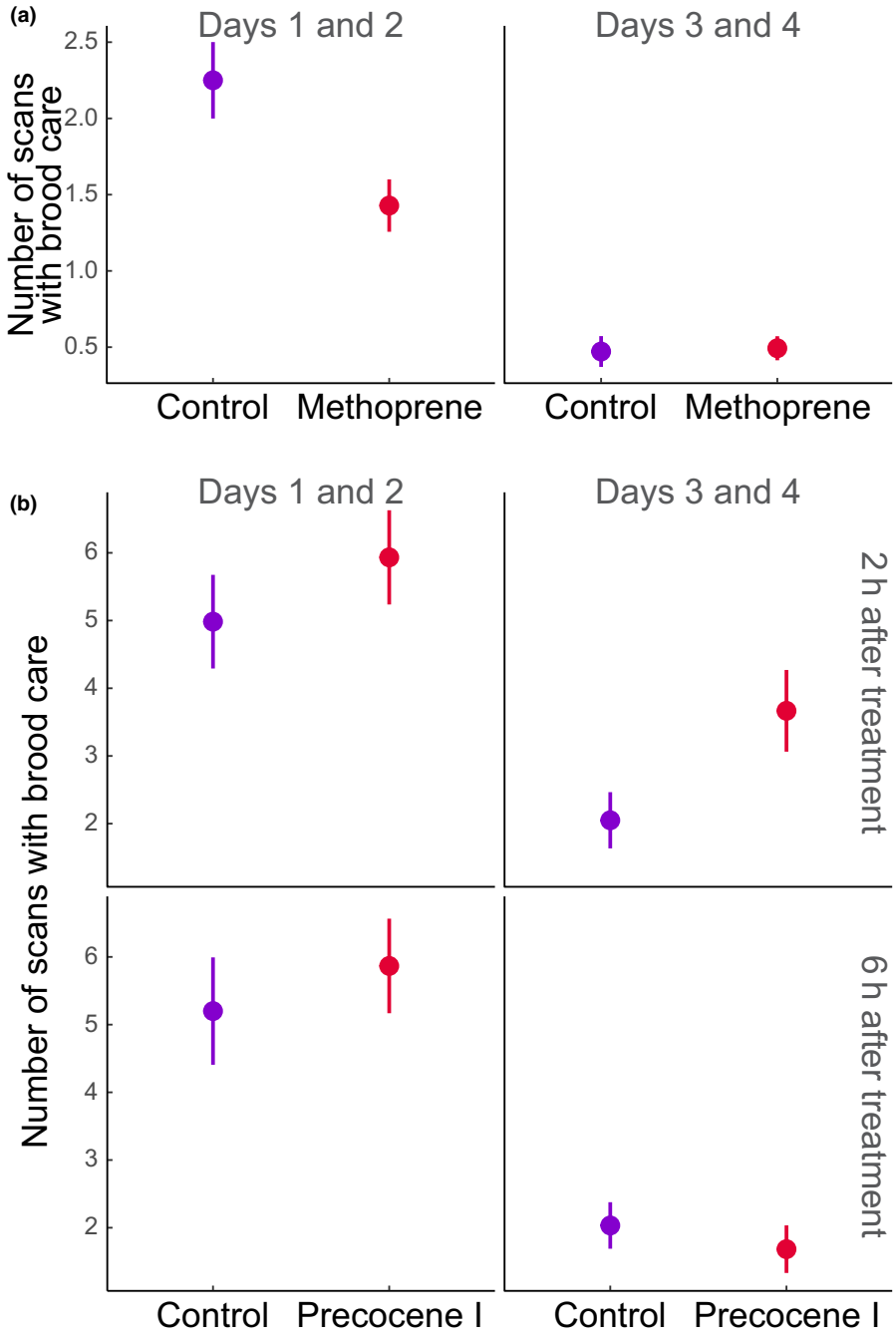

FIGURE 3 Possible role of JH in regulating the brood care behaviour of queens. (a) Founding queens treated with methoprene (JH analogue, $n=35$) showed reduced brood care levels compared with control queens (acetone, $n=35$) on Days 1 and 2 ($\chi^2=4.43$, $p=0.035$), but not on Days 3 and 4 ($\chi^2=1.05$, $p=0.31$). (b) Queens treated with precocene I (JH inhibitor, $n=30$) increased brood care levels compared with control queens (acetone, $n=30$) on Days 3 and 4 ($\chi^2=3.91$, $p=0.048$), but not on Days 1 and 2 ($\chi^2=2.34$, $p=0.13$). The effect on Days 3 and 4 was primarily driven by the difference between treated and control queens 2 h after treatment ($\chi^2=10.49$, $p=0.0012$), which was not detectable anymore 6 h after treatment ($\chi^2=0.41$, $p=0.52$).

queens and workers in various social insect species (Hartfelder et al., 2006; Libbrecht, Corona, et al., 2013; Mutti et al., 2011; Rembold et al., 1974; Röseler, 1976). In ants, the insulin-signalling pathway likely played a role in the emergence of reproductive division of labour (Chandra et al., 2018) from a subsocial ancestor that would alternate between reproduction and brood care phases (Hunt, 1999, 2012; West-Eberhard, 1987, 1996). These phases of the ancestral life cycle were controlled by the brood, which inhibited reproduction (Kelstrup et al., 2018). To investigate the effect of brood on the reproduction of founding and established queens, we quantified the effect of larvae on egg production over time in isolated *L. niger* queens before and after they produced workers. We found that, while the presence of larvae stimulated egg production in established queens (interaction between time and presence of

