Structural Pest Control Board
Research Advisory Panel

Neil Tsutsui Proposal

"RNAi Solutions for Low-impact Argentine Ant Management"

2025

Represented University:
UC Berkeley

Funds Requested: $329,710

Term: January 1, 2026 through June 30, 2028

Sponsored Projects Office

University of California, Berkeley
1608 Fourth Street, Suite 220
Berkeley, CA 94710-1749

Principal Investigator: Dr. Neil Tsutsui
Sponsoring Agency: California Department of Consumer Affairs
Project Title: RNAi Solutions for Low-impact Argentine ant Management

Please accept the enclosed proposal submitted on behalf of The Regents of the University of California Berkeley campus. Should this proposal be selected for funding, award documents should be issued using the information provided below.

Endorsed for the Regents by:

Bridget Zwimpfer
Contract and Grant Officer
Sponsored Projects Office

If you have any questions or need additional information regarding this proposal, please contact:

Bridget Zwimpfer
Contract and Grant Officer
Phone: (510) 664-9200
Fax: (510) 642-8236
Email: bzwimpfer@berkeley.edu

AWARDS SHOULD BE MADE TO:	CHECKS SHOULD BE MADE PAYABLE TO:
The Regents of the University of California c/o Sponsored Projects Office University of California, Berkeley 1608 Fourth Street, Suite 220 Berkeley, CA 94710-1749 email address for electronic awards: spoawards@berkeley.edu Main Office: (510) 642-0120 Fax: (510) 642-8236 Website: https://spo.berkeley.edu	**The Regents of the University of California** CHECKS SHOULD BE SENT TO: US Post Office Mailing Address: Contracts and Grants Administration P.O. Box 744695 Los Angeles, CA 90074 - 4695 Courier Delivery Address (UPS/FedEx/DHL): Bank of America Lockbox Services Lockbox 744695 2706 Media Center Drive Los Angeles, CA 90065-1733

ATTACHMENT 1

REQUIRED ATTACHMENT CHECKLIST

A complete proposal will consist of the items identified on the list below.

Complete this checklist to confirm that all items are contained with your proposal. Place a check mark or "✓" next to each item that you are submitting to the State. For your proposal to be responsive, in addition to your proposal, all required attachments must be returned. This checklist should be returned along with your proposal.

It is essential that the Cost Proposal be complete, thorough, and comply with content sequence requirements. The proposal must be typed and double-spaced on 8½ X 11 paper. All pages shall be consecutively numbered. All elements shall follow the sequence presented on the following checklist:

✓ Check	Attachment #	Attachment Name/Description	Form Provided	Completion Required
✓	Attachment 1	Required Attachment Checklist	YES	YES
✓	Attachment 2	Cost Proposal/Budget Display Sheets	YES	YES
✓	Attachment 3	Budget Narrative Form and Explanation of Costs	YES	YES
✓	Attachment 4	Proposer's References	YES	YES
✓	Attachment 5	Sample Agreement a) Project Summary and Scope of Work b) Schedule of Deliverables c) Key Personnel d) Authorized Representatives and Notices e) Use of Pre-existing Intellectual Property f) Current & Pending Support g) Third Party Confidential Information (if applicable) h) Budget Justification	YES	YES
✓	Attachment 6	Resumes (Curriculum Vitae) for Proposer, Proposer's staff involved in project, and all Subcontractors	NO	YES
✓	Attachment 7	Narrative of Research Objectives, as described in Rating/Scoring Criteria	NO	YES
✓	Attachment 8	Narrative of Project Direction (Work Plan and Work Schedule), as described in Rating/Scoring Criteria	NO	YES
✓	Attachment 9	Narrative of Qualifications, as described in "Minimum Qualifications for Proposers" and Rating/Scoring Criteria	NO	YES
✓	Attachment 10	Copy of current business license, professional certificates, or other credentials	NO	YES

ATTACHMENT 2

COST PROPOSAL/BUDGET DISPLAY
RESEARCH PROPOSAL

YEAR 1 – (for first 12 months)

Period of award
(i.e., 1/1/25-12/31/25)
Use separate sheet for each year

Period of award: 07/01/2026 -- 06/30/2028

Contractor: The Regents of the University of California (PI: Tsutsui, Neil)

Project Title/Description: RNAi Solutions for Low-impact Argentine ant Management

Description	Hours	Rate	Total
PERONNEL SERVICES			
1. PI Neil Tsutsui	87	141.45	12,306
2. Postdoc TBN	2088	34.31	71,632
		Total Salaries	83,938
		Total Benefits	12,843
		Total Personnel Services (A)	96,781
SUBCONTRACTOR SERVICES			
		Total Subcontractor Services (B)	
OTHER SERVICES			
1. Classification			
2. Classification			
3. Classification			
		Total Other Services (C)	
OPERATING EXPENSES			
1. Supplies and Expense			20,860
2. Travel In-State			
3. Travel Out-of-State			
4. Equipment			
5. Other Costs -- GAEL			1,972
		Total Operating Expenses (D)	22,832
Total Personnel and Operating (Add A through D)			119,613
Indirect Costs (detail)			41,865
TOTAL COSTS – Year 1 (for the first 12 months)			161,478

ATTACHMENT 2, Cont.

COST PROPOSAL/BUDGET
DISPLAY RESEARCH PROPOSAL
YEAR 2 – (for months 13 thru 24)

Period of award
(i.e., 1/1/25-12/31/25)
Use separate sheet for each year

Period of award: 07/01/2026 -- 06/30/2028

Contractor: The Regents of the University of California (PI: Tsutsui, Neil)

Project Title/Description: RNAi Solutions for Low-impact Argentine ant Management

Description	Hours	Rate	Total
PERONNEL SERVICES			
1. PI Neil Tsutsui	87	147.82	12,860
2. Postdoc TBN	2088	36.82	76,881
		Total Salaries	89,741
		Total Benefits	13,731
		Total Personnel Services (A)	103,472
SUBCONTRACTOR SERVICES			
		Total Subcontractor Services (B)	
OTHER SERVICES			
1. Classification			
2. Classification			
3. Classification			
		Total Other Services (C)	
OPERATING EXPENSES			
1. Supplies and Expense			16,380
2. Travel In-State			2,655
3. Travel Out-of-State			
4. Equipment			
5. Other Costs -- GAEL			2,109
		Total Operating Expenses (D)	21,144
Total Personnel and Operating (Add A through D)			124,616
Indirect Costs (detail)			43,616
TOTAL COSTS – Year 1 (for the first 12 months)			168,232

ATTACHMENT 2, Cont.

COST PROPOSAL/BUDGET DISPLAY
RESEARCH PROPOSAL
COMBINED YEARS (months 01-24)

Period of award
(i.e., 1/1/25-12/31/25)
Use separate sheet for each year

Period of award: 07/01/2026 -- 06/30/2028

Contractor: The Regents of the University of California (PI: Tsutsui, Neil)

Project Title/Description: RNAi Solutions for Low-impact Argentine ant Management

Description	Hours	Rate	Total
PERONNEL SERVICES			
1. PI Neil Tsutsui	194		25,166
2. Postdoc TBN	4176		148,513
		Total Salaries	173,679
		Total Benefits	26,574
		Total Personnel Services (A)	200,253
SUBCONTRACTOR SERVICES			
		Total Subcontractor Services (B)	
OTHER SERVICES			
1. Classification			
2. Classification			
3. Classification			
		Total Other Services (C)	
OPERATING EXPENSES			
1. Supplies and Expense			37,240
2. Travel In-State			2,655
3. Travel Out-of-State			
4. Equipment			
5. Other Costs -- GAEL			4,081
		Total Operating Expenses (D)	43,976
Total Personnel and Operating (Add A through D)			244,229
Indirect Costs (detail)			85,481
TOTAL COSTS – Year 1 (for the first 12 months)			329,710

ATTACHMENT 3,

BUDGET NARRATIVE FORM AND EXPLANATION OF COSTS:

Explain the need for individual staff, budgeted travel, equipment, subcontracts and consultants:

The Principal Investigator, Neil Tsutsui, will provide strategic oversight, supervise all aspects of the project, and lead reporting and dissemination. A full-time postdoctoral researcher will conduct molecular experiments, manage daily operations, and mentor undergraduate assistants. Travel funds are requested for one project member to present findings at the Entomological Society of America's annual meeting each year, facilitating professional feedback and knowledge exchange. No subcontracts or consultants are budgeted. One laptop computer is requested to support data analysis and field/lab coordination.

Please explain how the costs were arrived at:

Personnel costs for the PI and Post-doctoral Researcher are based on University of California salary scales and actual projected effort (0.5 summer months for the PI, 12 months/year for the postdoc). Fringe benefits were calculated using UC Berkeley's composite benefit rates. Travel costs are based on current airfare, lodging, registration, and per diem rates for conference destinations in 2026 and 2027. Materials and supplies reflect vendor quotes or historical purchase prices for RNAi reagents, lab consumables, and ant colony maintenance needs. The MacBook Air computer price includes projected sales tax and is based on Apple's educational pricing.

Please explain why the rates are considered reasonable and/or appropriate in your opinion:

All personnel costs reflect established academic compensation norms. The Post-doctoral Researcher salary follows UC's published pay scale for exempt Step IV researchers. Travel and lodging rates reflect actual market pricing for relevant destinations, with modest inflation adjustment. Lab supply costs are based on prior purchases or verified vendor prices. The computer requested is a mid-range model appropriate for the computing needs of data management, analysis, and writing.

Are costs based on industry standard or other basis of measurement? Please explain:

All personnel salaries, benefits, and indirect costs are governed by UC's standard rate agreements. Supply and equipment costs are based on pricing from common scientific vendors and current market rates. Travel costs are aligned with industry and UC travel standards. The combination of internal policy guidance and publicly available benchmarks ensures that all costs are reasonable and appropriate for the proposed work.

ATTACHMENT 4

PROPOSER REFERENCES

1. Please attach three letters of reference on company letterhead.
2. List below three references of similar types of services performed, as described in the description of services, within the last five years. If three references cannot be provided, please explain why on an attached sheet of paper.

REFERENCE 1	
Name of Firm	University of California, San Diego
Address	Department of Ecology, Behavior, and Evolution, One Shields Avenue, La Jolla, CA
Contact Person	Prof. David Holway
Telephone Number	(858) 342-2771
Dates of Service	2017 - present
Value or Cost of Service	$991,310.00

Brief Description of Service Provided: Prof. Holway and Prof. Tsutsui have collaborated for the past eight years on a research project, funded by the National Science Foundation, Long-term Research in Environmental Biology (NSF-LTREB) program, funded initially in 2017 for $391,313 and renewed in 2022 for $599,997. For this project, the invasive Argentine ant was eradicated from Santa Cruz Island (CA) and we are closely studying how the ecosystem is recovering, in terms of species recolonization, accumulation of genomic diversity, re-establishment of ecological function. This type of comprehensive analysis of ecological recovery has never been done before.

REFERENCE 2	
Name of Firm	Commonwealth Scientific and Industrial Research Organisation (CSIRO)
Address	Australia
Contact Person	Dr. Benjamin Hoffmann
Telephone Number	+61 (0)418 820 718
Dates of Service	2021 - present
Value or Cost of Service	$2,933,984

Brief Description of Service Provided: CSIRO is the national research agency of Australia. Dr. Hoffmann and Prof. Tsutsui have collaborated since 2021 on a research project, funded by the US Dept of Defense, funded initially for $654,433 and just renewed in 2025 for $1,254,430. The first grant was to develop proof-of-principle for RNAi as an effective species-specific insecticide. After successfully accomplishing this goal, we were awarded a second competitive grant, starting later in 2025, to scale up RNAi for future field tests and deployment.

REFERENCE 3	
Name of Firm	California Academy of Sciences
Address	San Francisco, CA
Contact Person	Dr. Brian Fisher
Telephone Number	(415) 722-6487
Dates of Service	2016 - 2020
Value or Cost of Service	$830,933

Brief Description of Service Provided: Dr. Fisher and Prof. Tsutsui collaborated for five years on a research project, funded by the National Science Foundation, Integrative Organismal Systems (NSF-IOS) program. For the Tsutsui portion of this project, we examined the evolution of cuticular hydrocarbon (CHC) pheromones and tested how they are used in ant recognition behavior and maintaining the cohesiveness of colonies. For the Fisher portion of the research, we launched the "Backyard Biodiversity Project", which exposed millions of people ant biology via YouTube videos, provided educational content to tens of thousands of people through displays at science-themed conventions and events, and gave first-hand experience with insects and biology to hundreds of K-12 students through classroom visits.

UNIVERSITY OF CALIFORNIA, SAN DIEGO UCSD

BERKELEY • DAVIS • IRVINE • LOS ANGELES • MERCED • RIVERSIDE • SAN DIEGO • SAN FRANCISCO SANTA BARBARA • SANTA CRUZ

DR. DAVID A. HOLWAY, Professor of Biology
ECOLOGY, BEHAVIOR AND EVOLUTION
TELEPHONE: (858) 822-5206
FAX: (858) 534-7108
EMAIL: dholway@ucsd.edu

SCHOOL OF BIOLOGICAL SCIENCES
9500 GILMAN DRIVE
LA JOLLA, CALIFORNIA 92093-0116

Structural Pest Control Board
2005 Evergreen Street, Ste. 1500
Sacramento, CA 95815

18 June 2025

Dear Structural Pest Control Board,

It is a pleasure to write a letter of recommendation in support of Dr. Neil D. Tsutsui's application for funding from the Structural Pest Control Board of the State of California. I have known Dr. Tsutsui since 1997, and we have collaborated on research projects throughout this 30-year period and made two research trips to Argentina together. We have co-authored 11 publications throughout this period, Dr. Tsutsui was co-PI on a previous NSF LTREB award (on which I was PI), and our successful work together on that project led to our second, currently active, NSF LTREB award.

Our past research collaborations focused on the behavior, ecology and genetics of the Argentine ant, a widespread, abundant and damaging insect pest. This research has primarily focused on the underlying causes of behavioral variation (especially intraspecific aggression) between native and introduced populations and how such variation may be related to the success of the Argentine ant in its introduced range. Other collaborations on this topic addressed the behavior and genetics of opposing Argentine ant supercolonies in southern California - work that was featured in part on the National Public Radio show *Radiolab*.

Our current collaboration is funded by Long-term Research in Environmental Biology Program at NSF and tests the capacity of native ant assemblages to recover structural (genetic diversity, species diversity) and functional (trophic position, ecological function) attributes following landscape-scale removal of the non-native Argentine ant from an island ecosystem. By contributing his molecular expertise to this project, Dr. Tsutsui and I hope to assess the rate and degree to which recolonizing ant species form assemblages composed of species that do not differ in terms of their colony structure and

population structure from ant populations that have never experienced displacement by the Argentine ant. Our labs are also collaborating on phylogenomics projects to test hypotheses about carpenter ant dispersal, phylogeography, and species boundaries on the California Channel Islands.

In working with Dr. Tsutsui in the field (both in Argentina and in California) as well as in the lab, I am consistently impressed by his creativity, enthusiasm, insightfulness, diligence, and follow-through. Throughout these years of work together, Dr. Tsutsui has consistently completed his research objectives on time and within budget.

These qualities have made my collaborations with Dr. Tsutsui extremely satisfying. Every project that we have worked on together has resulted in a publication in a leading, peer-reviewed journal (e.g., most recently in 2024 in *Evolution*). Based on these experiences, I am confident that the work proposed in Dr. Tsutsui's current proposal will generate carefully researched and exciting discoveries. This research not only has clear relevance to pest management, but given its ingenuity and novelty, I am confident that it will also yield important contributions to basic science.

Sincerely,

David Holway

Myrmex Pty Ltd

A.C.N. 683096497

14 CABMAN CT
BAKEWELL NT
NORTHERN TERRITORY, AUSTRALIA 0832
PH +61 (0)418 820 718

Date: 15 July 2025

Structural Pest Control Board
2005 Evergreen Street, Ste 1500
Sacramento, CA 95815

Dear Structural Pest Control Board,

I am happy to write a letter of support for Prof. Neil Tsutsui's proposal to the California Structural Pest Control Board on the development of RNA interference (RNAi) as a new method for controlling pest ants.

I have known Neil for over twenty years, and for the past several years while I was employed as a Principal Research Scientist at Australia's national research agency, the Commonwealth Scientific and Industrial Research Agency, Dr. Tsutsui and I collaborated on a project funded by the US Department of Defense (DOD) to develop proof of concept data that RNAi could be used successfully in a liquid feeding protocol to affect invasive ants. Among the species targeted was the Argentine ant (*Linepithema humile*), which is a prominent structural pest in California.

Our work was highly successful, such that targeting the PBAN gene by feeding specifically designed dsRNA constructs in sugar water to Argentine ants resulted in significant, dose-dependent mortality. *The new discoveries and formulations produced during that research project overcame the long-standing challenges that have plagued this field for decades, preventing the development of RNAi as a tool for invasive ant control and eradication.* Subsequently, Dr. Tsutsui expanded the team of collaborators and succeeded obtaining a follow-up grant from the US DOD to perform experiments that will decrease cost and allow this technology to be scaled up for use at practical, landscape scales for several of the world's most damaging invasive ant species. The research he describes in his proposal to the California Structural Pest Control Board perfectly complements this DOD-funded research, and together will significantly advance this technology toward a final product.

Having worked with Dr. Tsutsui on these complex international collaborations, I have seen first-hand his ability to guide large research teams to complete research objectives on schedule and under budget. I am confident that his leadership will produce practical, real-world benefits while also yielding new scientific discoveries and pointing the way forward for innovative new methods for applying genomic techniques to structural pest control. I whole-hearted encourage you to support this research proposal to the California Structural Pest Control Board.

Please feel free to contact me with any questions that you may have.

Sincerely,

Benjamin Hoffmann

Director, Myrmex Pty Ltd
ben.hoffmann.myrmex@gmail.com

Adjunct Senior Research Fellow, Research Institute for the Environment and Livelihoods, Charles Darwin University, Darwin
Affiliate, Plant and Environmental Protection Sciences, University of Hawaii, Honolulu

CALIFORNIA ACADEMY OF SCIENCES

July 24, 2025

California Structural Pest Control Board

RE: Letter of Support for Dr. Neil D. Tsutsui

Dear Review Committee,

It is a pleasure to offer my strongest support for **Dr. Neil D. Tsutsui's** proposal to the California Structural Pest Control Board, which seeks to advance the use of RNA interference (RNAi) as an innovative and precise method for invasive ant control. Having known and collaborated with Neil for over two decades, I can say without hesitation that he brings a rare combination of intellectual rigor, creativity, and deep ecological insight to every project he leads.

Neil and I previously co-led a multi-year NSF-funded research initiative that supported students, postdocs, and staff across our institutions. He not only played a key leadership role but also ensured the project's success through strategic hiring, inclusive management, and exemplary scientific guidance. These same qualities, leadership, scientific integrity, and foresight will no doubt ensure the success of the research he is proposing.

Dr. Tsutsui's scientific contributions have already reshaped our understanding of invasive ant dynamics. His work uniquely bridges molecular biology, ecology, and behavior, making him exceptionally well-suited to refine RNAi approaches for targeted and applied pest control. Given the complexity of RNAi technology and its potential ecological ramifications, a scientist must lead this work with a deep understanding of both molecular methods and ant biology. Neil is that scientist.

Equally important is Neil's role as an educator and mentor. His dedication to training the next generation of scientists is unparalleled. Through formal coursework and field-based training, Neil is known for engaging deeply with students, often transforming their perspectives and sharpening their research questions. I have seen firsthand how students, especially those from underrepresented backgrounds, thrive under his mentorship.

In summary, I wholeheartedly recommend Dr. Tsutsui. He is a gifted scientist, an outstanding mentor, and a visionary leader in entomological research. Supporting his proposal will not only advance pest control strategies for California but also help build a more sustainable and scientifically grounded approach to managing invasive species.

55 Concourse Drive, San Francisco, CA 94118

With best regards,

Brian L. Fisher
Curator & Chair of Entomology
California Academy of Sciences
55 Concourse Drive, San Francisco, CA 94118
bfisher@calacademy.org

STATE OF CALIFORNIA
STANDARD AGREEMENT
STD 213 (Rev 06/03)

ATTACHMENT 5 – SAMPLE AGREEMENT

AGREEMENT NUMBER	
REGISTRATION NUMBER	

1. This Agreement is entered into between the State Agency and the Contractor named below:

STATE AGENCY'S NAME
Department of Consumer Affairs, Structural Pest Control Board

CONTRACTOR'S NAME
The Regents of the University of California

2. The term of this Agreement is: July 1, 2026 through June 30, 2028

3. The maximum amount of this Agreement is: $329,710

4. The parties agree to comply with the terms and conditions of the following exhibits which are by this reference made a part of the Agreement.

Exhibit A – **A7**: A–Scope of Work; A1–Deliverables; A2–Key Personnel; A3–Authorized Representatives; A4–Use of Intellectual Property; A5–Resumes; A6–Current & Pending Support; A7-Third Party Confidential Information (if applicable)	page(s) 2-13
Exhibit B – B–Budget; B1–Budget Justification; B2– Subawardee Budgets (if applicable); B3–Invoice Elements	page(s) 14-19
Exhibit C* – University Terms and Conditions	UTC-518

Check mark additional Exhibits below, and attach applicable Exhibits or provide internet link:

☐ **Exhibit D** – Additional Requirements Associated with Funding Sources	page(s)
☐ **Exhibit E** – Special Conditions for Security of Confidential Information	page(s)
☐ **Exhibit F** – Access to State Facilities or Computing Resources	page(s)
☐ **Exhibit G** – Negotiated Alternate UTC Terms	page(s)

Items shown with an Asterisk (*), are hereby incorporated by reference and made part of this agreement as if attached hereto. These documents can be viewed at http://www.dgs.ca.gov/ols/Resources/ModelContractLanguageUniversities.aspx.

IN WITNESS WHEREOF, this Agreement has been executed by the parties hereto.

CONTRACTOR

CONTRACTOR'S NAME *(if other than an individual, state whether a corporation, partnership, etc.)*
The Regents of the University of California

BY *(Authorized Signature)* ✍ | DATE SIGNED *(Do not type)*

PRINTED NAME AND TITLE OF PERSON SIGNING
Bridget Zwimpfer, Contract and Grant Officer

ADDRESS
1608 Fourth Street, Suite 220, Berkeley, CA 94710-1749

STATE OF CALIFORNIA

AGENCY NAME
Department of Consumer Affairs, Structural Pest Control Board

BY *(Authorized Signature)* ✍ | DATE SIGNED *(Do not type)*

PRINTED NAME AND TITLE OF PERSON SIGNING

ADDRESS
1625 N. Market Blvd., Suite S-103
Sacramento, CA 95834

California Department of General Services Use Only

Exhibit A – Scope of Work

Project Summary & Scope of Work

☒ Contract ☐ Grant

PI Name: Neil D. Tsutsui .

Project Title: "RNAi Solutions for Low-impact Argentine ant Management"

Project Summary/Abstract

Structural pest control remains heavily reliant on broad-spectrum insecticides, which often cause unintended harm to non-target species, contaminate soil and water, and contribute to toxicant avoidance and resistance in pest populations. The Argentine ant (Linepithema humile), one of California's most problematic structural pests, exemplifies these challenges. In response, this project proposes a low-impact, species-specific control method based on RNA interference (RNAi), a naturally occurring immune response that can be co-opted to silence essential genes and induce mortality in target pest species.

Building on successful proof-of-concept work previously funded by the U.S. Department of Defense, this research will focus on improving RNAi efficacy and scalability by quantifying how double-stranded RNA (dsRNA) length affects RNAi-mediated mortality, as well as testing two promising delivery additives, chitosan and carbon quantum dots (CQDs), that may protect dsRNA molecules and enhance their uptake. Results will inform scalable RNAi bait formulations that maintain effectiveness in field-relevant conditions.

This project aligns with the goals of the 2023 California Sustainable Pest Management Roadmap and has the potential to transform structural pest control by replacing toxic insecticides with gene-targeted, environmentally benign approaches. To support adoption of these innovations, the project includes an outreach plan that will produce educational materials, deliver presentations to pest management professionals, and coordinate with UC IPM to ensure consistency with existing best practices.

If Third-Party Confidential Information is to be provided by the State: NOT APPLICABLE

☐ Performance of the Scope of Work is anticipated to involve use of third-party Confidential Information and is subject to the terms of this Agreement; ***OR***

☐ A separate CNDA between the University and third-party is required by the third-party and is incorporated in this Agreement as Exhibit A7, Third Party Confidential Information.

Scope of Work

This two-year project will evaluate RNAi-based control strategies for Argentine ants by developing and testing stabilized bait formulations using chitosan and CQDs. Each objective builds toward practical application and field readiness.

Objective 1: Determine optimal length of dsRNA constructs. We will synthesize and compare long (100-200 bp) vs. short (19-24 bp) dsRNA constructs targeting the PBAN gene, a gene previously validated to induce mortality in Argentine ants. Feeding assays will evaluate differences in gene silencing and survival, with the goal of identifying cost-effective constructs suitable for large-scale production.

Objective 2: Test palatability and toxicity of chitosan and carbon quantum dot (CQD) additives. Before testing RNAi efficacy, we will evaluate the standalone palatability and toxicity of chitosan and CQDs by incorporating them into sugar-based ant baits at multiple concentrations. A two-choice feeding assay will quantify total consumption and subsequent mortality (if any). Additives that are well-tolerated and do not deter feeding will advance to RNAi efficacy testing.

Objective 3: Quantify whether chitosan additive increases efficacy of RNAi. We will test whether adding chitosan, a naturally derived substance often used in drug delivery, can help improve the effectiveness of RNAi in Argentine ants. Chitosan may help protect dsRNA from degradation and make it easier for ants to absorb the molecules. After confirming that ants readily consume sugar water containing chitosan and that it is not toxic (Objective 2), we will compare ants treated with dsRNA alone to those treated with dsRNA+chitosan to see if chitosan improves gene silencing and increases mortality.

Objective 4: **Test whether CQD additive increases efficacy of RNAi**. Carbon quantum dots (CQDs) are nanoscale particles that may help carry dsRNA into insect cells more effectively, making RNAi treatments more potent. To explore their potential in Argentine ants, we will synthesized CQDs from poly(ethylene glycol) and polyethylenimine and verify that CQDs are both palatable and non-toxic when mixed with sugar water (Objective 2). For this Objective, we will assess whether adding CQDs to dsRNA treatments improves gene silencing and increases ant mortality compared to dsRNA alone.

Objective 5: **Determine impact of chitosan and carbon quantum dots together on efficacy of RNAi**. Regardless of whether chitosan or CQDs show significant individual effects on RNAi enhancement, we will conduct a final experiment testing a combination of both additives. This approach allows us to detect potential additive or synergistic effects that may only emerge when both delivery enhancers are present together in the bait formulation.

Experimental Overview

All treatments will be tested using standardized feeding assays with field-collected Argentine ants maintained in the laboratory. Ants will be assigned to replicate groups and fed dsRNA-containing diets for 24 hours, followed by 7 days of monitoring for mortality and behavioral changes. Gene expression knockdown will be quantified by qPCR at 24

and 72 hours post-exposure. Appropriate statistical tests (ANOVA, GLMs, nonparametric tests) will be used to assess treatment effects.

Benchmarks for success will include achieving statistically significant increases in mortality and gene knockdown in ants treated with dsRNA formulations containing chitosan and/or CQDs compared to dsRNA alone, as well as confirming the palatability and non-toxicity of each additive prior to combination experiments. Success will also be measured by the reproducibility of results across replicates and the generation of outreach materials suitable for pest management professionals. A potential challenge is that chitosan or CQDs may not significantly enhance RNAi efficacy relative to dsRNA alone. However, such outcomes would still provide valuable guidance by narrowing the field of candidate additives and informing future optimization strategies that explore alternative delivery enhancements.

Timeline

Time Period	*Major Activities and Milestones*
Months 1–6	*Collect and prepare lab ant colonies; synthesize dsRNA constructs (short and long); prepare CQD and chitosan formulations; begin Obj. 1 and 2 experiments.*
Months 7-12	*Complete experiments and gene expression analysis for Obj. 1 and 2. Initiate data analysis for Obj. 1 and 2. Initiate experiments for Obj. 3 and 4.*
Months 13–18	*Complete experiments and gene expression analysis for Obj. 3 and 4. Perform data analysis for Obj. 3 and 4. Initiate experiments for Obj. 5.*
Months 19–24	*Finalize any last data collection and complete data analysis; manuscript and report preparation; develop factsheet and video for outreach; present results at professional meetings*

Public Engagement

Educational materials (e.g., fact sheets, short videos) summarizing ant biology, bait behavior, and RNAi strategies will be developed for pest control professionals. Outreach will include at least one live presentation or webinar and coordination with UC IPM and professional associations to ensure alignment with existing pest management resources.

Exhibit A1 - Deliverables

SCHEDULE OF DELIVERABLES

List all items that will be delivered to the State under the proposed Scope of Work. Include all reports, including draft reports for State review, and any other Deliverables, if requested by the State and agreed to by the Parties.

If use of any Deliverable is restricted or is anticipated to contain preexisting Intellectual Property with any restricted use, it will be clearly identified in Exhibit A4, Use of Preexisting Intellectual Property.

Unless otherwise directed by the State, the University Principal Investigator shall submit all Deliverables to the State Contract Project Manager, identified in Exhibit A3, Authorized Representatives.

Deliverable	Description	Due Date
Interim Progress Report #1	Report on postdoc hiring, setup, and initial dsRNA length testing results	12/31/2026
Interim Progress Report #2	Report on dsRNA length study and start of chitosan trials	7/1/2027
Interim Progress Report #3	Report on chitosan trials and start of CQD experiments	12/31/2027
Outreach Factsheet	Plain-language resource on Argentine ant biology and RNAi	5/1/2028
Informational Video	Educational video for pest professionals on Argentine ants and RNAi	5/1/2028
Interim Progress Report #4	Report on CQD results and early data from combined additive experiments	7/1/2028
Oral Progress Presentation #1	SPCB Board presentation (upon invitation from SPCB)	2027 (TBD)
Draft Scientific Manuscript	Manuscript for submission to peer-reviewed journal	6/30/2028
Final Project Report	Full report of all experimental results and conclusions	9/30/2028
Oral Progress Presentation #2	SPCB Board presentation (upon invitation from SPCB)	2028 (TBD)

Exhibit A2 – Key Personnel

KEY PERSONNEL

List Key Personnel as defined in the Agreement starting with the PI, by last name, first name followed by Co-PIs. Then list all other Key Personnel in alphabetical order by last name. For each individual listed include his/her name, institutional affiliation, and role on the proposed project. Use additional consecutively numbered pages as necessary.

Last Name, First Name	Institutional Affiliation	Role on Project
PI:		
Tsutsui, Neil D.	*UC Berkeley*	*Overall project guidance and management, experimental design, data analysis , guide post-doc and students, manuscript writing, reporting and deliverables.*
Co-PI(s) – if applicable: None		
Other Key Personnel (if applicable):		
Post-doctoral Researcher, to be hired	*UC Berkeley*	*Molecular and experimental research, data analysis, manuscript writing, guide students, coordinate with PI and collaborators.*

Exhibit A3 – Authorized Representatives

AUTHORIZED REPRESENTATIVES AND NOTICES

The following individuals are the authorized representatives for the State and the University under this Agreement. Any official Notices issued under the terms of this Agreement shall be addressed to the Authorized Official identified below, unless otherwise identified in the Agreement.

State Agency Contacts Agency Name: <Agency Name>	**University Contacts** University Name: The Regents of the University of California
Contract Project Manager (Technical) Name: <Name> <Title> Address: <Department> <Address> <City,State,Zip> Telephone: <Telephone#> Fax: <Fax#, if available> Email: <EmailAddress>	***Principal Investigator*** Name: Neil Tsutsui Professor Address: Environmental Science, Policy & Mngmt 5057 Valley Life Sciences Bldg Berkeley, CA 94720-3204 Telephone: (510) 642-0542 Fax: Email: ntsutsui@berkeley.edu Designees to certify invoices under Section 14 of Exhibit C on behalf of PI: 1. <Name>, <Title>, <EmailAddress> 2. <Name>, <Title>, <EmailAddress> 3. <Name>, <Title>, <EmailAddress>
Authorized Official (contract officer) Name: <Name> <Title> Address: <Department> <Address> <City,State,Zip> Telephone: <Telephone#> Fax: <Fax#, if available> Email: <EmailAddress> ***Send notices to (if different):*** Name: <Name> <Title> Address: <Department> <Address> <City,State,Zip> Telephone: <Telephone#>	***Authorized Official*** Name: Bridget Zwimpfer Contract and Grant Officer Address: Sponsored Projects Officer 1608 Fourth Street, Suite 220 Berkeley, CA 94710-1749 Telephone: 510-642-0120 Fax: Email: bzwimpfer@berkeley.edu ***Send notices to (if different):*** Name: <Name> <Title> Address: <Department> <Address> <City,State,Zip> Telephone: <Telephone#>

Email: <EmailAddress>	Email: <EmailAddress>
Administrative Contact Name: <Name> <Title> Address: <Department> <Address> <City,State,Zip> Telephone: <Telephone#> Fax: <Fax#, if available> Email: <EmailAddress>	***Administrative Contact*** Name: Bridget Zwimpfer Contract and Grant Officer Address: Sponsored Projects Officer 1608 Fourth Street, Suite 220 Berkeley, CA 94710-1749 Telephone: 510-642-0120 Fax: Email: bzwimpfer@berkeley.edu
Financial Contact/Accounting Name: <Name> <Title> Address: <Department> <Address> <City,State,Zip> Telephone: <Telephone#> Fax: <Fax#, if available> Email: <EmailAddress>	***Authorized Financial Contact/Invoicing*** Name: Beata Najman Director Address: Contracts and Grants Accounting 1608 Fourth Street, Suite 201 Berkeley, CA 94710-1103 Telephone: 510-643-4246 Fax: Email: CGAawards@berkeley.edu Designees for invoice certification in accordance with Section 14 of Exhibit C on behalf of the Financial Contact: 1. <Name>, <Title>, <EmailAddress> 2. <Name>, <Title>, <EmailAddress> 3. <Name>, <Title>, <EmailAddress>

Exhibit A4 – Use of Intellectual Property

USE OF INTELLECTUAL PROPERTY

If either Party will be using any third-party or pre-existing intellectual property (including, but not limited to data, copyrighted works, known patents, trademarks, service marks and trade secrets) "IP" with restrictions on use, then list all such IP and the nature of the restriction below. If no third-party or pre-existing IP will be used, check "none" in this section.

A. State: Preexisting IP to be provided to the University from the State or a third party for use in the performance in the Scope of Work.

☒ None or ☐ List:

Owner (Name of State Agency or 3rd Party)	Description	Nature of restriction:

B. University: Restrictions in Preexisting IP included in Deliverables identified in Exhibit A1, Deliverables.

☒ None or ☐ List:

Owner (Name of University or 3rd Party)	Description	Nature of restriction:

C. Anticipated restrictions on use of Project Data.

If the University PI anticipates that any of the Project Data generated during the performance of the Scope of Work will have a restriction on use (such as subject identifying information in a data set) then list all such anticipated restrictions below. If there are no restrictions anticipated in the Project Data, then check "None" in this section.

☒ None or ☐ List:

Owner (University or 3rd Party)	Description	Nature of Restriction:

Exhibit A5 – Resume/Biosketch

NEIL DURIE TSUTSUI
Professor and Michelbacher Chair

University of California, Berkeley
Dept. of Environmental Science, Policy, & Management
130 Mulford Hall, #3114
Berkeley CA 94720-3114

ntsutsui@berkeley.edu
(510) 684-5572
https://nature.berkeley.edu/tsutsuilab

EDUCATION

1994. BA, Biology, specialization in Marine Science — Boston University
2000. PhD, Biology. Advisor: Ted J. Case — UC San Diego, Department of Biology

PROFESSIONAL APPOINTMENTS

UC Berkeley, Department of Environmental Science, Policy & Management
- 2022-present. Director, UC Berkeley Central Sierra Field Research Stations
- 2017-present. Abraham & Martha Michelbacher Chair of Systematic Entomology
- 2014-present. Professor
- 2007-present. Faculty Affiliate, Essig Museum of Entomology
- 2010-2012, 2014-2015. Vice Chair for Instruction
- 2009-2014. Associate Professor
- 2007-2009. Assistant Professor

UC Irvine, Department of Ecology and Evolutionary Biology
- 2003-2007. Assistant Professor

UC Davis, Section of Evolution and Ecology, Center for Population Biology
- 2000-2003. Post-doctoral Associate. Advisor: Richard K. Grosberg

10 RELEVANT PUBLICATIONS (of 81 total)

Ferger, K. & **N. D. Tsutsui.** 2025. Social organization is associated with relaxed selection on worker genes in highly polygyne ants. ECOLOGY & EVOLUTION 15:e71696.

Cash, E. I., M. Escalona, P. S. Ward, R. Sahasrabudhe, C. Miller, E. Toffelmier, C. Fairbairn, W. Seligmann, H. B. Shaffer, and **N. D. Tsutsui**. 2024. Reference genome of the kidnapper ant, *Polyergus mexicanus*. J. HEREDITY esae047.

Ward, P. S., E. I. Cash, K. Ferger, M. Escalona, R. Sahasrabudhe, C. Miller, E. Toffelmier, C. Fairbairn, W. Seligmann, H. B. Shaffer, and **N. D. Tsutsui**. 2023. Reference genome of the bicolored carpenter ant, *Camponotus vicinus*. J. HEREDITY 115(1):120-129.

Tonione, M. A., K. Bi, and **N. D. Tsutsui**. 2020. Transcriptomic signatures of cold adaptation and heat stress in the thermally sensitive winter ant (*Prenolepis imparis*). PLOS ONE **15**(10): e0239558.

Cridland, J., S. R. Ramírez, C. Dean, A. Sciligo, and **N. D. Tsutsui**. 2018. Genome sequencing of museum specimens reveals rapid changes in genetic composition of honey bees in California. GENOME BIOLOGY & EVOLUTION **10**(2):458-472.

Smith, C. D., *et al.* 2011. The draft genome of the globally widespread and invasive Argentine ant (*Linepithema humile*). PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA **108**:5673-5678. * **N. D. Tsutsui** – corresponding author.

van Wilgenburg, E., C. W. Torres, and **N. D. Tsutsui**. 2010. The global expansion of a single ant supercolony. EVOLUTIONARY APPLICATIONS **3**:136-143.

Tsutsui, N. D., A. V. Suarez, J. C. Spagna, and J. S. Johnston. 2008. The evolution of genome size in ants. BMC EVOLUTIONARY BIOLOGY **8**:64.