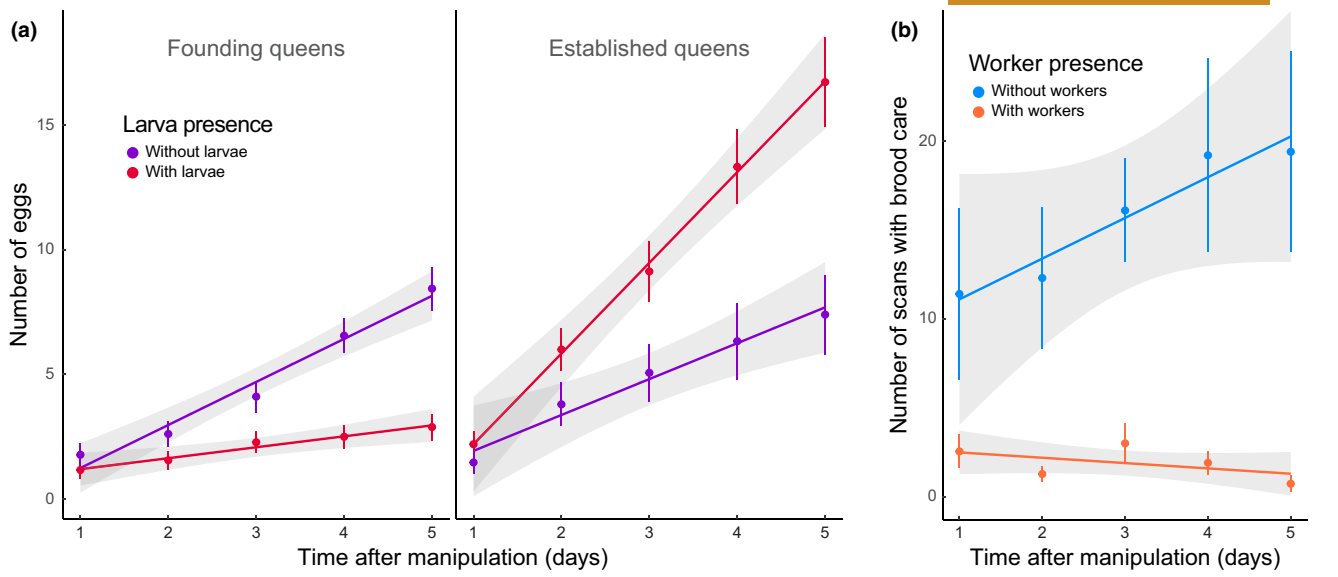

FIGURE 4 Founding queens may express an ancestral physiological response to brood and workers drive the queen specialization in another ant species. (a) Founding queens (left) kept with larvae ($n=18$) produced less eggs than founding queens kept without larvae ($n=18$; interaction between time and presence of larvae: $\chi^2=68.84$, $p<0.0001$; main effect of the presence of larvae: $\chi^2=16.47$, $p<0.0001$), while established queens (right) kept with larvae ($n=15$) produced more eggs than established queens kept without larvae ($n=15$; interaction between time and presence of larvae: $\chi^2=50.02$, $p<0.0001$; main effect of the presence of larvae: $\chi^2=9.76$, $p=0.0018$). Note that both founding and established queens were kept without workers for the duration of the experiment. (b) Established queens of the ant *Temnothorax nylanderi* expressed more brood care behaviour when kept without ($n=10$) than with workers ($n=11$; $\chi^2=32.23$, $p<0.0001$). Coloured dots and error bars represent means and standard errors, respectively.

larvae: $\chi^2=50.02$, $p<0.0001$; main effect of the presence of larvae: $\chi^2=9.76$, $p=0.0018$; Figure 4a), it had the reverse effect and inhibited egg production in founding queens (interaction between time and presence of larvae: $\chi^2=68.84$, $p<0.0001$; main effect of the presence of larvae: $\chi^2=16.47$, $p<0.0001$; Figure 4a). Because larvae inhibit their egg production, founding queens show an ancestral physiological response to the presence of brood, and are thus more similar in that respect to the subsocial ancestor of ants than to established queens. That founding and established queens show a different response to brood presence in terms of egg production may also stem from differences between these queens in their physiological condition and in the cost of expressing brood care behaviour.

3.9 | Workers induce the queen specialization in multiple species of ants

After finding that founding queens may express ancestral traits, we hypothesized that the social control of queen specialization may not be specific to *L. niger*, but rather occur across the phylogeny of ants. To test this, we used field-collected mature colonies of *Temnothorax nylanderi* (which belongs to a different subfamily from *L. niger*) and experimentally manipulated the presence of workers. In addition to worker presence affecting queen brood care changes over time (interaction between time and worker presence: $\chi^2=3.96$, $p=0.047$), we found that queens without workers generally expressed more brood care than queens with workers ($\chi^2=32.23$, $p<0.0001$, Figure 4b). This result shows that workers maintain queen specialization in *T. nylanderi* as well, and together with previous reports of social effects on queen behaviour (Cassill, 2002; Ortiz-Alvarado & Rivera-Marchand, 2020; Woodard et al., 2013), suggests that it may be a common feature in social insects (but see (Chouvenc & Su, 2017; Rüppell et al., 2002)).

4 | DISCUSSION

Social insect colonies are often viewed as superorganisms because they are intricate, complex biological systems that operate through the coordinated efforts of specialized individuals (Boomsma & Gawne, 2018; Szathmáry & Smith, 1995; Wheeler, 1911). While numerous studies explored the division of labour between fertile queens that specialize in egg production and functionally sterile workers that perform all non-reproductive tasks (Corona et al., 2016; Kronauer & Libbrecht, 2018; Libbrecht, Oxley, et al., 2013), our understanding of the establishment of this division of labour during the ontogeny of superorganisms remains limited. Specifically, it is crucial to elucidate the factors and mechanisms governing the behavioural shift from pluripotent founding queens that display a range of reproductive and non-reproductive behaviours to specialized established queens that exclusively prioritize egg laying. Our study reveals that (i) the presence of workers drives queen specialization in egg production by inhibiting brood care in a dose-dependent manner, (ii) the mere presence of worker cues does not trigger the queen specialization, (iii) this specialization is reversible upon worker removal in at least two species of ants, even after several years of specialization,

and (iv) founding queens may express ancestral behaviour and physiology.