Whitfield, C. W., S. K. Behura, S. H. Berlocher, A. G. Clark, S. Johnston, W. S. Sheppard, D. Smith, A. V. Suarez, D. Weaver, and **N. D. Tsutsui**. 2006. Thrice out of Africa: Ancient and recent expansions of the honey bee, *Apis mellifera*. SCIENCE **314**:642-645.

Tsutsui, N. D., A. V. Suarez, D. A. Holway, and T. J. Case. 2000. Reduced genetic variation and the success of an invasive species. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA **97**:5948-5953.

GRANT SUPPORT

>$6.5 million from federal & state agencies, including NSF, USDA, and DOD.

TEACHING EXPERIENCE & MENTORSHIP

MENTORING

13 PhD students. 14 Post-doctoral researchers. Hundreds of undergraduate researchers.

COURSES TAUGHT

UC-Berkeley

Insect Behavior (ESPM150, ESPM142), 12 semesters.
Molecular Approaches to Environmental Problem Solving (ESPM 192), 4 semesters.
Advanced Chemical Ecology (ESPM290), 6 semesters.
Senior Seminar-Conservation & Resource Studies (ESPM194), 3 semesters.
Environmental Science Forum (ESPM 201C), 1 semester.

UC-Irvine

Graduate Core in Evolution, 1 quarter.
Advanced Evolutionary Biology (BS168), 3 quarters.
Freshman Seminar (BS2B), "Social behavior of ants", 4 quarters.

INVITED PRESENTATIONS & PROFESSIONAL MEETINGS

78 invited seminar and conference presentations since 1999.

SERVICE (partial)

COMMITTEE MEMBER

EAST BAY REGIONAL PARK DISTRICT, Parks Advisory Committee. Appointed by Contra Costa County Board of Supervisors.
Chair, 2024. Vice-chair, 2023. Member, 2017–2024.

PANEL MEMBER:

4 NATIONAL SCIENCE FOUNDATION and 3 USDA grant review panels since 2007.

EDITORIAL BOARDS:

BMC ECOLOGY. Associate Editor, 2011–present.
BIOLOGICAL INVASIONS. Associate Editor, 2011–present.
AUSTRALIAN JOURNAL OF ZOOLOGY. Associate Editor, 2007–2012.

REVIEWER (manuscripts and grant proposals, excluding panel service above)**:**

Several hundred from July 2003–present.

EXTENSIVE COMMITTEE ADDITIONAL SERVICE TO UC SYSTEM, COLLEGE OF NATURAL RESOURCES, AND DEPT OF ENVIRONMENTAL SCIENCE, POLICY, & MANAGEMENT.

Exhibit A6 – Current & Pending Support

CURRENT & PENDING SUPPORT

University will provide current & pending support information for Key Personnel identified in Exhibit A2 at time of proposal and upon request from State agency. The "Proposed Project" is this application that is submitted to the State. Add pages as needed.

PI: Neil Tsutsui

Status:	Award # (if available)	Source (name of the sponsor)	Project Title	Start Date	End Date
Proposed Project	NA	California Structural Pest Control Board	RNAi Solutions for Low-impact Argentine ant Management	7/1/2026	6/30/2028
CURRENT	RC25-4645	Dept of Defense, SERDP	From the Lab to the Field:Continued RNAi Development for Invasive Ant Eradication	8/1/2026	7/31/2029
CURRENT	WC-2501JC	California Wildlife Conservation Board	Saghen Creek Field Station Facilities Improvement Project	8/1/2024	9/30/2026
CURRENT	NSF-LTREB 2203151	National Science Foundation, Long-term Research in Environmental Biology	Large-scale Removal of Introduced Ants as a Test of Community Reassembly	5/1/2022	4/30/2027

Exhibit A7

Third Party Confidential Information

Confidential Nondisclosure Agreement

(Identified in Exhibit A, Scope of Work – will be incorporated, if applicable)

If the Scope of Work requires the provision of third party confidential information to either the State or the Universities, then any requirement of the third party in the use and disposition of the confidential information will be listed below. The third party may require a separate Confidential Nondisclosure Agreement (CNDA) as a requirement to use the confidential information. Any CNDA will be identified in this Exhibit A7.

NOT APPLICABLE

Exhibit B2 – Budget

Name: The Regents of the University of California **Exhibit B2**

Principal Investigator (Last, First): Tsutsui, Neil

COMPOSITE SUBAWARDEE BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
07/01/2026 to 06/30/2028

	From:	7/1/2026	7/1/2027	7/1/2027	
	To:	6/30/2027	6/30/2028	6/30/2028	
BUDGET CATEGORY		**Year 1**	**Year 2**	**Year 3**	**TOTAL**
PERSONNEL: *Salary and fringe benefits.*		$96,781	$103,472	$0	$200,253
TRAVEL		$0	$2,655	$0	$2,655
MATERIALS & SUPPLIES		$20,860	$16,380	$0	$37,240
EQUIPMENT		$0	$0	$0	$0
CONSULTANT		$0	$0	$0	$0
SUBRECIPIENT		$0	$0	$0	$0
OTHER DIRECT COSTS (ODC)	*Subject to IDC Calc*				
ODC #1 -- GAEL	*Y*	$1,972	$2,109	$0	$4,081
ODC #2	*Y*	$0	$0	$0	$0
ODC #3	*Y*	$0	$0	$0	$0
ODC #4	*Y*	$0	$0	$0	$0
ODC #5	*Y*	$0	$0	$0	$0
ODC #6	*Y*	$0	$0	$0	$0
TOTAL DIRECT COSTS		$119,613	$124,616	**$0**	**$244,229**
Indirect (F&A) Costs	**F&A Base**				
Rate 35%	*MTDC **	*$119,613*	*$124,616*	*$0*	*$224,229*
		$41,865	$43,616	**$0**	**$85,481**
TOTAL COSTS PER YEAR		$161,478	$168,232	**$0**	
TOTAL COSTS FOR PROPOSED PROJECT PERIOD					**329,710**

* MTDC = Modified Total Direct Cost

JUSTIFICATION. *See Exhibit B1 - Follow the budget justification instructions.*

Project Period Budget Flexibility (lesser of % or Amount)

Prior approval required for budget changes between approved budget categories above the thresholds identified.

% **10.00%**

or

Amount *$10,000*

EXHIBIT B1

BUDGET NARRATIVE

Personnel

Name: Neil Tsutsui

Role on Project: Principal Investigator.

PI Tsutsui will be responsible for overall strategy and project management. Tsutsui will guide the research performed by the Post-doctoral researcher and will work with the Post-doctoral researcher on experimental design, data collection, data analysis and visualization, trouble-shooting experimental approaches, and will write the first draft of manuscripts. Tsutsui will provide budgetary oversight, track all expeditures, and will compile and prepare all materials for reports. Prof. Tsutsui is requesting 0.5 months of summer salary support per year. The amount requested is based on 0.5 month equivalent of his 9-month salary paid by the University of California.

Fringe Benefits:

Name: Post-doctoral Researcher, to be hired.

Role on Project:

A post-doctoral researcher will be hired to oversee and organize the day-to-day operations of the proposed research and will train and oversee the undergraduate researchers. The post-doctoral researcher will perform field work to collect insect colonies for the proposed research, establish and maintain the colonies in the lab, and assemble a team of undergraduates to assist with ongoing care of the colonies (feeding, cleaning, etc). The post-doctoral researcher will also perform the hands-on genetic and molecular research, including ordering reagents, preparing and executing RNAi experiments, collecting and analyzing data, and contribute to the writing of all manuscripts. The post-doc will work closely with the undergraduate researchers, providing training, mentorship, and guidance throughout the duration of the project. The postdoc will commit 12 months per year (100% time) to this project in both years. Post-doctoral salary is based on the compensation scale for Step IV exempt post-doctoral researchers at the University of California, effective 03/01/2020, published online at:
https://apo.berkeley.edu/2019-20-academic-salary-scales

Fringe Benefits:

Employee benefits were estimated using the UC Composite Benefits Rates agreed upon by the University of California Office of the President, published online at:
https://spo.berkeley.edu/policy/benefits/benefits.html

Travel

We are requesting expenses for travel to one scientific conference in year 2 (Entomological Society of America, or equivalent). This travel will allow the PI or Post-doctoral researcher to present current results to world experts and receive feedback and assistance with any problems that may arise. At the same time, the conference attendee will be able to attend research presentations on relevant topics and utilize this knowledge to improve the ongoing research.

The most recent registration cost for this conference was $795 (https://entsoc.org/events/annual-meeting). We also include 4 nights of lodging and estimated round trip airfare from SFO to the conference location each year (Orlando, FL in 2027). We estimated this airfare using current ticket costs plus 5% increase per year. We also request a per diem rate of $65 for meals each day ($15 breakfast, $20 lunch, $30 dinner; 5 days).

2027:
Registration: $800
Airfare (economy): $530 (Orlando, FL)
Lodging: $1000 ($250/night)
Meals: $325
TOTAL: $2655

Materials and Supplies
We are requesting $20,860 in Year 1 and $16,380 in Year 2 to purchase materials, supplies, and consumables for the proposed RNAi experiments. These amounts are based on actual current prices for the necessary items or invoices for recent purchases that we have made. A spreadsheet listing items, vendor number, pricing, and units purchased per year is attached at the end of Exhibit B-1.

Computing: In Year 1, we are requesting $1200 + $870 sales tax = $2070 for one 13" Apple MacBook Air, 16GB Unified Memory, 512GB SSD Storage (included in above figure). As per State Contracting Manual section 7.29, this item will be assigned an inventory number and tracked by the UC Berkeley College of Natural Resources. The cost of this item is included in the Materials and Supplies figure above.

Equipment
None.

Consultant Costs
None.

Subawardee Costs
None.

Other Direct Costs
None.

Rent
None.

Indirect (F&A) Costs
Indirect costs are based on University-negotiated rates with the State of California and are applied at a rate of 35.0%. Indirect costs are applied using the Modified Total Direct Cost (MTDC) formula, per rate agreement dated April 10, 2024. Modified total direct costs exclude equipment, capital expenditures, charges for patient care, student tuition remission, rental costs

of off-site facilities, scholarships, and fellowships, participant support costs and the portion of each subgrant and subcontract in excess of $25,000.

Name (product number)		Unit price	# Units Year 1	# Units Year 2	SUBTOTALS BY YEAR Year 1	SUBTOTALS BY YEAR Year 2	**Vendor total**
1. Integrated DNA Technologies							
1a. Custom dicer substrate short interfering RNA constructs		$10,296.00	0.5	0.5	$5,148	$5,148	
	Subtotal				**$5,148**	**$5,148**	**$10,296**
2. New England Biolabs							
2a. NEBNext® High-Fidelity 2X PCR Master Mix, 250 rxn (M0541L)		$405.00	3	3	$1,215	$1,215	
2b. T4 DNA Ligase (M0202L)		$281.00	2	2	$562	$562	
2c. WarmStart® RTx Reverse Transcriptase		$322.00	2	2	$644	$644	
2d. DNA Polymerase I, Large (Klenow) Fragment (M0210L)		$293.00	2	2	$586	$586	
	Subtotal				**$3,007**	**$3,007**	**$6,014**
3. Qiagen							
3a. QIAamp Fast DNA Tissue Kit (51404)		$288.00	2	1	$576	$288	
3b. QIAquick PCR Purification Kit, 250 (28106)		$640.00	2	2	$1,280	$1,280	
	Subtotal				**$1,856**	**$1,568**	**$3,424**
4. Sigma-Aldrich							
4a. Chloroquine diphosphate (C-6628-25g)		$62.24	1	1	$62	$62	
4b. Hexadecyltrimethylammonium bromide (H6269-100g)		$28.94	2	1	$58	$29	
4c. Potato glucose agar microbiology (110300500)		$117.81	2	1	$236	$118	
4d. Sodium acetate (279293-1g)		$75.22	2	1	$150	$75	
4e. Polyethylene glycol, 400 (25322-68-3, 1L)		$46.60	1	1	$47	$47	
4f. Sodium sulfate (239313-500g)		$75.70	2	1	$151	$76	
4g. Polyethylenimine, branched (408727, 250ml)		$171.00	2	1	$342	$171	
4h. Chitosan, low molecular weight (448869)		$298.00	1	1	$298	$298	
	Subtotal				**$1,344**	**$876**	**$2,220**
5. Thermo Fisher Scientific (chemicals)							
5a. Applied Biosystems Power SYBR Green RNA-to-CT (50591795)		$672.36	1	1	$672	$672	
5b. Invitrogen Lipofectamine RNAiMAX transfection reagent (13778150)		$800.62	1	1	$801	$801	
5c. Epoch DNA/RNA mini spin columns (1920-250)		$201.06	2	1	$402	$201	
5d. Invitrogen Qubit RNA HS Assay kit (Q32855)		$319.76	1	1	$320	$320	
5e. MEGAscript RNAi kit (AM1626)		$650.65	1	1	$651	$651	
	Subtotal				**$2,846**	**$2,644**	**$5,490**
6. EMD Millipore							
6a. siRNAs		$665.00	1	1	$665	$665	
	Subtotal				**$665**	**$665**	**$1,330**

7. Life Technologies							
7a. Qbit RNA HS Assay kit (Q32855)		$352.36	1	1	$352	$352	
	Subtotal				**$352**	**$352**	**$705**
9. Ecology Supplies							
9a. 5 dram plastic snap cap vials		$0.32	10	0	$3	$0	
9b. USNM Unit Trays		$2.95	6	0	$18	$0	
9c. Insect aspirators		$19.99	5	1	$100	$20	
	Subtotal				**$121**	**$20**	**$141**
10. Fuel Cell Earth LLC							
10a. Teflon PTFE DISP 30, 500ml (DISP30-500)		$90.00	1	1	$90	$90	
	Subtotal				**$90**	**$90**	**$180**
11. AddGene Inc							
11a. GFP::L4440 (plasmid) (11335) + $25 shipping on all orders		$113.00	2	0	$226	$0	
	Subtotal				**$226**	**$0**	**$226**
12. UCB DNA sequencing facility							
12a. 200 reactions/year (quantification & sequencing)		$7.50	200	50	$1,500	$375	
	Subtotal				**$1,500**	**$375**	**$1,875**
13. UCB Computational Genomics Resource Lab							
13a. Two memberships/year (4 minimum per lab)		$3,270.00	0.5	0.5	$1,635	$1,635	
	Subtotal				**$1,635**	**$1,635**	**$3,270**
14. Apple Computers							
14a. 13" MacBook Air + sales tax		$2,070.00	1	0	$2,070	$0	
	Subtotal				**$2,070**	**$0**	**$2,070**
					YEAR 1	YEAR 2	**TOTAL**
	OVERALL TOTAL				$20,860	$16,380	**$37,240**
							$37,240

Prices reflect current cost, and may change in the future. Products may be substituted based on availability, cost, or research needs.

Exhibit B3 – Invoice Elements

Invoice and Detailed Transaction Ledger Elements

In accordance with Section 14 of Exhibit C – Payment and Invoicing, the invoice, summary report and/or transaction/payroll ledger shall be certified by the University's Financial Contact and the PI (or their respective designees).

Summary Invoice – includes either on the invoice or in a separate summary document – by approved budget category (Exhibit B) – expenditures for the invoice period, approved budget, cumulative expenditures and budget balance available[1]

- Personnel
- Equipment
- Travel
- Subawardee – Consultants
- Subawardee – Subcontract/Subrecipients
- Materials & Supplies
- Other Direct Costs
    - TOTAL DIRECT COSTS (if available from system)
- Indirect Costs
    - TOTAL

Detailed transaction ledger and/or payroll ledger for the invoice period [2]

- Univ Fund OR Agency Award # (to connect to invoice summary)
- Invoice/Report Period (matching invoice summary)
- GL Account/Object Code
- Doc Type (or subledger reference)
- Transaction Reference#
- Transaction Description, Vendor and/or Employee Name
- Transaction Posting Date
- Time Worked
- Transaction Amount

[1] If this information is not on the invoice or summary attachment, it may be included in a detailed transaction ledger.

[2] For salaries and wages, these elements are anticipated to be included in the detailed transaction ledger. If all elements are not contained in the transaction ledger, then a separate payroll ledger may be provided with the required elements.

Exhibit C – University Terms and Conditions

CMA (AB20) State/University Model Agreement Terms & Conditions 518

https://www.ucop.edu/research-policy-analysis-coordination/_files/cma_documents/exhibit-c_utc-220_feb_2020.pdf

ATTACHMENT 6 – Curriculum Vitae

NEIL DURIE TSUTSUI

Professor and Michelbacher Chair of Systematic Entomology

University of California, Berkeley
Dept. of Environmental Science, Policy, & Management
130 Mulford Hall, #3114
Berkeley CA 94720-3114

ntsutsui@berkeley.edu
(510) 684-5572
https://nature.berkeley.edu/tsutsuilab

EDUCATION

1994. BA, Biology, specialization in Marine Science
Boston University

2000. PhD, Biology. Advisor: Ted J. Case
UC San Diego – Department of Biology

PROFESSIONAL APPOINTMENTS

UC Berkeley
Department of Environmental Science, Policy & Management

2022-present. Director, UC Berkeley Central Sierra Field Research Stations
2017-present. Abraham & Martha Michelbacher Chair of Systematic Entomology
2018-present. Faculty Affiliate, Berkeley Food Institute
2018-present. Faculty Affiliate, Berkeley Institute for People, Parks, & Biodiversity
2007-present. Faculty Affiliate, Essig Museum of Entomology
2014-present. Professor
2010-2012, 2014-2015. Vice Chair for Instruction
2009-2014. Associate Professor
2007-2009. Assistant Professor

UC Irvine
Department of Ecology and Evolutionary Biology

2003-2007. Assistant Professor

UC Davis
Section of Evolution and Ecology, Center for Population Biology

2000-2003. Post-doctoral Associate. Advisor: Richard K. Grosberg

PUBLICATIONS

82. Whyte, B. A., E. I. Cash, and **N. D. Tsutsui**. *In revision.* Colony discrimination and competition in the eusocial trematode, *Himasthla rhigedana*. BEHAVIORAL ECOLOGY & SOCIOBIOLOGY.
81. Ferger, K. & **N. D. Tsutsui.** 2025. Social organization is associated with relaxed selection on worker genes in highly polygyne ants. ECOLOGY & EVOLUTION 15:e71696. http://dx.doi.org/10.1002/ece3.71696
80. Cash, E. I., P. S. Ward, M. Escalona, R. Sahasrabudhe, C. Miller, E. Toffelmier, C. Fairbairn, W. Seligmann, H. B. Shaffer, and **N. D. Tsutsui**. 2024. Genome assembly of the winter ant, *Prenolepis imparis*. J. HEREDITY esae066.

79. Cash, E. I., M. Escalona, P. S. Ward, R. Sahasrabudhe, C. Miller, E. Toffelmier, C. Fairbairn, W. Seligmann, H. B. Shaffer, and **N. D. Tsutsui**. 2024. Reference genome of the kidnapper ant, *Polyergus mexicanus*. J. HEREDITY esae047. https://doi.org/10.1093/jhered/esae047
78. Naughton, I., **N. D. Tsutsui**, P. S. Ward, and D. A. Holway. 2024. An assemblage-level comparison of genetic diversity and population genetic structure between island and mainland ant populations. EVOLUTION qpae103. https://doi.org/10.1093/evolut/qpae103
77. Felden, A., Baty, J. W., Chapple, D. G., Paris, C., Gruber, M. A. M., Haywood, J., Suarez, A. V., **Tsutsui, N. D.**, and P. J. Lester. 2024. Variable viral loads and immune response in an invasive ant's native and introduced ranges. DIVERSITY & DISTRIBUTIONS 00:13867. https://doi.org/10.1111/ddi.13867
76. Adams, S. A., A. Gurajapu, A. Qiang, M. Gerbaulet, S. Schulz, **N. D. Tsutsui**, S. R. Ramírez, and R. G. Gillespie. 2024. Chemical species recognition in an adaptive radiation of Hawaiian *Tetragnatha* spiders (Araneae: Tetragnathidae). PROCEEDINGS OF THE ROYAL SOCIETY OF LONDON B. **291**: 20232340. https://doi.org/10.1098/rspb.2023.2340
75. Ward, P. S., E. I. Cash, K. Ferger, M. Escalona, R. Sahasrabudhe, C. Miller, E. Toffelmier, C. Fairbairn, W. Seligmann, H. B. Shaffer, and **N. D. Tsutsui**. 2023. Reference genome of the bicolored carpenter ant, *Camponotus vicinus*. J. HEREDITY 115(1):120-129. https://doi.org/10.1093/jhered/esad055
74. Whyte, B. A., R. Sandidge, J. Buellesbach, E. I. Cash, K. J. Scheckel, J. D. Gibson, and **N. D. Tsutsui**. 2023. The role of body size and cuticular hydrocarbons in the desiccation resistance of invasive Argentine ants (*Linepithema humile*). J. EXPERIMENTAL BIOLOGY **226**(16): jeb245578. https://doi.org/10.1242/jeb.245578
73. Tonione, M. A., K. Bi, R. R. Dunn, A. Lucky, and **N. D. Tsutsui**. 2022. Phylogeography and population genetics of a widespread cold-adapted ant, *Prenolepis imparis*. MOLECULAR ECOLOGY **31**(18):4884-4899. https://doi.org/10.1111/mec.16624
72. van Wilgenburg, E., M. Mariotta, and **N. D. Tsutsui**. 2022. The effect of diet on colony recognition and cuticular hydrocarbon profiles of the invasive Argentine ant, *Linepithema humile*. INSECTS **13**(4):335. https://doi.org/10.3390/insects13040335
71. Maccaro, J. J., B. A. Whyte, and **N. D. Tsutsui**. 2020. The ant who cried wolf? Short-term repeated exposure to alarm pheromone reduces behavioral response in Argentine ants. INSECTS **11**(12):871. https://doi.org/10.3390/insects11120871
70. Tonione, M. A., K. Bi, and **N. D. Tsutsui**. 2020. Transcriptomic signatures of cold adaptation and heat stress in the thermally sensitive winter ant (*Prenolepis imparis*). PLOS ONE **15**(10): e0239558. https://doi.org/10.1371/journal.pone.0239558
69. Sánchez-Restrepo, A. F., L. Chifflet, V. A. Confalonieri, **N. D. Tsutsui,** M. A. Pesquero, and L. A. Calcaterra. 2020. Species delimitation and molecular

phylogenetics of South American fire ant decapitating flies (Diptera: Phoridae: *Pseudacteon*). PLoS ONE **15**(7): e0236086. https://doi.org/10.1371/journal.pone.0236086

68. Adams, S. A. and **N. D. Tsutsui**. 2020. The evolution of species recognition labels in insects. PHILOSOPHICAL TRANSACTIONS ROYAL SOCIETY LONDON B. **375:** 20190476. https://doi.org/10.1098/rstb.2019.0476
67. Tonione, M. A., S. Cho, G. Richmond, C. Irian, **N. D. Tsutsui**. 2020. Thermal acclimation ability differs between high- and low- elevation populations of the winter ant, *Prenolepis imparis*. ECOLOGY & EVOLUTION **8**:11385-11398. https://doi.org/10.1002/ece3.6229
66. Naughton, I., C. Boser, **N. D. Tsutsui**, and D. A. Holway. 2020. Direct evidence of native ant displacement by the Argentine ant in island ecosystems. BIOLOGICAL INVASIONS **22**:681-691. https://doi.org/10.1007/s10530-019-02121-7
65. Felden, A., C. I. Paris, D. G. Chapple, A. V. Suarez, **N. D. Tsutsui**, P. J. Lester, and M. A. M. Gruber. 2019. Native and introduced Argentine ant populations are characterised by distinct transcriptomic signatures associated with behaviour and immunity. NEOBIOTA. **49**:105-126. https://doi.org/10.3897/neobiota.49.36086
64. Buellesbach, J., B. A. Whyte, B., E. Cash, J. D. Gibson, K. J. Scheckel, R. Sandidge, and **N. D. Tsutsui**. 2018. Desiccation resistance and micro-climate adaptation: Cuticular hydrocarbon signatures of different Argentine ant supercolonies across California. JOURNAL OF CHEMICAL ECOLOGY **44**:1101-1114. https://doi-org.libproxy.berkeley.edu/10.1007/s10886-018-1029-y
63. Torres, C. W., M. A. Tonione, S. R. Ramírez, J. R. Sapp, and **N. D. Tsutsui**. 2018. Genetic and chemical divergence among host races of a socially parasitic ant. ECOLOGY & EVOLUTION. **8**:11385-11398. http://dx.doi.org/10.1002/ece3.4547
62. Felden, A., C. I. Paris, D. G. Chapple, J. Haywood, A. V. Suarez, **N. D. Tsutsui**, P. J. Lester, and M. A. M. Gruber. 2018. Behavioural variation and plasticity along an invasive ant introduction pathway. JOURNAL OF ANIMAL ECOLOGY **87**(6):1653-1666. https://doi.org/10.1111/1365-2656.12886
61. **Tsutsui, N. D.** 2018. Understanding the nature of ant cognition by studying ant cognition in Nature (Box). *Animal Cognition*, Nereida Bueno-Guerra, Federica Amici (eds). Cambridge University Press.
60. **Tsutsui, N. D.** 2018. Strangers in a strange land: *Biological Invasions and Animal Behavior* (Book review). BIOSCIENCE **68**(3):223-224. https://doi.org/10.1093/biosci/bix160
59. Cridland, J., S. R. Ramírez, C. Dean, A. Sciligo, and **N. D. Tsutsui**. 2018. Genome sequencing of museum specimens reveals rapid changes in genetic composition of honey bees in California. GENOME BIOLOGY & EVOLUTION **10**(2):458-472. https://doi.org/10.1093/gbe/evy007

58. Cridland, J., **N. D. Tsutsui**, and S. R. Ramírez. 2017. The complex demographic history and evolutionary origin of the western honey bee, *Apis mellifera*. GENOME BIOLOGY & EVOLUTION **9**(2):457–472. https://doi.org/10.1093/gbe/evx009
57. Mathis, K. A. and **N. D. Tsutsui.** 2016. Dead ant walking: A myrmecophilous beetle predator uses parasitoid host location cues to selective prey on parasitized ants. PROCEEDINGS OF THE ROYAL SOCIETY OF LONDON, B **283**:20161281. https://doi.org/10.1098/rspb.2016.1281
56. Mathis, K. A. and **N. D. Tsutsui.** 2016. Cuticular hydrocarbon cues are used for host acceptance by *Pseudacteon* spp. phorid flies that attack *Azteca sericeasur* ants. JOURNAL OF CHEMICAL ECOLOGY **42**:286-293. 10.1007/s10886-016-0694-y
55. Emery, V. J. and **N. D. Tsutsui.** 2016. Differential sharing of chemical cues by social parasites versus social mutualists in a three-species symbiosis. JOURNAL OF CHEMICAL ECOLOGY **42**:277-285. 10.1007/s10886-016-0692-0
54. Torres, C. W. and **N. D. Tsutsui.** 2016. The effect of social parasitism by *Polyergus breviceps* on the nestmate recognition system of its host, *Formica altipetens*. PLOS ONE **11**(2): e0147498. https://doi.org/10.1371/journal.pone.0147498
53. **Tsutsui, N. D.** 2014. Controlling the spread of Argentine ants. VOICE OF THE PCOC. Fall:10-11.
52. Tillberg, C.V, Edmonds, B., Freauff, A., Hanisch, P., Paris, C., Smith, C.R., **Tsutsui, N. D.**, Wills, B.D., Wittman, S.E., and A.V Suarez. 2014. Foraging ecology of the tropical giant hunting ant *Dinoponera australis* (Hymenoptera: Formicidae) – Evaluating mechanisms for high abundance. BIOTROPICA **46**(2):229-237. https://doi.org/10.1111/btp.12097
51. **Tsutsui, N. D.** 2013. Dissecting ant recognition systems in the age of genomics. BIOLOGY LETTERS **9**:20130416.
50. Lewis V., Moore, S., Tabuchi, R., Sutherland, A., Choe, D.-H., and **N. D. Tsutsui**. 2013. Researchers combat the resurgence of bed bug in behavioral studies and monitor trials. CALIFORNIA AGRICULTURE **67**:172-178.
49. Simola, D. F., Wissler, L., Donahue, G., Waterhouse, R.M., Helmkampf, M., Roux, J., Nygaard, S., Glastad, K.M., Hagen, D.E., Viljakainen, L., Reese, J.T., Hunt, B.G., Graur, D., Elhaik, E., Kriventseva, E.V., Wen, J., Parker, B.J., Cash, E., Privman, E., Childers, C.P., Muñoz-Torres, M.C., Boomsma, J.J., Bornberg-Bauer, E., Currie, C., Elsik, C.G., Suen, G., Goodisman, M.A.D., Keller, L., Liebig, J., Rawls, A., Reinberg, D., Smith, C.D., Smith, C.R., **Tsutsui, N.**, Wurm, Y., Zdobnov, E.M., Berger, S.L. and J. Gadau. 2013. Social insect genomes exhibit dramatic evolution in gene composition and regulation while preserving regulatory features linked to sociality. GENOME RESEARCH **23**:1235-1247.
48. Emery, V. J. and **N. D. Tsutsui**. 2013. Recognition in a social symbiosis: Chemical phenotypes and nestmate recognition behaviors of Neotropical parabiotic ants. PLOS ONE **8**(2): e56492.

47. Choe, D.-H., D. B. Villafuerte, and **N. D. Tsutsui**. 2012. Trail pheromones of the Argentine ant, *Linepithema humile* (Mayr) (Hymenoptera: Formicidae). PLoS ONE **7**(9):e45016.
46. Johnson, B. R., E. van Wilgenburg, and **N. D. Tsutsui**. 2012. Nestmate recognition in social insects is sometimes more complex than an individual based decision to accept or reject (Commentary). BEHAVIORAL ECOLOGY AND SOCIOBIOLOGY **66**:343-346.
45. Choe, D.-H., S. R. Ramírez, and **N. D. Tsutsui**. 2012. A silica gel-based method for extracting insect surface hydrocarbons. JOURNAL OF CHEMICAL ECOLOGY **38**:176-187. DOI 10.1007/s10886-012-0074-1.
44. van Wilgenburg, E., A. Felden, D.-H. Choe, R. Sulc, J. Luo, K. J. Shea, M. A. Elgar, and **N. D. Tsutsui**. 2012. Learning and discrimination of cuticular hydrocarbons in a social insect. BIOLOGY LETTERS **8**:17-20.
43. Ramírez, S. R., T. Eltz, M. K. Fujiwara, G. Gerlach, B. Goldman-Huertas, **N. D. Tsutsui**, and N. E. Pierce. 2011. Asynchronous diversification in a specialized plant-pollinator mutualism. SCIENCE. **333**: 1742-1746.
42. Smith, C. R., A. V. Suarez, **N. D. Tsutsui**, S. E. Wittman, B. Edmonds, A. Freauff, and C. V. Tillberg. 2011. Nutritional asymmetries are related to division of labor in a queenless ant. PLoS ONE **6**:e24011.
41. Johnson, B. R. and **N. D. Tsutsui**. 2011. Taxonomically restricted genes are associated with the evolution of sociality in the honey bee. BMC GENETICS **12**:164.
40. Abbot, P. *et al.* 2011. Inclusive fitness theory and eusociality. NATURE **466**:1057-1062. (Brief communications arising).
39. Suen, G., *et al.* 2011. The genome sequence of the leaf-cutter ant, *Atta cephalotes*, reveals insights into its obligate symbiotic lifestyle. PLoS GENETICS **7**:e1002007.
38. Smith, C. R., *et al.* 2011. A draft genome of the red harvester ant, *Pogonomyrmex barbatus*: a model for reproductive division of labor and social complexity. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA **108**:5667-5672.
37. Smith, C. D., *et al.* 2011. The draft genome of the globally widespread and invasive Argentine ant (*Linepithema humile*). PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA **108**:5673-5678. * **N. D. Tsutsui** – corresponding author.
36. Johnson, B. R., E. van Wilgenburg, and **N. D. Tsutsui**. 2011. Nestmate recognition in social insects: overcoming physiological constraints with collective decision making. BEHAVIORAL ECOLOGY AND SOCIOBIOLOGY **65**:935-944.
35. Franks, S. J., P. D. Pratt, and **N. D. Tsutsui**. 2010. The genetic consequences of a demographic bottleneck in an introduced biological control insect. CONSERVATION GENETICS **12**:201-211.
34. Ramírez, S. R., T. Eltz, F. Fritzsch, R. Pemberton, E. G. Pringle, and **N. D. Tsutsui**. 2010. Intraspecific geographic variation of fragrances acquired by orchid bees in native and introduced populations. JOURNAL OF CHEMICAL ECOLOGY **36**:873-884.
33. van Wilgenburg, E., R. Sulc, K. J. Shea, and **N. D. Tsutsui**. 2010. Deciphering the chemical basis of nestmate recognition. JOURNAL OF CHEMICAL ECOLOGY **36**:751-758.
32. Marsico, T. D., J. W. Burt, E. K. Espeland, G. Gilchrist, M. A. Jamieson, L. Lindstrom, S. Swope, G. Roderick, M. Szucs, and **N. D. Tsutsui**. 2010. Underutilized resources for studying the evolution of invasive species during their introduction, establishment and lag phases. EVOLUTIONARY APPLICATIONS **3**:203-219.
31. Vanderhoeven, S., C. Brown, C. Tepolt, **N. D. Tsutsui**, V. Vanparys, S. Atkinson, G. Mahy, and A. Monty. 2010. Linking concepts in invasion biology: Network analysis

shows what has been studied most and helps identification of knowledge gaps. EVOLUTIONARY APPLICATIONS **3**:193-202.

30. van Wilgenburg, E., C. W. Torres, and **N. D. Tsutsui**. 2010. The global expansion of a single ant supercolony. EVOLUTIONARY APPLICATIONS **3**:136-143.
29. Suarez, A. V., T. McGlynn, and **N. D. Tsutsui**. 2010. Biogeographic patterns of invasive species. In: Lach, L., C. Parr, and K. Abbott, eds., ANT ECOLOGY, pp. 233-244 (Peer-reviewed book chapter).
28. van Wilgenburg, E., J. Clémencet, and **N. D. Tsutsui**. 2010. Experience influences aggressive behaviour in the Argentine ant. BIOLOGY LETTERS **6**:152-155.
27. Brandt, M., E. van Wilgenburg, R. Sulc, K. J. Shea, and **N. D. Tsutsui**. 2009. The scent of supercolonies: The discovery, synthesis and behavioral verification of ant colony recognition cues. BMC BIOLOGY **7**:71.
26. Brandt, M., E. van Wilgenburg, and **N. D. Tsutsui**. 2009. Global-scale analyses of chemical ecology and population genetics in the invasive Argentine ant. MOLECULAR ECOLOGY **18**:997-1005.
25. Cooper, M. L., K. M. Daane, E. H. Nelson, L. G. Varela, M. C. Battany, **N. D. Tsutsui**, and M. K. Rust. 2008. Liquid baits manage Argentine ants in coastal vineyards. CALIFORNIA AGRICULTURE **62**:177-183. (Peer-reviewed extension article).
24. Spagna, J. C., A. I. Vakis, S. N. Patek, X. Zhang, C. Schmidt, **N. D. Tsutsui**, and A. V. Suarez. 2008. Phylogeny, scaling, and the generation of extreme forces in trap-jaw ants. JOURNAL OF EXPERIMENTAL BIOLOGY **211**:2358-2368.
23. Suarez, A. V., D. A. Holway, and **N. D. Tsutsui**. 2008. Genetics and behavior of a colonizing species: The invasive Argentine ant. AMERICAN NATURALIST **172**:S72-S84.
22. Calcaterra, L. A., A. Delgado, and **N. D. Tsutsui**. 2008. Activity patterns and parasitism rates of fire ant decapitating flies (Diptera: Phoridae: *Pseudacteon* spp.) in their native Argentina. ANNALS OF THE ENTOMOLOGICAL SOCIETY OF AMERICA **101**:539-550.
21. **Tsutsui, N. D.**, A. V. Suarez, J. C. Spagna, and J. S. Johnston. 2008. The evolution of genome size in ants. BMC EVOLUTIONARY BIOLOGY **8**:64.
20. Suarez, A. V. and **N. D. Tsutsui**. 2008. The evolutionary consequences of biological invasions. MOLECULAR ECOLOGY **17**:351-360.
19. Torres, C. W., M. Brandt, and **N. D. Tsutsui**. 2007. The role of cuticular hydrocarbons as chemical cues for nestmate recognition in the invasive Argentine ant (*Linepithema humile*). INSECTES SOCIAUX **54**:363-373.
18. Calcaterra, L. A., R. Vander Meer, J. P. Pitts, J. P. Livore, and **N. D. Tsutsui**. 2007. Survey of *Solenopsis* fire ants and their parasitoid flies (Diptera: Phoridae: *Pseudacteon*) in central Chile and central western Argentina. ANNALS OF THE ENTOMOLOGICAL SOCIETY OF AMERICA **100**:512-521.
17. Thomas, M. L., C. M. Payne-Makrisa, A. V. Suarez, **N. D. Tsutsui**, and D. A. Holway. 2007. Contact between supercolonies elevates aggression in Argentine ants. INSECTES SOCIAUX **54**: 225-233.
16. Thomas, M., C. M. Payne-Makrisa, A. V. Suarez, **N. D. Tsutsui**, and D. A. Holway. 2006. When supercolonies collide: Territorial aggression in an invasive and unicolonial social insect. MOLECULAR ECOLOGY **15**:4303-4315.
15. The Honeybee Genome Sequencing Consortium. 2006. Insights into social insects from the genome of the honeybee *Apis mellifera*. NATURE **443**:931-949.