Our finding that queen specialization is under the strict control of the social environment is inconsistent with the traditional view of social insect queens as being intrinsically specialized in egg production (Hölldobler & Wilson, 1990; Wilson, 1971), but rather indicates that their specialization is dependent on social conditions and is more flexible than typically assumed. We argue that it is not sufficient to study caste differentiation (Ashby et al., 2016; Cameron et al., 2013; Collins et al., 2020; Corona et al., 2016; Genzoni et al., 2023; Libbrecht et al., 2011; Libbrecht, Corona, et al., 2013; Libbrecht, Oxley, et al., 2013; Montagna et al., 2015; Mutti et al., 2011; Psalti & Libbrecht, 2020; Schultner et al., 2023; Schwander et al., 2008; Schwander & Keller, 2008; Wheeler et al., 2006) and/or compare adult queens and workers (Bonasio et al., 2012; Chandra et al., 2018; Corona et al., 2007, 2013; Feldmeyer et al., 2014; Grozinger et al., 2007; Kronauer & Libbrecht, 2018; Libbrecht, Oxley, et al., 2013; Patalano et al., 2015) to fully understand division of labour in insect societies. It is necessary to also investigate the transition from pluripotent to specialized queens, as well as the maintenance of the queen specialization. Our result that queen specialization is flexible and context-dependent in two species of ants echoes previous reports that social insect species with lower degrees of morphological differentiation between queens and workers tend to exhibit behavioural flexibility within the queen caste, including in the expression of non-reproductive behaviours (Gustilo et al., 2023; Lorenzi & Turillazzi, 1986; Richards, 1971; Shell & Rehan, 2018; Theraulaz et al., 1990; Woodard et al., 2013). That established queens retain the ability to express brood care (i.e., they retain their behavioural pluripotency) raises semantic doubts on whether social insect queens are truly specialized. One may argue that because they keep the ability to express other tasks, queens show apparent, rather than true specialization. This certainly needs to be taken into account when considering insect societies with reproductive division of labour as the epitome of specialization.

Several hypotheses could explain how workers influence the behaviour of queens. The first hypothesis is that queens show an active behavioural response to the presence of workers. However, this hypothesis makes the prediction that queens would modify their behaviour in response to the mere presence of workers cues, which is inconsistent with our results. The second hypothesis is that the effect of workers on queen behaviour requires close interactions between queens and workers, for example to allow an exchange of fluids via trophallaxis. Indeed, trophallactic fluids not only contain food, but also molecules that influence behaviour (LeBoeuf et al., 2016; Meurville & LeBoeuf, 2021). Our finding that the effect of workers on queen behaviour disappeared when we prevented close interactions between queens and workers is consistent with this hypothesis, but it remains unclear whether the freshly emerged workers used in the experimental manipulations of the social environment actually engaged in trophallaxis with the queens. The third hypothesis is that the queen specialization in egg production is a mere consequence of workers performing all other, non-reproductive tasks. This hypothesis is supported by all our findings, in particular, that workers show a dose-dependent inhibition of queen brood care behaviour, that cues of worker presence are not sufficient to drive queen specialization, and that established queens revert to expressing brood care relatively rapidly upon removal of their workers. According to this hypothesis, the workers reduce the larval needs by providing care to the larvae, up to the point when the larvae receive all the care they need from the workers, and the queens stop providing brood care. We thus propose that division of labour between queens and workers may be regulated similarly as the threshold-based models of behavioural division of labour that exists within the worker force (Oldroyd & Fewell, 2007; Waddington et al., 2010; Weidenmüller, 2004). In these models of division of labour, workers have distinct thresholds for task-specific stimuli, and they only respond when a stimulus exceeds their threshold, which in turn reduces the stimulus for the other workers (Bonabeau et al., 1997; Duarte et al., 2012; Robinson, 1987a, 1992). Therefore, queen specialization may simply be explained by queens having a higher response threshold to stimuli associated with non-reproductive tasks (e.g. cues emitted by the brood) than workers.