14. Whitfield, C. W., S. K. Behura, S. H. Berlocher, A. G. Clark, S. Johnston, W. S. Sheppard, D. Smith, A. V. Suarez, D. Weaver, and **N. D. Tsutsui**. 2006. Thrice out of Africa: Ancient and recent expansions of the honey bee, *Apis mellifera*. SCIENCE **314**:642-645.
13. Thomas, M., **N. D. Tsutsui**, and D. A. Holway. 2005. Intraspecific competition influences the symmetry and intensity of aggression in the invasive Argentine ant. BEHAVIORAL ECOLOGY **16**:672-481.
12. **Tsutsui, N. D.** 2004. Scents of self: The expression component of self/non-self recognition systems. ANNALES ZOOLOGICI FENNICI **41**:713-727.
11. Suarez, A. V. and **N. D. Tsutsui**. 2004. The value of museum collections for research and society. BIOSCIENCE **54**:66-74.
10. **Tsutsui, N. D.**, S. N. Kauppinen, A. F. Oyafuso, and R. K. Grosberg. 2003b. The distribution and evolutionary history of *Wolbachia* infection in native and introduced populations of the invasive Argentine ant (*Linepithema humile*). MOLECULAR ECOLOGY **12**:3057-3068.
9. **Tsutsui, N. D.** and A. V. Suarez. 2003. The colony structure and population biology of invasive ants. CONSERVATION BIOLOGY **17**:48-58.
8. **Tsutsui, N. D.**, A. V. Suarez, and R. K. Grosberg. 2003. Genetic diversity, asymmetrical aggression, and recognition in a widespread invasive species. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA **100**:1078-1083.
7. Suarez, A. V., D. A. Holway, D. Liang, **N. D. Tsutsui**, and T. J. Case. 2002. Spatio-temporal patterns of intraspecific aggression in the invasive Argentine ant. ANIMAL BEHAVIOUR **64**:697-708.
6. Holway, D. A., L. Lach, A. V. Suarez, **N. D. Tsutsui**, and T. J. Case. 2002. The ecological causes and consequences of ant invasions. ANNUAL REVIEW OF ECOLOGY AND SYSTEMATICS **33**:181-233.
5. Suarez, A. V., M. Benard, **N. D. Tsutsui**, T. A. Blackledge, K. Copren, E. M. Sarnat, A. L. Wild, W. M. Getz, P. T. Starks, K. Will, P. J. Palsboll, M. E. Hauber, C. Moritz, and A. D. Richman. 2002. Conflicts around a study of Mexican crops. NATURE **417**:897. (*Correspondence*).
4. **Tsutsui, N. D.**, A. V. Suarez, D. A. Holway, and T. J. Case. 2001. Relationships among native and introduced populations of the Argentine ant, *Linepithema humile* and the source of introduced populations. MOLECULAR ECOLOGY **10**:2151-2161.
3. **Tsutsui, N. D.** and T. J. Case. 2001. Population genetics and colony structure of the Argentine ant (*Linepithema humile*) in its native and introduced ranges. EVOLUTION **55**:976-985.
2. **Tsutsui, N. D.**, A. V. Suarez, D. A. Holway, and T. J. Case. 2000. Reduced genetic variation and the success of an invasive species. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA **97**:5948-5953.
1. Suarez, A. V., **N. D. Tsutsui**, D. A. Holway, and T.J. Case. 1999. Behavioral and genetic differentiation between native and introduced populations of the Argentine ant. BIOLOGICAL INVASIONS **1**:43-53.

GRANT SUPPORT

(* currently active grants)

* US DEPT. OF DEFENSE, STRATEGIC ENVIRONMENTAL RESEARCH AND DEVELOPMENT PROGRAM (SERDP), "*From the lab to the field: Continued RNAi development for invasive ant eradication*", Tsutsui, N. D. (PI), and B. Hoffmann, M. Melzer, T. Walsh. **$1,254,530** (UCB **$624,250**).
* State of California, Wildlife Conservation Board, Proposition 68, "*Sagehen Creek Field Station Facilities Improvement Project*". **$203,276**
* NATIONAL SCIENCE FOUNDATION, LONG-TERM RESEARCH IN ENVIRONMENTAL BIOLOGY (LTREB), Holway, D. A. (PI), Tsutsui as co-PI. "*LTREB Collaborative Proposal: Large-scale removal of introduced ants as a test of community reassembly*" (renewal). Aug. 2022 – April 2027. **$599,997** (UCB **$300,002**).

NATIONAL SCIENCE FOUNDATION, DIVISION OF BIOLOGICAL INFRASTRUCTURE (DBI), "*Collaborative Research: Digitization TCN: Extending Anthophila research through image and trait digitization (Big-Bee)*", Seltmann, K. (PI), Tsutsui and eight others as co-PI. Sept. 2021 – Aug. 2024. **$1,288,241** (UCB **$235,963**).

UC Natural Reserve System, Environmental Monitoring and Data Science, "*Wireless mesh radio network upgrade for Sagehen Creek Field Station*". 2024. **$5,191**

UC Natural Reserve System, Critical Needs Infrastructure, "*Snowblower for Sagehen Creek Field Station*". 2024. **$4,000**

UC Berkeley, Be Smart About Safety, "*Snowmobile for emergency transport at Sagehen Creek Field Station*". 2024. **$19,065**

CALIFORNIA CONSERVATION GENOMICS PROJECT, "*Conservation genomics of three California ant species*", Tsutsui, N.D. (PI) and P.S. Ward. Nov. 2020 – Jan. 2024, **$78,298** (UCB **$45,645**).

US DEPT. OF DEFENSE, STRATEGIC ENVIRONMENTAL RESEARCH AND DEVELOPMENT PROGRAM (SERDP), "*RNAi development for invasive ant eradication*", Tsutsui, N. D. (PI), and B. Hoffmann, T. Walsh. May 2021 – April 2024. **$654,433** ($592,433 + $62,000 Supplement) (UCB **$378,763**).

CALIFORNIA STRUCTURAL PEST CONTROL BOARD, "*Diet and colony structure of two emerging invasive pest ants*", October 2018 – August 2022. **$146,325**.

NATIONAL SCIENCE FOUNDATION, LONG-TERM RESEARCH IN ENVIRONMENTAL BIOLOGY (LTREB), Holway, D. A. (PI), Tsutsui as co-PI. "*LTREB Collaborative Proposal: Large-scale removal of introduced ants as a test of community reassembly*". April 2017 – April 2022. **$391,313** (UCB **$177,548**).

NATIONAL SCIENCE FOUNDATION, IOS-SDS, "*Collaborative Research: Scents of self: How trade-offs shape self/non-self recognition cues in a supercolonial insect*", Feb. 2016 – Jan. 2020. **$830,933** (UCB **$605,479)**. (ranked #1 in panel)

US DEPARTMENT OF AGRICULTURE, NIFA-AFRI. "*Using functional genomics to link behavior, chemical ecology and chemoreception in a widespread and damaging agricultural pest*" Feb. 2016 – Jan. 2019. **$498,083**.

UC BERKELEY BAKAR FELLOW, "*Deploying insect pheromones to control a widespread and damaging insect*", July 2012 – June 2017. **$250,000**.

UC MEXUS, (K. Mathis, co-PI), "*Host Selection and Successful Parasitism p of the aggressive arboreal ant* Azteca instabilis *by decapitating phorid flies (Genus:* Pseudacteon*) in dynamic coffee agroecosystems*", Jan. 2012 – Dec. 2013. **$11,852**.

GORDON AND BETTY MOORE FOUNDATION, "*Using genomics, isotopes and pollen to illuminate the past and predict the future of California bees*", Nov. 2011 – Oct. 2014. **$379,339**.

CALIFORNIA STRUCTURAL PEST CONTROL BOARD, "*Bedbug detection using airborne pheromone cues*". Feb. 2011 – Feb. 2012. **$116,650**.

DEFINING WISDOM, University of Chicago Arete Initiative and the John Templeton Foundation. "*The wisdom of the ant: The role of experience in sociality and aggression*". Oct. 2008 – Sept. 2010. **$100,000**.

US DEPARTMENT OF AGRICULTURE, NRI-CSREES 2008-35302-04680. "*An integrative analysis of altered social behavior in the invasive Argentine ant (*Linepithema humile*)*". Sept. 2008 – Aug. 2011. **$425,000**.

COMMITTEE ON RESEARCH, FACULTY RESEARCH GRANT (UC-BERKELEY), "*A preliminary analysis of candidate genes for aggression in the invasive Argentine ant*". 2007 – 2008. **$5,000**.

AUSTRALIAN RESEARCH COUNCIL, "*Sociality and a sense of smell: Receptor organ evolution in ants*". (PI: Mark A. Elgar, University of Melbourne). 2008 – 2010. **$225,000**.

CALIFORNIA STRUCTURAL PEST CONTROL BOARD, "*The role of genetics and cuticular hydrocarbons in Argentine ant aggression*". (Co-Investigator: Ken Shea, UC-Irvine, Department of Chemistry). Sept. 2005 – Aug. 2008. **$312,037**.

US DEPARTMENT OF AGRICULTURE, NRI-CSREES 2004-35302-14865. "*The genetic and biochemical basis of altered social behavior in the invasive Argentine ant (*Linepithema humile*)*" (ranked #2 of 117). Sept. 2004 – Aug. 2007. **$325,000**.

TEACHING & MENTORSHIP

GRADUATE STUDENTS ADVISED:

Eleanor Terner (Ph.D.; 2025–present)
Rachel Weinberg (Ph.D.; 2019–present)
Amelia Harvey (Ph.D.; 2017–present)
Kelsey Scheckel (Ph.D.; 2014–2022)
Rebecca Sandidge (Ph.D.; 2008–2018)
Kaitlyn Mathis (Ph.D.; 2009–2015)
Candice Torres (Ph.D.; 2005–2012)
Kailey Ferger (Ph.D.; 2022–present)
Brian Whyte (Ph.D.; 2015–2022)
Nina Pak (Ph.D.; 2017–2021)
Maria Tonione (Ph.D.; 2012–2018)
Tara Madsen-Steigmeyer (Ph.D.; 2011–2014)
Virginia Emery (Ph.D.; 2009–2013)

POST-DOCTORAL RESEARCHERS ADVISED:

Dr. Ida Naughton (2023–present)
Dr. Elizabeth Cash (2016–2023)
Dr. Jan Buellesbach (2016–2018)
Dr. Santiago Ramirez (2008–2013)
Dr. Dong-Hwan Choe (2009–2011)
Dr. Johanna Clemencet (2006–2007)
Dr. Megan McPhee (2003–2005)
Dr. Tyler Larsen (2024–present)
Dr. Maria Tonione (2018–2020)
Dr. Joshua Gibson (2016–2017)
Dr. Kaustubh Gokhale (2014–2016)
Dr. Brian Johnson (2009–2011)
Dr. Ellen van Wilgenburg (2006–2010)
Dr. Miriam Brandt (2005–2007)

GRADUATE STUDENT COMMITTEE MEMBER

Qualifying exam and/or dissertation committee for 49 PhD students.

UNDERGRADUATES MENTORED:

>100 since 2003.

COURSES TAUGHT:

UC-Berkeley

Insect Behavior (ESPM 142; Lecture only), Spring 2025. (ESPM 142; Lecture + Lab), Fall 2022, 2023. (ESPM 150, ESPM 142; Lecture + Discussion), Fall 2008-2012; 2015-2017, 2019-2020.

Current Topics in Social Insect Behavior (ESPM 242), Spring 2025.

Recent Discoveries of Unusual Reproductive Systems in Insects (ESPM 290), Spring 2023.

Inversions, Supergenes, and Other Genetic Oddities (ESPM 290), Spring 2023.

RNA Interference in Insect Model Systems (ESPM 290), Spring 2022.

Molecular Approaches to Environmental Problem Solving (ESPM 192**)**, Spring 2015, 2016, 2018, 2019.

Advanced Chemical Ecology (ESPM 290), Spr 2014, Fall 2014, 2016, Spr 2020, 2021.

Senior Seminar-Conservation & Resource Studies (ESPM 194), Spring 2011 (two classes), Spring 2012.

Genetics and Genomics of Insect Behavior (ESPM 290), Spring 2009.

Environmental Science Forum (ESPM 201C**)**, Spring 2008.

UC-Irvine

Graduate Core in Evolution, Winter 2007.

Advanced Evolutionary Biology (BS168), Spring 2004, 2005, 2006.

Freshman Seminar (BS2B), "Social behavior of ants", Spring 2004, 2005, 2006, 2007.

Faculty Appreciation Award – Alpha Epsilon Delta (AED) Pre-Medical Honor Society, Spring 2006.

INVITED PRESENTATIONS & PROFESSIONAL MEETINGS

76 departmental seminars and conference talks since 2003. Includes two graduate student invited talks and one keynote.

SERVICE

SERVICE TO UC SYSTEM:

Chair – Ten-year review committee, UC Education Abroad Program (UCEAP), "*Tropical Biology and Conservation*", Monteverde, Costa Rica. 2023.

Member – Faculty advisory committee, UC Education Abroad Program (UCEAP). 2013–2018.

SERVICE TO CAMPUS:

Faculty Director, UC BERKELEY Central Sierra Field Research Stations. July 2022–present.

Member – Berkeley Institute for Global Change Biology. 2010–2020.

Chair – Search Committee, Sagehen Creek Field Station, Reserve Director, Jan.–May 2024.

Chair – Search Committee, Sagehen Creek Field Station, Reserve Director, Sept.–Dec. 2023.

Member – Search Committee. UCB Academic Coordinator for UC Natural Reserve System. Dec. 2022–March 2023.

Member –Sagehen Creek Field Station Advisory Committee, February 2021–July 2022.

Member – Search Committee, Sagehen Creek Field Station, Station Manager, February – June 2021.
Member – UC BERKELEY, Hill Campus, Long Range Development Plan Committee, 2019–2021.
Member, Academic Senate co – Committee on Courses of Instruction (COCI). 2014–2018.
- Sciences subcommittee. 2014–2015.
- Variances subcommittee. 2015–2016.

Member – Academic Senate Advisory Committee, Student Information Systems. 2016–2018.
Member – *Ad hoc* campus tenure review committee – 2010, 2014.
Member – Ecological Preserve and Open Space committee. 2006–2007 (UCI).
Member – Phi Beta Kappa nominating committee. 2004–2007 (UCI).

SERVICE TO SCHOOL/COLLEGE:

Member – RCNR Endowed Chairs committee. July 2023–present.
Member – Search Committee, Executive Director for UC Berkeley Institute for People, Parks, and Biodiversity. June–October 2022.
Member – Search Committee, Executive Director for UC Berkeley Institute for People, Parks, and Biodiversity. Nov 2021–April 2022.
Member – RCNR Executive Committee, Sept 2019–Oct. 2021.
Member – RCNR Committee on Centers and Facilities, Nov 2020–Jan 2021.
Faculty Judge – College of Natural Resources Gradfest Symposium. 2011.
Faculty Judge – College of Natural Resources Honor Symposium. 2010, 2015.
Member – Biological Sciences Executive Committee. Elected, 2006–2008 (UCI).
Member – Crystal Cove Research Cottage committee. 2005–2006 (UCI).

SERVICE TO THE DEPARTMENT:

Member – Student Endowment Allocation Committee. 2022–present.
Member – Molecular Environmental Biology advising committee. 2008–present.
Chair – *Ad hoc* personnel action committee. 2007, 2009, 2012, 2016, 2021, 2023.
Member – Search Committee, Assistant Professor in Forest Health. 2023–2024.
Member – Graduate admissions committee. Division of Organisms & Environment. 2022–2023.
Chair – Diversity, Equity, and Inclusion (DEI) committee, Mentoring working group. 2020–2021.
Member – DEI Committee. 2019–2021.
Member – Philomathia Prize selection committee. 2021.
Member – Search Committee, Assistant Professor in Race, Culture, and Environment. 2016.
Vice Chair for Instruction. 2010–2012, 2014–2015.
Chair – Undergraduate Programs Committee. 2010–2012, 2014–2015.
Member – ESPM Departmental Vision committee. 2012.
Member – Graduate student advising committee. 2008–present.
Member – Assistant Professor in Global Change Organismal Biology search committee. 2010.
Member – Environmental Science undergraduate major committee. 2008–2010.
Member – Environmental Science undergraduate major transition committee. 2010–2011.

Chair – Safety Committee. 2009.
Member – Graduate admissions committee. 2009–2010.
Chair – Graduate student awards committee. 2007–2008.
Chair – Molecular Analytical Facility Steering committee. 2006–2007. (UCI)
Member – Search Committee, Assistant Professor in Ecology. 2 positions; 2005–2006. (UCI)
Member – Departmental web-page committee. 2003–2007. (UCI)
Member – *Ad hoc* personnel merit review committee; 5 times from 2003–2007. (UCI)

REVIEWER (manuscripts and grant proposals, excluding panel service below): Several hundred from July 2003–present
Journals, including: *The American Naturalist, Animal Behaviour, Annals of the Entomological Society of America, Behavioral Ecology, Behavioral Ecology & Sociobiology, Biology Letters, BMC Ecology, Conservation Biology, Diversity & Distributions, Ecological Entomology, Ecology, Ecology Letters, eLife, Evolution, Evolutionary Applications, Evolutionary Ecology Research, Genetics, Insectes Sociaux, Insects, Journal Chemical of Ecology, Naturwissenschaften, Molecular Ecology, Oecologia, PLoS One, Proceedings of the National Academy of Sciences, Scientific Reports.*
Funding agencies, including: *The National Science Foundation, US Department of Agriculture, National Institute for Climate Change Research (US Dept of Energy), Natural Environment Research Council.*

SERVICE TO COMMUNITY
EAST BAY REGIONAL PARK DISTRICT, Parks Advisory Committee. Appointed by Contra Costa County Board of Supervisors.
Chair (elected), January–December 2024.
Vice-chair (elected), January 2022–December 2023.
Member, June 2017– December 2024.
SIERRA CLUB, West Contra Costa group.
Vice-chair (elected), May 2020–April 2022.
ENVIRONMENTAL QUALITY COMMITTEE, City of El Cerrito.
Chair (elected), May 2020–April 2022.
Member, Aug. 2017–February 2023.

EDITORIAL BOARDS:
BMC ECOLOGY. Associate Editor, 2011–present.
BIOLOGICAL INVASIONS. Associate Editor, 2011–2024.
PLOS GENETICS. Guest Editor, 2013.
AUSTRALIAN JOURNAL OF ZOOLOGY. Associate Editor, 2007–2012.

PANEL MEMBER:
NATIONAL SCIENCE FOUNDATION – IOB Behavioral Systems. 2011, 2013.
NATIONAL SCIENCE FOUNDATION – DEB Phylobiogeography. 2008.
NATIONAL SCIENCE FOUNDATION – Doctoral Dissertation Improvement Grants. 2007.
US DEPARTMENT OF AGRICULTURE – Arthropod & Nematode Biology: Organismal & Population Biology. 2007, 2009.

US DEPARTMENT OF AGRICULTURE – ARS, OSQR: Ants and Termites. 2009.

CO-FOUNDER

BACKYARD BIODIVERSITY PROJECT. 2016–present.
BAY AREA ANT GROUP (BAAG). 2007–2020.

COMMITTEE MEMBER:

NATIONAL ECOLOGICAL OBSERVATORY NETWORK (NEON) DESIGN CONSORTIUM, Invasive Species subcommittee. 2004–2005.

MEETING CO-ORGANIZER

California Population & Evolutionary Genetics meeting (CALPEG), December 2003.

MEMBERSHIPS

MEMBER:

American Association for the Advancement of Science. 2016–present.
Sigma Xi Honor Society. 2005–present.
Phi Beta Kappa Honor Society. 1994–present.
American Federation of Teachers. 2019–2023.
Entomology Society of America. Intermittently since 2007.
American Society of Parasitologists. 2019–2020.
Society for the Study of Evolution. 1997–2005, 2019-2020.
UC-Irvine, Institute for Genomics and Bioinformatics. 2003–2007.
American Society of Ichthyologists and Herpetologists. 1998–2000.

ATTACHMENT 7 – Narrative of Research Objectives

A. Briefly state what the research described in this application is intended to accomplish and what hypotheses or research questions are to be tested.

While conventional insecticides remain the primary tools for structural pest management, they are often broad-spectrum, environmentally persistent, and can drive the evolution of insecticide resistance. In contrast, RNA interference (RNAi) is a natural biological process that uses double-stranded RNA (dsRNA) molecules to selectively switch off genes essential for an organism's survival. Although RNAi-based methods have shown promise in agriculture, their application to structural pest control has been limited by delivery challenges. This project aims to develop RNAi as a novel, species-specific strategy to control Argentine ants by testing the hypothesis that combining protective carriers (chitosan nanoparticles and carbon quantum dots) with optimally designed dsRNA constructs will significantly improve gene silencing and ant mortality. The research will identify the most effective dsRNA length, assess whether chitosan or carbon quantum dots improve stability and uptake, and evaluate whether combining them further increases efficacy. By overcoming these barriers, the project will establish RNAi as a scalable, precise, and environmentally responsible alternative to conventional insecticides. Although the target species for the research proposed here is the Argentine ant, once optimized, this technology could potentially be extended to any insect pest.

B. Briefly sketch the background of the present proposal, critically evaluating existing knowledge and identifying gaps that this project is intended to fill.

RNA interference has emerged over the past two decades as a powerful tool for managing agricultural pests, offering an approach that can be tailored to target specific species without harming beneficial insects or other non-target organisms. In particular, RNAi has been successfully commercialized in crops like corn, where plants expressing dsRNA suppress essential genes in pests such as corn rootworm, demonstrating that gene silencing can be a reliable and safe pest control strategy. However, translating this success to non-crop contexts has proven more difficult. Unlike transgenic plants, which continuously produce dsRNA, structural pest control requires delivering dsRNA through baits that must survive harsh digestive environments and reach target tissues in insects with different feeding behaviors. Our recent studies have shown that feeding purified dsRNA to ants can induce mortality, but outcomes have been inconsistent due to degradation in the gut, limited cellular uptake, and insufficient persistence. Although preliminary experiments, including work in the Tsutsui lab funded by the Department of Defense, have demonstrated proof of concept in Argentine ants, no standardized formulation has yet overcome these barriers reliably. In particular, the potential of scalable, low-cost carriers such as chitosan and carbon quantum dots to improve dsRNA stability and delivery in ants remains unexplored. This project is designed to fill these knowledge gaps by systematically testing and optimizing these delivery strategies to establish a practical RNAi platform for structural pest management. In addition, we proposed development of informational outreach materials for pest control professionals that will be delivered as factsheets and presentations at relevant conferences and trainings.

C. Focus the study on the prioritized need(s) identified in the solicitation notice criteria.

This proposal directly addresses the prioritized needs outlined in the SPCB-25-01 solicitation by advancing new treatment methods within the framework of integrated pest management (IPM) for ants, a key target group specified by the Board. The research focuses on developing species-specific, RNA interference (RNAi)-based baits that align with California's policy goals to reduce reliance on conventional broad-spectrum insecticides, which pose risks to public health, non-target species, and the environment.

By combining advances in genomic tools with affordable, scalable delivery systems (chitosan and carbon quantum dots), this project will fill a critical gap in structural pest management: the lack of effective, selective technologies for Argentine ant control in urban and structural settings. Because the approach is designed to integrate seamlessly with existing IPM strategies, leveraging baiting systems and behavioral ecology of target ants, it offers a practical pathway to reduce pesticide use, support sustainable pest management, and advance public outreach by demonstrating new science-based alternatives.

This research responds to the Board's explicit emphasis on developing innovative technologies that promote IPM, reduce high-risk pesticide applications, and support adoption by pest management professionals. Successful outcomes will also create an adaptable platform technology that could potentially be extended to other structural pests, including other ant species, cockroaches, and termites.

ATTACHMENT 8

2. NARRATIVE OF PROJECT DIRECTION (WORK PLAN AND WORK SCHEDULE)

RNAi SOLUTIONS FOR LOW-IMPACT ARGENTINE ANT MANAGEMENT

2A. Research Design and Procedures

Objectives

Genomic technologies have revolutionized the biological sciences, including agriculture, human health, natural resource management, and our understanding of fundamental biological processes. However, this genomic revolution has had little, if any, role in the field of structural pest control. In recent years, my lab has been refining a powerful new genomic technique, based on RNA interference (RNAi), to control or eradicate invasive ants by turning their own immune system against themselves. RNAi-based pest control has previously been developed and applied in agricultural settings as part of modern IPM approaches for controlling crop pests, so safety and efficacy have been previously established. This technology is designed in a species-specific manner, so it is harmless to non-target organisms, such as pollinators, pets, and people. A previous grant to my lab from the US Department of Defense (DOD) supported our successful proof of concept, and a newly awarded grant from the same program will allow us to advance this technology by screening new genomic targets in several different ant species, testing alternative designs, and assessing methods of delivery. Below, I propose a complementary research project that will develop formulations to increase efficacy and drive down cost. The research I propose here has no overlap with my currently funded projects, but it fills essential gaps in our knowledge that will be necessary for scaling up this technology for broader use. Although we focus here on Argentine ants, once we have developed and optimized our approach and formulation, re-focusing this technology on different target ant species (or even non-ant insects) should be straightforward.

In 2023, the *Sustainable Pest Management Work Group* published "*Accelerating Sustainable Pest Management: A Roadmap for California*" as a guiding document to advance the development of more sustainable, less toxic methods of pest control, particularly in urban and agricultural systems. This document gathers together an abundance of useful information and points the way forward for pest control in California. Three crucial points made in the *Roadmap* are:

1) The use of non-agricultural pesticides is extremely high, and risks to the environment and human health are a serious concern. As noted in the SPM Roadmap Executive Summary, "*Based on limited current data, nonagricultural uses account for between 35-55 percent of pesticide sales (pounds sold), 16-19 percent of reported pesticide use (pounds applied primarily by licensed applicators), and 65-75 percent of reported pesticide-related illnesses*", and these figures are likely substantial underestimates.
2) **Goal #1** in this document is that, by 2050, California will have "…*eliminated the use of Priority Pesticides by transitioning to sustainable pest management practices*".
3) **Keystone Action C** in this document includes, "*Expand funding and infrastructure for urban SPM research, innovation, and outreach*".

The research that I describe here addresses these goals by advancing the development of cutting edge, low toxicity, species-specific pest management. RNAi-based pest management is exactly the type of new technology that, when incorporated into a portfolio of Sustainable Pest Management approaches, will enable California to achieve Goal #1.

Likewise, the research that I describe here meets several of the needs expressed in the Structural Pest Control Board RFP, including:

- Development of novel control methods for important structural pests such as Argentine ants
- Reduction in the use of traditional chemical pesticides in and around structures
- Exploration of low-toxicity, targeted pest management strategies
- Minimization of environmental and non-target impacts from pest control practices
- Scientific advancement to support the future of structural pest control regulation and best practices

The research I propose here is focused on advancing and developing RNAi as a new approach for structural pest control. Specifically, I propose the following objectives and hypotheses:

Objective 1) Determine optimal length of dsRNA constructs.

Hypothesis 1: Longer dsRNA constructs (100-200 nucleotides) will induce RNAi more effectively than short constructs (19-24 nucleotides).

Objective 2) Test palatability and toxicity of chitosan and carbon quantum dot (CQD) additives.

Hypothesis 2: Both chitosan and CQDs will be palatable and non-toxic to Argentine ant workers.

Objective 3) Quantify whether chitosan additive increases efficacy of RNAi.

Hypothesis 3: Adding chitosan to the dsRNA formulation will increase efficacy of RNAi (compared to controls).

Objective 4) Test whether CQD additive increases efficacy of RNAi.

Hypothesis 4: Adding CQDs to the dsRNA formulation will increase efficacy of RNAi (compared to controls).

Objective 5) Determine impact of chitosan and carbon quantum dots together on efficacy of RNAi.

Hypothesis 5: Formulations with chitosan and CQD together will produce stronger RNAi than formulations with either alone.

Background

Although insects are, by far, the most diverse and abundant animals on earth, only a small number of insect species have become structural pests. Ants are consistently responsible for many of the most chronic and severe pest infestations in California, and the Argentine ant (*Linepithema humile*) has historically been the most common ant species that pest control professionals are called upon to address.

Ants can be difficult to control for a variety of reasons: 1) the vast majority of individuals are non-reproductive workers, so their elimination does not directly impact reproductive output, 2) some species have colonies with many queens, making it difficult to eliminate all reproductive individuals (and making it easy to introduce viable propagules to new locations), 3) they display

surprisingly complex behaviors, such as learned insecticide avoidance (Zanola et al. 2024) and, 4) they have the potential to evolve insecticide resistance (Pu & Chung 2024), thus reducing the efficacy of conventional control strategies.

At the same time, some features of ant biology present opportunities for the development of new controls, including: 1) the strong attraction of many ant species (including Argentine ants) to liquid baits (Cooper et al. 2008), 2) the ability of workers to consume food then distribute it to other colony members, including queens and young, via regurgitation (aka trophallaxis; LeBoeuf 2017), 3) ease of handling in the laboratory setting, which enables large, well-replicated experiments, and 4) relatively well-developed genomic resources, including many sequenced reference genomes and relatively small genome sizes (Tsutsui et al. 2008; Smith et al. 2011).

One of the primary concerns associated with current methods of control pests, including ants, is the toxicity of conventional insecticides. These insecticides can persist in soil, water, and air, and there are potential negative effects on non-target organisms, including pollinators, pets, and humans. In this context, an ideal insecticide would display a very narrow range of toxicity, producing mortality only in the targeted species, and be harmless to all other species.

In recent years, we have developed a new approach to controlling invasive ants in a species-specific manner. At the most basic level, this technology works by turning the ant's own immune system against itself, turning off (or "silencing") genes that are essential for the ant's survival. More specifically, we have designed double-stranded RNA (dsRNA) molecules that, when fed to ants in sugar solution, initiate the RNA interference (RNAi) immune response, turning off expression of an essential gene, leading to death of the ants (see "*What is RNA interference (RNAi)?*", below).

We have recently completed a project, funded by a grant from the US Department of Defense, Strategic Environmental Research and Development Program (DOD-SERDP), to develop a proof of concept for this new technology, showing that it produces mortality in several different invasive ant species, including the Argentine ant (Figure 1). After successfully accomplishing this goal, we were recently awarded a second grant from DOD-SERDP to increase efficacy by: 1) identifying new gene targets, 2) testing new structural variants, 3) developing a high-output bacterial expression system, and 4) testing liquid bait delivery systems. The research that I describe in this proposal complements and extends this DOD-funded research and will fill several important knowledge gaps that are current barriers to successful RNAi-based ant control. Moreover, the results of this research will inform RNAi-based insect control generally, accelerating the use of these methods for a variety of different structural pests as well as, potentially, other insect pests and vectors of human and livestock diseases.

What is RNA interference (RNAi)?

All organisms possess immune systems that recognize and target viral genetic material. Some types of viruses have genomes that are made of double-stranded RNA (dsRNA). When cells detect dsRNA inside themselves they perceive this as a viral infection and turn on the RNAi immune response. This immune response destroys the dsRNA, but also seeks out and destroys any other genetic material that resembles this dsRNA. We have constructed dsRNA molecules that contain parts of the ant's own genome within them. When ants consume these synthetic dsRNA molecules, the immune response is tricked into attacking the ant's own genetic material. Because we insert sequences of genes that are essential for the ant's survival, the immune response shuts down necessary biological processes, leading to death of the ants. Moreover,

because we can select gene sequences that are unique to a specific ant species, mortality only occurs in the target species; non-target organisms remain unharmed if they consume the dsRNA because they do not possess these sequences in their genomes.

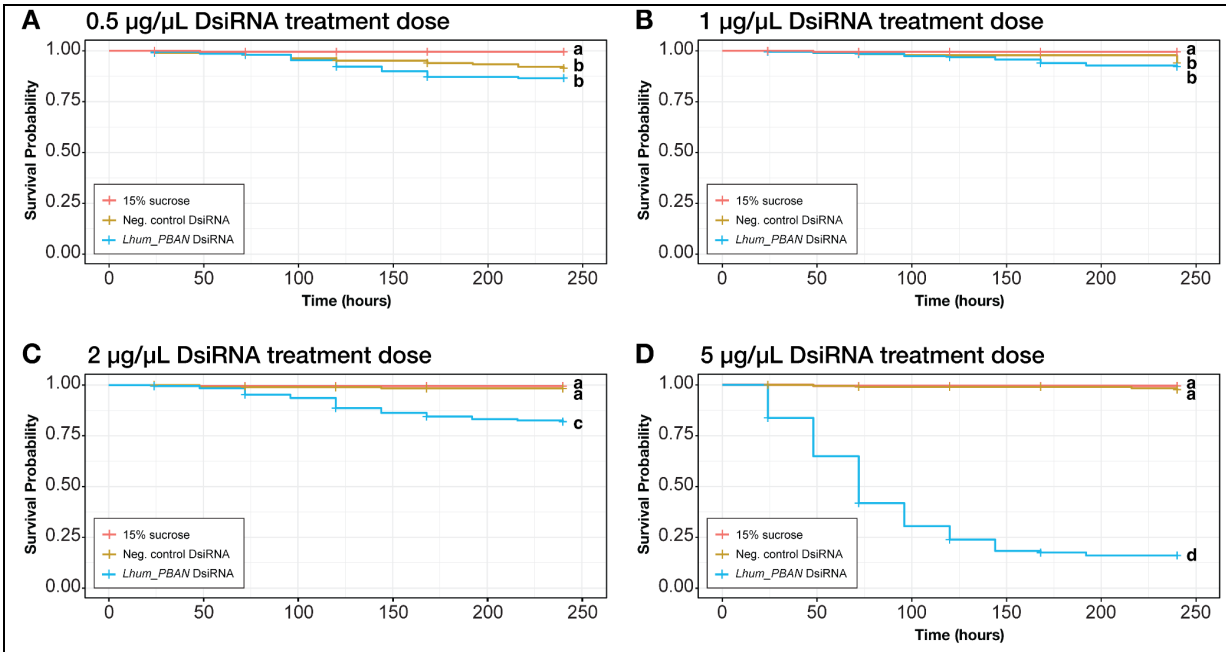

Figure 1. Example of successful RNAi-mediated mortality in the Argentine ant. When low concentrations of dsRNA are fed to Argentine ant workers (A and B), survival is not affected. At a concentration of 2ug/uL, mortality increases to about 20% (C). However, at a dsRNA concentration of 5ug/mL, we achieve ~80% mortality (blue line in part D) compared to negative controls (brown and red lines). The dose-dependent effect seen here shows that this is a direct effect of the dsRNA feeding. (DsiRNA refers to the specific design of the dsRNA)

The current state of RNAi technology for pest control

RNA interference (RNAi) has emerged as a powerful and increasingly practical tool for insect pest control in the agricultural sector. Several crop protection strategies now leverage RNAi to selectively silence essential genes in pest insects, resulting in reduced survival, reproduction, or feeding. For example, transgenic corn expressing dsRNA targeting the western corn rootworm (*Diabrotica virgifera virgifera*) has demonstrated effective root protection and received regulatory approval in multiple countries (e.g. Fishilevich et al. 2016; Darlington et al. 2022). Sprayable RNAi formulations are also under development, offering a non-transgenic alternative for managing pests such as the Colorado potato beetle (*Leptinotarsa decemlineata*)(reviewed in Hanamasagar et al. 2024). These successes highlight the potential of RNAi as a species-specific, environmentally benign method for controlling harmful insects, while minimizing risks to beneficial species and reducing reliance on conventional chemical insecticides.

A few papers have been published on the use of RNAi for the control of ants, but the results are mixed and inconsistent. While some studies have reported successful gene knockdown and measurable phenotypic effects in certain ant species, others have found limited efficacy, low mortality, or variable uptake of dsRNA. Factors contributing to this inconsistency include differences in dsRNA delivery methods, target gene selection, and species-specific RNAi

sensitivity. As noted in Liu & Yang (2025), achieving reliable RNAi-induced mortality in ants has been challenging, and further research is needed to optimize delivery systems and identify more consistent gene targets.

Our previous research has made several key advances that have set the stage for the research proposed here. Most importantly, we have shown that we can trigger Argentine ant mortality in a dose-dependent manner using dsRNA that targets the PBAN gene (Pheromone Biosynthesis Activating Neuropeptide). The survival curves in Figure 1 show that, at the low doses of 0.5 and 1ug/uL, there is minimal ant mortality through time (Figures 1A, 1B). However, once the dsRNA concentration is increased to 2ug/uL (blue line in Figure 1C), survival is significantly lower compared to controls (brown and red lines). Moreover, at dsRNA concentration of 5 ug/uL (Figure 1D, blue line), there is significant and substantial mortality of Argentine ant workers compared to controls (brown and red lines). **Amazingly, 240 hours after feeding, the RNAi-mediated silencing of the PBAN gene resulted in 80% mortality of Argentine ant workers (Figure 1D)**, compared to virtually no mortality in control ants that were fed just 15% sucrose (red line) and control ants fed 15% sucrose+dsRNA that matches no gene sequence (brown line).

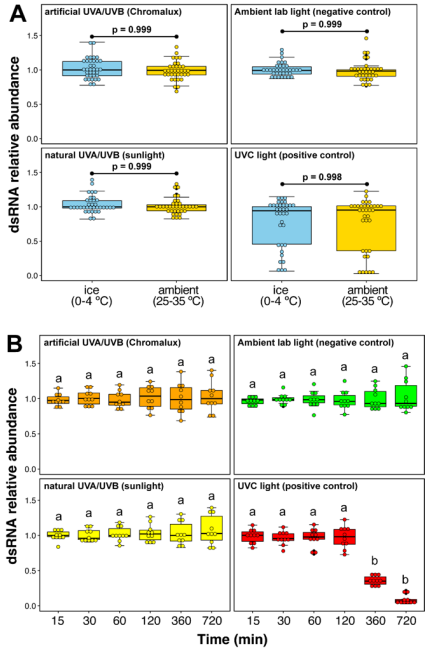

Figure 2. dsRNA is not degraded by UV light at either cold or warm temperatures. (A) Temperature effects of cool (ice, 0-4 ºC) and warm (ambient, 25-35 ºC) treatments showed no significant differences in dsRNA degradation across any of the four UV treatments, and (B) UV treatment only showed significant dsRNA degradation at 6 h (360 min) and 12 h (720 min) exposure times for the UVC treatment (positive control, red boxplots). All other UV treatments and exposure times showed no significant differences in dsRNA degradation.

In separate experiments, we have also shown that dsRNA is able to persist under both sunlight and UVA+UVB light, at both cold and ambient temperatures (Figure 2). These results show that dsRNA is sufficiently robust to be deployed in real-world environments without degrading.

Thus, we are currently at a pivotal stage in the development of this technology. We have shown that it works, but the current cost is far higher than is acceptable for a viable, deployable product. To achieve a realistic cost and scale, we need to increase efficacy substantially.

If successful, our proposed research will solve several major problems of current insecticide-based approaches for ant control, including:

1. Harm to non-target organisms. Conventional insecticides are non-specific, potentially harming or killing insects and other arthropods generally. One of the greatest strengths of our approach is that it is specifically designed to target gene sequences that are unique to Argentine ants, and that are absent in non-target organisms (such as pollinating insects). RNAi can be easily designed to only harm the targeted species.
2. Contamination of air, soil, and water. Chemical insecticides can persist in soil and water, extending the time and spatial extent of their off-target harm. Because our approach uses biological molecules (dsRNA), any residual material will degrade through the natural action of bacteria, fungi, and other decomposers.