Several lines of evidence indicate that the behavioural flexibility and physiological response to brood of the subsocial ancestor of ants are still apparent during colony foundation. First, the diverse behavioural repertoire of founding queens can be found in many species of ants (Augustin et al., 2011; Brown, 1999; Cassill, 2002; Dejean & Lachaud, 1992; Haskins & Haskins, 1955; Helms Cahan & Fewell, 2004; Jeanson & Fewell, 2008; Wheeler, 1932, 1933). Second, our results suggest that the brood care behaviour expressed by founding queens may be regulated by the JH pathway, a very conserved and pleiotropic hormonal pathway with ancestral functions (Robinson & Vargo, 1997). Third, we find that larvae inhibit egg production in founding queens, indicating that the physiological response to brood of founding queens parallels that of the subsocial ancestor of ants (Kelstrup et al., 2018). The similarities between founding queens and the subsocial ancestor of ants echo recent reports of molecular and structural parallels between pluripotent stem cells in multicellular organisms and their unicellular ancestors (Brunet & King, 2017; Mikhailov et al., 2009; Naumann & Burkhardt, 2019; Sogabe et al., 2019). Not having access to the subsocial ancestor of ants has hampered the study of the evolution of ant sociality (Kronauer & Libbrecht, 2018), and investigating pluripotent founding queens may provide a window into these ancestral functions. In that perspective, the transition from pluripotency to specialization that underlies colony foundation may even mirror the evolutionary transition from subsocial pluripotency to division of labour between specialized castes.

Finally, we report that queen specialization is reversible in two ant species that diverged more than 100 million years ago (Borowiec et al., 2020). Finding such flexibility in the queen caste is particularly unexpected in *L. niger* and *T. nylanderi*, as both are derived ant species with high degrees of social complexity and differentiation between the queen and worker castes. Future research should explore the flexibility of queen specialization in other species of ants,

including species where queens found their colonies dependently (i.e. with the help of workers) and thus are not required to express non-reproductive behaviours at the founding stage of the colony cycle. We propose that, although it remained relatively unnoticed so far that workers continuously maintain queen specialization, this social control may actually be a common feature of ant colonies, and possibly of other insect societies (Cassill, 2002; Ortiz-Alvarado & Rivera-Marchand, 2020; Woodard et al., 2013). By revealing such an underappreciated feature of division of labour and behavioural specialization, our study could reshape our understanding of the evolution and functioning of insect societies.

AUTHOR CONTRIBUTIONS

RL conceived the ideas and designed the methodology; VM, MNP, MC, EF, E-MT, FR, JG, LM, MR, DU and RL collected the data; VM, MNP, MC and RL analysed the data; VM, MNP, MC and RL led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

All data used in this study are publicly available from the Dryad Digital Repository https://doi.org/10.5061/dryad.pnvx0k6wd (Majidifar et al., 2024), and the code for all analyses is provided as Supporting Information.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. One experimental tray with 12 observation arenas, which were recorded simultaneously with a single camera.

Figure S2. Resolution of the video when zooming in on one observation arena with one queen and five larvae.

Figure S3. Experimental setup to test the effect of workers separated from the queens with a circular wire mesh.

Figure S4. Brood production of 22 founding queens.

Figure S5. Effect of workers on the brood care behaviour of founding queens 20 h after the experimental manipulation.

Figure S6. Brood care behaviour of established queens (3 years and 2 months after their nuptial flight) in response to the sequential removal of their workers over the course of 6 days.

Figure S7. Proportion of unmated queens expressing brood care behaviour in the presence or absence of workers.

Figure S8. Workers induce queen specialization in a dose-dependent manner.

Figure S9. No effect of former presence of workers on the brood care behaviour of founding queens.

Figure S10. Effect of dead worker presence on brood care of founding queens.

Figure S11. Effect of precocene II on brood care behavior in founding queens.

Table S1. Information on the collection and keeping conditions of ant queens.

Table S2. Summary of the input data, syntax, and output of all statistical models.

Appendix S1. Fully annotated R script to run the data analyses conducted in the study.

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