3. Learned toxicant avoidance. Although the mechanisms that allow ants to eventually learn to avoid conventional insecticides are not well understood, it is clear that conventional insecticides are static, unchanging stimuli that can be easily learned and avoided (Zanola et al., 2024). Our approach is highly flexible, because new dsRNA constructs can be designed with different nucleotide sequences, lengths, 3-dimensional configurations, and molecular modifications, thus creating a "moving target" that is more difficult for organisms to learn to avoid.
4. Insecticide resistance. When a single type of insecticide is applied to a population that has many individuals, it is inevitable that resistance to the insecticide will eventually evolve (as has been seen in many agricultural settings; Pu & Chung 2024). Our approach is highly flexible, so that new dsRNA constructs can be easily designed to target different genes, thus shifting the targets of selective pressure when necessary and preventing resistance from evolving.

2B. Specific Objectives, Activities, and Timeline

Objective 1: Determine optimal length of dsRNA constructs.
When dsRNA viruses infect a cell, they inject their dsRNA genomes into it. The presence of this dsRNA is detected by the RNAi machinery in the cell, and proteins are mobilized to destroy the viral dsRNA and any other dsRNA that resembles it.

In our prior work, we successfully used short dsRNA constructs (19–24 base pairs long) to induce RNAi-mediated mortality in Argentine ants. These synthetic molecules are designed to resemble the products naturally generated by the Dicer enzyme during the RNAi process, and they integrate efficiently into the gene-silencing pathway. However, they are expensive to produce at scale due to the need for precise chemical synthesis and purification, making them impractical for broad application in the field.

A promising alternative is the use of longer dsRNA constructs, ranging from 100-600 base pairs. These molecules can be produced enzymatically *in vitro* or expressed in bacterial systems such as *E. coli*, enabling low-cost, high-volume production. When ingested by the target insect, these longer dsRNAs are cleaved by the insect's endogenous Dicer enzyme into multiple short fragments, which then trigger gene silencing. Although less precisely defined than synthetic siRNAs, long dsRNAs have shown comparable or even superior efficacy in other species (e.g. Shpak et al. 2017), and their scalability makes them especially attractive for use in pest control.

In this objective, we will compare the efficacy of longer dsRNA constructs to that of the short dsRNAs that we have already validated. Our goal is to determine whether long-form dsRNA can achieve similar RNAi-induced mortality in Argentine ants, while significantly reducing production costs. This work will inform the development of practical, field-ready formulations for RNAi-based ant control.

Objectives 2-5: Testing chitosan and carbon quantum dot (CQD) additives.
When a feeding protocol is used to trigger RNAi, the dsRNA molecules must traverse a diverse set of challenges to before getting inside the cells where the RNAi machinery exists. The digestive system in particular is a challenging environment, with a low pH and digestive enzymes that can degrade dsRNA molecules before they reach their target cells. To prevent

degradation, several different types of molecules have been discovered that can protect dsRNA molecules. Two of the most cost effective are chitosan and carbon quantum dots (CQDs).

For Objective 2, we will test palatability and toxicity of these two additives using a dual-choice feeder protocol (Wagner et al. 2025). Although our ultimate goal is to induce mortality via RNAi, if either additive by itself causes mortality then it would be no improvement over a broad-spectrum insecticide. Next, if either additive (or both) is confirmed as acceptable, we will test the ability of each to trigger RNAi-induced mortality when combined with the dsRNA.

Chitosan is a naturally derived, biodegradable polysaccharide obtained from chitin, which is found in the exoskeletons of crustaceans and insects. Commercially, most chitosan is derived as a byproduct of the shrimp, crab, and lobster industry, but fungal sources are also available. Chitosan is currently used in a large number of commercial applications, including in drug formulations to decrease stomach irritation, as well as in cosmetics, textiles, and many other products (Kumar 2000; Bakshi et al. 2020). Chitosan is approved by the US Food and Drug Administration (FDA) for wound treatment and nutritional use, and is classified as "generally recognized as safe" as a food ingredient. It has also become one of the most widely studied materials for enhancing RNA interference (RNAi) efficiency in insects. Chitosan carries a positive charge, which enables it to bind the negatively charged phosphate backbone of dsRNA molecules through electrostatic interactions (He et al. 2017; Zhou et al. 2023). This binding forms chitosan–dsRNA nanoparticles, sometimes referred to as "nanoshields", that protect the dsRNA from degradation by nucleases in the insect gut and hemolymph. This protective effect prolongs the persistence of dsRNA after ingestion and increases the probability that it reaches the target cells intact.

Mechanistically, chitosan also facilitates cellular uptake. The positively charged nanoparticles can interact with negatively charged cell membranes, promoting endocytosis and enhancing intracellular delivery of the dsRNA. In practice, chitosan is easy to use: it can be dissolved under mild acidic conditions, mixed with dsRNA in aqueous solutions, and spontaneously forms complexes without requiring elaborate equipment. Because of its biocompatibility and low toxicity (Muzzarelli et al. 1999), chitosan is attractive for agricultural RNAi applications. Over the past decade, it has been studied in a range of insect species, including the fall armyworm (*Spodoptera frugiperda*; Gurusamy et al. 2020), the malaria mosquito, *Anopheles gambiae* (Zhang et al. 2010), and the whitefly, *Bemisia tabaci* (Keppanan et al. 2024), and has been shown to significantly improve gene silencing efficiency compared to naked dsRNA. While some optimization is needed for different insects and delivery methods (e.g., droplet feeding, leaf coating, or microinjection), chitosan is among the best-characterized carriers for insect RNAi and is considered a practical, scalable approach for research and pest management.

Carbon quantum dots (CQDs) are tiny, carbon-based nanomaterials, generally less than 10 nanometers in diameter, that have emerged as promising carriers to enhance RNA interference (RNAi) in insects (Lim et al. 2015). In RNAi applications, CQDs are typically positively charged, which allows them to electrostatically bind dsRNA molecules and form stable CQD–dsRNA complexes. This binding offers two major benefits: it protects dsRNA from degradation by nucleases in the insect digestive system and hemolymph, and it facilitates cellular uptake by promoting endocytosis through interactions with negatively charged cell membranes.

Despite the technical-sounding name, CQDs are easy and inexpensive to make in the lab, and the protocol for binding CQDs to dsRNA is straightforward (e.g. Bulgarella et al 2023). CQDs can be easily synthesized from inexpensive precursors such as poly(ethylene glycol)

(PEG) and polyethylenimine (PEI) by simply heating them in a microwave. CQDs are a newer class of delivery system compared to chitosan or liposomes, but they have been successfully tested in insects such as *Aedes aegypti* (Das et al. 2015) and *Bemisia tabaci* (Kaur et al. 2020), where CQD–dsRNA complexes significantly improved RNAi efficiency over naked dsRNA.

To test and optimize the use of chitosan and CQDs in RNAi, we will first test each for palatability and toxicity (Obj. 2), then combine each with dsRNA to determine whether they increase efficacy of RNAi, both individually (Obj. 3 and 4) and together (Obj. 5).

Relationship to other projects and grants

We currently have one other grant to study RNAi in invasive ants, from the US Department of Defense (DOD). The DOD is funding this research because they are deeply interested in eradicating invasive ants from lands they control in the Indo-Pacific where endangered species are also present. Conventional insecticides cannot be used in these settings because of the potential collateral damage to protected, non-target species. This is a collaborative research project with Commonwealth Scientific and Industrial Research Organisation (CSIRO; Australia's federal research agency) and the University of Hawaii.

Although this DOD-funded project is also about the development of RNAi for the control and eradication of ants, there is no overlap between the research for that project and the objectives described in this SPCB proposal. Specifically, the objectives of the DOD grant are to: 1) identify new gene targets, 2) test new structures of dsRNA constructs, 3) develop a bacterial expression system, and 4) test efficacy of hydrogel liquid bait delivery systems. The DOD-funded research also targets several different species of ants, only one of which is the Argentine ant. The research described in this SPCB proposal tests several potential methods for increasing the efficacy of RNAi in Argentine ants that complement the experiments for the DOD project.

Although the research objectives described here do not overlap with the DOD-funded project, it is worth noting that there are synergies that will be enjoyed by having both lines of research performed at the same time. Since both projects will require the collection and maintenance of Argentine ant colonies, these resources and infrastructure can be shared by both projects. In addition, the staff hired to perform this proposed research will be able to collaborate and coordinate with the staff and students hired for the DOD project, enabling the pooling of shared knowledge, help with experimental design and troubleshooting, and coordination of research activities to optimize the use of time and funds. Ultimately, the discoveries produced by this SPCB project may be combined with the discoveries from the DOD project, accelerating the transition of this technology from the lab to the field.

Timeline and Milestones. To accomplish the proposed objectives, the project will proceed in sequential phases over the 24-month funding period:

1. **Phase 1 (Months 1–6):** Setup of lab colonies, setup of dsRNA length experiment (Objective 1) and palatability/toxicity assays for additives (Objective 2), and initiate experiments.
2. **Phase 2 (Months 7–12):** Complete feeding trials, gene expression analyses for Objectives 1 and 2, and perform data analysis. Setup and launch experiments for Objectives 3 (chitosan) and 4 (CQDs).
3. **Phase 3 (Months 13-18):** Complete Objectives 3 and 4 and perform data analysis. Setup and launch Objective 5 experiment (chitosan and CQD combined).

4. **Phase 4 (Months 19–24):** Perform data analysis for Objective 5. Complete any other remaining data analysis, synthesize of result, prepare manuscript, and prepare final report to SPCB.

Time Period	*Major Activities and Milestones*
Months 1–6	*Collect and prepare lab ant colonies; synthesize dsRNA constructs (short and long); prepare CQD and chitosan formulations; begin Obj. 1 and 2 experiments.*
Months 7-12	*Complete experiments and gene expression analysis for Obj. 1 and 2. Initiate data analysis for Obj. 1 and 2. Initiate experiments for Obj. 3 and 4.*
Months 13–18	*Complete experiments and gene expression analysis for Obj. 3 and 4. Perform data analysis for Obj. 3 and 4. Initiate experiments for Obj. 5.*
Months 19–24	*Finalize any last data collection and complete data analysis; manuscript and report preparation; develop factsheet and video for outreach; present results at professional meetings*

2C. Data Collection, Analysis, and Interpretation

2C.1. Methods

Ant collection and maintenance. Argentine ants will be collected from established populations in California using 5-gallon buckets containing natural nesting substrate (e.g., soil, decaying wood, and leaf litter). In the lab, ants and their substrate will be transferred into plastic holding bins (58.4 × 41.3 × 15.2 cm, Sterilite) coated internally with PTFE fluoropolymer (Fluon) to prevent escape. After an overnight acclimation period, colonies will be moved into laboratory colony bins with artificial nest chambers (plaster-lined Petri dishes), red acetate light filters, and conical feeding vials containing 15% sucrose and water. Ants will be induced to migrate into the lab colonies via simulated flooding, using paper bridges and water drip to encourage transport of queens, workers, and brood. Colonies will be maintained on sucrose and water *ad libitum*, supplemented twice weekly with artificial diets (Bhatkar & Whitcomb 1970).

dsRNA Preparation. We have previously had success targeting the Pheromone Biosynthesis Activating Neuropeptide (PBAN) gene using RNAi, so we will use this as a comparison for the new approaches tested here. Our previous research used custom, short dsRNAs to trigger RNAi. To test the efficacy of long dsRNAs (Objective 1), we will synthesize dsRNA targeting the PBAN gene via *in vitro* transcription using the MEGAscript RNAi kit (Thermo Fisher Scientific) and purify the products by phenol-chloroform extraction. The integrity and concentration of dsRNA will be confirmed by agarose gel electrophoresis and spectrophotometry (NanoDrop). If the DOD-supported bacterial expression system comes online during the course of this project, we will switch to that approach for dsRNA production.

Initial chitosan and CQD palatability and toxicity experiments (no RNAi). Previous research has shown that Argentine ants are largely insensitive to dietary additives, including various insecticides (e.g. Wagner et al. 2025a) and bitter substances (e.g. Wagner et al. 2025b). This trait suggests that Argentine ants will readily consume sugar solutions that contain chitosan and CQDs. Nevertheless, a necessary first step in assessing whether chitosan and/or CQDs can be effectively used to enhance RNAi is verification of their palatability and low toxicity to Argentine ants. To assess this, we will perform a two-choice feeding assay incorporating a dose-response design. This method, adapted from Wagner et al. (2025c), allows quantification of feeding preferences and behavioral deterrence across a range of concentrations, as well as evaluation of sublethal or lethal effects over time.

Worker ants will be starved for 24 hours and then placed in Fluon-lined foraging arenas (n=50 ants per trial). Each arena will contain two dual-choice feeding stations (as in Wagner et al. 2025a) containing control solution (15% sucrose) on one side and treatment solution (15% sucrose supplemented with chitosan, CQDs, or both) on the other. For each additive type, we will test a minimum of three concentrations (0.01%, 0.1%, and 1.0% w/v). For combination treatments, we will use a factorial design crossing two concentrations of chitosan and CQDs (e.g., 0.01% and 0.1%) to explore additive or synergistic effects.

For each feeding trial, we will collect: 1) total volume consumed of each solution, to quantify palatability and 2) mortality at 24 and 48 hours, to assess toxicity. To compare the volume of liquid consumed from the treatment and control solutions in the palatability assays, we will use a paired t-test if assumptions of normality are met, or a Wilcoxon signed-rank test if the data are non-normally distributed, with each replicate trial providing a matched pair of consumption values. Mortality data will be analyzed using Cox proportional hazards models (for survival time) or GLMs with binomial error (for proportion dead at timepoints), including additive and concentration as predictors. Dose–response curves will be fitted using the drc package in R (Ritz et al. 2015) to estimate palatability or toxicity thresholds (e.g., EC50, LC50) for each additive. Where appropriate, we will assess interactions between chitosan and CQDs using factorial ANOVA or GLMMs.

Preparation of Delivery Formulations for RNAi

dsRNA length. We will prepare short and long dsRNAs for RNAi trial as described above, in "dsRNA preparation".

Chitosan-dsRNA Nanoparticles. Chitosan-dsRNA nanoparticles will be prepared using ionic gelation, following a modified protocol from Zhang et al. (2010) and optimized for RNAi delivery in Argentine ants. Low molecular weight chitosan (Sigma-Aldrich) will be dissolved in 0.1% v/v acetic acid to a final concentration of 0.02% w/v, and the pH will be adjusted to 5.5 using 1 M NaOH to ensure solubility and promote nanoparticle formation. The chitosan solution will be filtered through a 0.45 μm membrane to remove particulates and equilibrated at room temperature for 30 minutes before use.

Double-stranded RNA (dsRNA) will be synthesized as described in previous sections and diluted to a working concentration in nuclease-free water. For nanoparticle formation, dsRNA will be added dropwise to the chitosan solution at a weight ratio of 1:3 (dsRNA:chitosan) while gently stirring to ensure homogenous mixing. The mixture will be incubated at room temperature

for 30 minutes to allow ionic complexation between the positively charged chitosan and negatively charged dsRNA.

Nanoparticle formation will be confirmed by visual inspection for turbidity, followed by agarose gel electrophoresis to assess dsRNA encapsulation and mobility shift. The final chitosan–dsRNA complexes will be used immediately in feeding assays or stored on ice for no more than 6 hours to preserve stability. All formulations will be prepared fresh on the day of feeding.These will be fed to Argentine ant workers to test for silencing of the PBAN gene as in "RNAi feeding assays", below.

Carbon Quantum Dot (CQD)–dsRNA Complexes. CQD–dsRNA conjugates will be prepared following the method of Bulgarella et al. (2023), adapted from Kaur et al. (2020). A CQD stock solution will be synthesized by combining 9 mL of poly(ethylene glycol) (PEG, Sigma Aldrich, Missouri, USA), 3 mL of nuclease-free water (Invitrogen, Massachusetts, USA), and 2 mL of a 50 mg/mL solution of branched polyethylenimine (average Mw ~25,000 by LS, average Mn ~10,000 by GPC; Sigma Aldrich). This 14 mL mixture will be heated in a microwave in short bursts for a total of 3 minutes and then allowed to cool. Volumes of this stock will be diluted 1:10, 1:100, and 1:200 prior to conjugation with dsRNA. These dilutions will be assessed on gel retardation assays confirming dsRNA binding.

CQD-dsRNA conjugates will be prepared at three dsRNA concentrations: 0.5 μg/μL, 1 μg/μL, and 5 μg/μL. For the 0.5 μg/μL treatment, 3.5 mg of dsRNA will be diluted to 7 mL in 50 mM sodium sulfate (Sigma Aldrich) and mixed with 7 mL of 1:200 diluted CQD. For 1 μg/μL, 7 mg of dsRNA in 7 mL sodium sulfate will be combined with 7 mL of 1:100 diluted CQD. For 5 μg/μL, 35 mg of dsRNA in 7 mL sodium sulfate will be combined with 7 mL of 1:10 diluted CQD. Mixtures will form a precipitate, which will be centrifuged at 7,000 g for 10 minutes. The resulting pellets will be resuspended by pipetting and gentle sonication in 1 mL of 15% sugar water per tube and then pooled to yield 7 mL per treatment. These will then be fed to Argentine ant workers to test for silencing of the PBAN gene as in "RNAi feeding assays", below.

Lipofectamine Formulation (control). Lipofectamine RNAiMAX (Thermo Fisher) will be prepared according to the manufacturer's protocol. Briefly, Lipofectamine reagent will be diluted in Opti-MEM and combined with dsRNA at a 1:2 volume ratio. The mixture will incubate for 20 minutes to allow lipoplex formation.

Feeding Assays. Ant diet will consist of 15% w/v sterile sucrose solution mixed with the respective additive or control. dsRNA formulations will be incorporated by gentle stirring to a final dsRNA concentration of 5 μg/μL and final sucrose concentration of 15% w/v. Each treatment will be prepared fresh on the day of feeding.

Before treatment, ants will be food-deprived for 24 hours to increase feeding motivation. Each replicate group will be provided the dsRNA-containing diet *ad libitum* for 24 hours in covered feeding chambers. After the exposure period, ants will be transferred to clean containers with standard diet and monitored for mortality.

Assessment of RNAi Efficacy

Gene Expression Analysis. At 24 and 72 hours post-feeding, five ants per replicate (15 ants per treatment) will be collected, flash-frozen in liquid nitrogen, and stored at –80°C. Total RNA will

be extracted using TRIzol reagent and reverse-transcribed to cDNA. Target gene expression will be quantified by qPCR using SYBR Green chemistry, normalized to a housekeeping gene, and compared among treatments. Relative expression levels will be analyzed by ANOVA followed by Tukey's *post hoc* tests.

Mortality and Behavior. Ant mortality will be recorded every 24 hours for seven days post-treatment. Dead ants will be removed and counted. Ants exhibiting severe paralysis or loss of coordinated movement will be classified as moribund. Behavioral observations (locomotion, grooming, feeding) will be recorded daily using a standardized scoring rubric to detect sublethal effects. Daily mortality proportions will be analyzed using generalized linear models (GLMs) with binomial error structure. Cumulative mortality by day 7 will be compared among treatments using logistic regression. Behavioral scores will be analyzed by nonparametric Kruskal-Wallis tests followed by Dunn's post hoc comparisons.

2C.2. Data Analysis and Use

This project will generate multiple types of data, including: 1) behavioral data from feeding assays (initial choice, feeding duration), 2) physiological and survival data (mortality, moribund status), and 3) gene expression levels (qPCR).

Behavioral and feeding preference data will be analyzed using generalized linear mixed models (GLMMs), linear models (LMMs), and dose-response modeling (e.g., EC_{50} for deterrence). Mortality outcomes will be assessed with survival analyses (Kaplan–Meier and Cox models) and logistic regression to estimate LC_{50} values. Gene expression levels will be analyzed using ANOVA, normalized to housekeeping genes, with *post hoc* testing to compare between treatments. All statistical analyses will be conducted in R, using reproducible scripts.

The results will be used to evaluate the relative efficacy of chitosan, carbon quantum dots, and their combination in enhancing RNAi in Argentine ants. Specifically, the data will allow us to identify: 1) optimal additive concentrations that maximize gene knockdown, 2) behavioral and physiological indicators of RNAi success, and 3) candidate delivery strategies for practical field applications in species-specific ant control.

Objective 1) Determine optimal length of dsRNA constructs.
We will reject Hypothesis 1 if longer dsRNA constructs do not induce significantly higher levels of RNAi-induced mortality compared to short constructs.

Objective 2) Test palatability and toxicity of chitosan and carbon quantum dot (CQD) additives.
We will reject Hypothesis 2 if chitosan or CQDs are significantly less palatable or significantly more toxic to Argentine ant workers compared to controls (the hypothesis may be be rejected for only one or the other additive).

Objective 3) Quantify whether chitosan additive increases efficacy of RNAi.
We will reject Hypothesis 3 if adding chitosan to the dsRNA formulation does not significantly increase RNAi-induced mortality compared to control.

Objective 4) Test whether CQD additive increases efficacy of RNAi.

We will reject Hypothesis 4 if adding CQDs to the dsRNA formulation does not significantly increase RNAi-induced mortality compared to control.

Objective 5) Determine impact of chitosan and carbon quantum dots together on efficacy of RNAi.
We will reject Hypothesis 5 if formulations that contain both chitosan and CQD do not significantly increase RNAi-induced mortality compared to formulations with either additive alone.

Future directions
After the completion of this research project, we will be well-positioned to assess potential non-target effects. These next studies will test the impacts of feeding dsRNA formulations on an array of non-target species, including pollinators and other beneficial insects. The formulations used in this next step of research will be those that are most successful at inducing RNAi-triggered mortality in Argentine ants. These assays will help determine the degree of species-specificity and inform risk assessment for potential future field applications. We will seek funding for this research from the DOD Environmental Security Technology Certification Program (ESTCP) program, which is specifically set up to test these types of emerging technologies at secure, contained military installations. I will also seek follow-on funding from the National Science Foundation and US Dept of Agriculture, if appropriate funding programs are available.

2.D. Project Staffing and Monitoring

Principal Investigator: The proposed project will be led by Dr. Neil Tsutsui, who will be responsible for overall project leadership, research oversight, and compliance with project goals and reporting requirements. Dr. Tsutsui will contribute approximately 3.8% annual effort (0.5 person-months) and will guide all aspects of the research, including experimental design, data analysis, manuscript preparation, and coordination of deliverables. He will also supervise all project personnel and ensure adherence to the proposed work plan and timeline.

Post-doctoral researcher: A full-time post-doctoral researcher (100% time, to be hired) will manage the day-to-day operations of the project. This individual will carry out the majority of the experimental work, including field collection of Argentine ant colonies, maintenance of laboratory colonies, molecular and RNAi assays, and data collection and analysis. The postdoc will work closely with Dr. Tsutsui to refine methodologies, troubleshoot protocols, and prepare presentations and manuscripts. They will also be responsible for mentoring undergraduate assistants.

Undergraduate research assistants/work-study students: One to two undergraduate researchers will be recruited to assist with colony maintenance and routine laboratory tasks. These students will contribute approximately 5–10 hours per week during the academic year and summer sessions. While no salary support is requested for these positions, their involvement will provide valuable research training and capacity to support the project.

External collaborators (as needed): Consultation with formulation chemists and RNAi delivery specialists, particularly for chitosan and CQD complex optimization, will be integrated where appropriate.

Progress toward research objectives will be monitored through biweekly internal lab meetings, biweekly, one-on-one check-ins with the postdoctoral researcher, and regular documentation of research activities. Formal progress reports will be submitted to SPCB every six months as required, and Dr. Tsutsui will track all deliverables to ensure timely completion of the project milestones.

Project Management Plan

This project will be overseen by the Principal Investigator (PI), Dr. Neil Tsutsui, who will be responsible for all aspects of project direction, coordination, data integrity, and reporting. The PI brings extensive experience in insect behavior, molecular ecology, and RNA interference (RNAi), and has successfully managed many federally- and state-funded research projects. Biweekly lab meetings will be held to review progress, troubleshoot challenges, and coordinate upcoming tasks. Experimental milestones and timelines will be monitored using a shared digital project management platform (e.g., Asana, Notion), ensuring that tasks are completed on schedule and that responsibilities are clearly assigned to team members.

Reporting and Communication
The PI will submit interim and final reports to the Structural Pest Control Board as required. Findings will also be disseminated through conference presentations, peer-reviewed publications, and (if appropriate) outreach to pest control professionals. Progress will be benchmarked quarterly to ensure timely completion and budget adherence.

2E. Accountability and Institutional Oversight

All research conducted under this contract will comply with the University of California, Berkeley's policies governing sponsored research, including those related to research integrity, financial accountability, and reporting. The UC Berkeley Sponsored Projects Office (SPO) and Contracts and Grants Accounting (CGA) provide oversight to ensure that all expenditures, milestones, and deliverables conform to the terms of the agreement. The PI will be responsible for the scientific execution of the project, with institutional safeguards in place to ensure timely reporting, accurate financial management, and adherence to ethical standards. Final products, including technical reports and data summaries, will be submitted in accordance with SPCB requirements. All personnel involved in the project will have completed required university trainings in topics such as research ethics, sexual harassment prevention, laboratory and field safety, and cybersecurity, consistent with UC Berkeley's standards for responsible conduct of research.

Data Management and Integrity. All raw data (e.g., mortality counts, qPCR values, behavioral scores) will be entered into paper lab notebooks and shared spreadsheets with version control. Experimental protocols and outcomes will be documented in electronic lab notebooks. All qPCR,

statistical, and graphical analyses will be conducted in R, with scripts archived for reproducibility.

2F. Public Engagement and Outreach Plan

To support pest management professionals and encourage the adoption of integrated pest management practices, this project will include targeted outreach activities focused on Argentine ant biology and control strategies. We will prepare a plain-language, illustrated factsheet covering key aspects of ant diet, foraging behavior, colony structure, and how these factors influence bait acceptance and treatment success. This factsheet will be provided to the Structural Pest Control Board and made available to pest management professionals through professional associations and industry events. Additionally, we will develop a brief informational video describing ant biology and current best practices for Argentine ant management, concluding with an overview of RNA interference (RNAi) as a promising future technology. Project staff will offer at least one in-person presentation at pest control conference or training events. The Principal Investigator has previously presented at the Pest Control Operators of California (PCOC) annual conference and at professional trainings hosted by Target Specialty Products and will leverage these established relationships to share research updates and educational materials. Existing resources produced by UC Cooperative Extension and the UC Statewide IPM Program already provide high-quality foundational materials on Argentine ant management. The educational components proposed here will complement these materials by consolidating practical information specifically relevant to structural pest control professionals and by introducing RNAi-based control strategies as an emerging research area. Materials will be developed in consultation with Extension resources to avoid redundancy and to ensure alignment with current IPM best practices. These outreach efforts will also be offered to the Board for inclusion on its website or other communication channels at its discretion and will be made available to the public and interested stakeholders to build awareness of both established and emerging approaches to sustainable ant control.

2G. References

Bakshi, P.S., Selvakumar, D., Kadirvelu, K. and Kumar, N.S., 2020. Chitosan as an environment friendly biomaterial–a review on recent modifications and applications. *International Journal of Biological Macromolecules*, *150*, pp.1072-1083.

Bhatkar, A. and Whitcomb, W.H., 1970. Artificial diet for rearing various species of ants. *Florida Entomologist*, pp.229-232.

Bulgarella, M., Baty, J.W., McGruddy, R. and Lester, P.J. 2023. Gene silencing for invasive paper wasp management: Synthesized dsRNA can modify gene expression but did not affect mortality. *PLoS One*, *18*(1), p.e0279983.

Cooper, M. L., K. M. Daane, E. H. Nelson, L. G. Varela, M. C. Battany, N. D. Tsutsui, and M. K. Rust. 2008. Liquid baits manage Argentine ants in coastal vineyards. *California Agriculture* 62:177-183.

Darlington, M., Reinders, J.D., Sethi, A., Lu, A.L., Ramaseshadri, P., Fischer, J.R., Boeckman, C.J., Petrick, J.S., Roper, J.M., Narva, K.E. and Vélez, A.M., 2022. RNAi for western corn rootworm management: Lessons learned, challenges, and future directions. *Insects*, *13*(1), p.57.

Das, S., Debnath, N., Cui, Y., Unrine, J. and Palli, S.R., 2015. Chitosan, carbon quantum dot, and silica nanoparticle mediated dsRNA delivery for gene silencing in *Aedes aegypti*: a comparative analysis. *ACS Applied Materials & Interfaces*, *7*(35), pp.19530-19535.

Fishilevich, E., Vélez, A.M., Storer, N.P., Li, H., Bowling, A.J., Rangasamy, M., Worden, S.E., Narva, K.E. and Siegfried, B.D., 2016. RNAi as a management tool for the western corn rootworm, *Diabrotica virgifera virgifera*. *Pest Management Science*, *72*(9), pp.1652-1663.

Gurusamy, D., Mogilicherla, K. and Palli, S.R., 2020. Chitosan nanoparticles help double-stranded RNA escape from endosomes and improve RNA interference in the fall armyworm, *Spodoptera frugiperda*. *Archives of Insect Biochemistry and Physiology*, *104*(4), p.e21677.

Hanamasagar, Y., Ramesha, N.M., Mahapatra, S., Panigrahi, C.K., Vidhya, C.S., Agnihotri, N. and Satapathy, S.N., 2024. Advancing RNAi for sustainable insect management: targeted solutions for eco-friendly pest control. *Journal of Experimental Agriculture International*, *46*(6), pp.740-775.

Kaur, R., Gupta, M., Singh, S., Joshi, N. and Sharma, A., 2020. Enhancing RNAi efficiency to decipher the functional response of potential genes in *Bemisia tabaci* AsiaII-1 (Gennadius) through dsRNA feeding assays. *Frontiers in Physiology*, *11*, p.123.

Keppanan, R., Karuppannasamy, A., Nagaraja, B.C., Thiruvengadam, V., Kesavan, S., Dhawane, Y.A. and Ramasamy, A., 2024. Effectiveness of chitosan nanohydrogel mediated encapsulation of EcR dsRNA against the whitefly, *Bemisia tabaci* Asia-I (Gennedius)(Hemiptera: Aleyordidae). *Pesticide Biochemistry and Physiology*, *198*, p.105712.

Kaur, R., Gupta, M., Singh, S., Joshi, N. and Sharma, A., 2020. Enhancing RNAi efficiency to decipher the functional response of potential genes in *Bemisia tabaci* AsiaII-1 (Gennadius) through dsRNA feeding assays. *Frontiers in physiology*, *11*, p.123.

Kumar, M.N.R., 2000. A review of chitin and chitosan applications. *Reactive and Functional Polymers*, *46*(1), pp.1-27.

LeBoeuf, A.C., 2017. Trophallaxis. *Current Biology*, *27*(24), pp.R1299-R1300.

Lim, S.Y., Shen, W. and Gao, Z., 2015. Carbon quantum dots and their applications. *Chemical Society Reviews*, *44*(1), pp.362-381.

Liu, F.L.C., Tseng, S.P., Lin, C.C. and Yang, C.C.S., 2025. Advancing invasive ant management: Innovative and targeted strategies. *74*(2), pp.113-128.

Muzzarelli, R.A., Mattioli-Belmonte, M., Pugnaloni, A. and Biagini, G., 1999. Biochemistry, histology and clinical uses of chitins and chitosans in wound healing. *Exs*, *87*, pp.251-264.

Pu, J. and Chung, H., 2024. New and emerging mechanisms of insecticide resistance. *Current Opinion in Insect Science*, *63*, p.101184.

Ritz C., Baty F., Streibig J.C., Gerhard D., 2015. Dose-Response analysis using R. *PLOS ONE*, 10(e0146021).

Shpak, N., Manor, R., Abilevich, L.K., Mantal, O., Shavit, K., Aflalo, E.D., Toiber, D. and Sagi, A., 2017. Short versus long double-stranded RNA activation of a post-transcriptional gene knockdown pathway. *RNA biology*, *14*(12), pp.1766-1775.

Smith, C. D., *et al.* 2011. The draft genome of the globally widespread and invasive Argentine ant (*Linepithema humile*). *Proceedings of the National Academy of Sciences, USA* 108:5673-5678.

Tsutsui, N. D., A. V. Suarez, J. C. Spagna, and J. S. Johnston. 2008. The evolution of genome size in ants. *BMC Evolutionary Biology* 8:64.

Wagner, T., Vorjans, M., Garsi, E., Werneke, C., and Czaczkes, T.J. 2025a. Palatability of insecticides and protein in sugar solutions to Argentine ants. *Journal of Pest Science* 98:1335-1343.

Wagner, T., Paintner, R., Obermeier, M., and Czaczkes, T.J. 2025b. Invasive ants have a high bitterness tolerance. Chapter 3 of "*Exploiting learning and feeding preferences of invasive ants to inform control efforts".*

Wagner, T., Galante, H. and Czaczkes, T.J. 2025c. A high throughput and sensitive method for food preference assays in the Argentine ant. Chapter 3 of "*Exploiting learning and feeding preferences of invasive ants to inform control efforts".*

Zanola, D., Czaczkes, T.J. and Josens, R., 2025. Toxic bait abandonment by an invasive ant is driven by aversive memories. *Communications Biology*, *8*(1), p.486.

Zhang, X., Zhang, J. and Zhu, K.Y., 2010. Chitosan/double-stranded RNA nanoparticle-mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito (*Anopheles gambiae*). *Insect Molecular Biology*, *19*(5), pp.683-693.

Zhou, H., Wan, F., Jian, Y., Guo, F., Zhang, M., Shi, S., Yang, L., Li, S., Liu, Y. and Ding, W., 2023. Chitosan/dsRNA polyplex nanoparticles advance environmental RNA interference efficiency through activating clathrin-dependent endocytosis. *International Journal of Biological Macromolecules*, *253*, p.127021.

ATTACHMENT 9 – Narrative of Qualifications

A. Three prior peer-reviewed research projects

I have published more than 80 papers which have been collectively cited over 13,000 times, yielding an H-index of 42. At the end of Attachment 9, I have attached three relevant papers from throughout my career:

1) Tsutsui et al (2000; PNAS) is considered a landmark study in the fields of behavioral ecology and biological invasions, and has been cited over 1100 times. Web of Science lists this as the most cited paper on the Argentine ant of all time.

2) Smith et al (2011, PNAS) is the publication of the Argentine ant genome sequence, which was the second ant genome ever published. I co-led the Argentine ant Genome Sequencing Consortium with Dr. Chris Smith, and I am the senior author on this paper.

3) Ferger & Tsutsui (2025, Ecology & Evolution) is a recent publication in which my graduate student and I analyzed all published ant genomes and discovered that, surprisingly, in species with many queens (like Argentine ants), genes for worker traits are experiencing significantly reduced natural selection compared to genes for queen traits.

B. References, background material, relevant papers

I have studied the biology, behavior, evolution, and genetics of the Argentine ant for the past 25 years and have published dozens of peer-reviewed papers on topics relevant to the research proposed here. Subject areas include: ant genome sequences, ant population genetics, the causes and consequences of invasive ant success, chemical ecology (pheromone biology) of ants, and behavioral evolution. Please see the included CV for a complete list of publications.

Previous funding from the California Structural Pest Control Board supported my research published in the 18 papers listed below, and this support is acknowledged in these papers. I am happy to provide reprints upon request.

van Wilgenburg, E., M. Mariotta, and **N. D. Tsutsui**. 2022. The effect of diet on colony recognition and cuticular hydrocarbon profiles of the invasive Argentine ant, *Linepithema humile*. INSECTS **13**(4):335.

Lewis V., Moore, S., Tabuchi, R., Sutherland, A., Choe, D.-H., and **N. D. Tsutsui**. 2013. Researchers combat the resurgence of bed bug in behavioral studies and monitor trials. CALIFORNIA AGRICULTURE **67**:172-178.

Choe, D.-H., S. R. Ramírez, and **N. D. Tsutsui**. 2012. A silica gel-based method for extracting insect surface hydrocarbons. JOURNAL OF CHEMICAL ECOLOGY **38**:176-187.

Suarez, A. V., T. McGlynn, and **N. D. Tsutsui**. 2010. Biogeographic patterns of invasive species. In: Lach, L., C. Parr, and K. Abbott, eds., ANT ECOLOGY, pp. 233-244 (Peer-reviewed book chapter).

van Wilgenburg, E., R. Sulc, K. J. Shea, and **N. D. Tsutsui**. 2010. Deciphering the chemical basis of nestmate recognition. JOURNAL OF CHEMICAL ECOLOGY **36**:751-758.

van Wilgenburg, E., C. W. Torres, and **N. D. Tsutsui**. 2010. The global expansion of a single ant supercolony. EVOLUTIONARY APPLICATIONS **3**:136-143.

Franks, S. J., P. D. Pratt, and **N. D. Tsutsui**. 2010. The genetic consequences of a demographic bottleneck in an introduced biological control insect. CONSERVATION GENETICS **12**:201-211.

Brandt, M., E. van Wilgenburg, R. Sulc, K. J. Shea, and **N. D. Tsutsui**. 2009. The scent of supercolonies: The discovery, synthesis and behavioral verification of ant colony recognition cues. BMC BIOLOGY **7**:71.

Brandt, M., E. van Wilgenburg, and **N. D. Tsutsui**. 2009. Global-scale analyses of chemical ecology and population genetics in the invasive Argentine ant. MOLECULAR ECOLOGY **18**:997-1005.

Suarez, A. V. and **N. D. Tsutsui**. 2008. The evolutionary consequences of biological invasions. MOLECULAR ECOLOGY **17**:351-360.

Suarez, A. V., D. A. Holway, and **N. D. Tsutsui**. 2008. Genetics and behavior of a colonizing species: The invasive Argentine ant. AMERICAN NATURALIST **172**:S72-S84.

Calcaterra, L. A., A. Delgado, and **N. D. Tsutsui**. 2008. Activity patterns and parasitism rates of fire ant decapitating flies (Diptera: Phoridae: *Pseudacteon* spp.) in their native Argentina. ANNALS OF THE ENTOMOLOGICAL SOCIETY OF AMERICA **101**:539-550.

Tsutsui, N. D., A. V. Suarez, J. C. Spagna, and J. S. Johnston. 2008. The evolution of genome size in ants. BMC EVOLUTIONARY BIOLOGY **8**:64.

Torres, C. W., M. Brandt, and **N. D. Tsutsui**. 2007. The role of cuticular hydrocarbons as chemical cues for nestmate recognition in the invasive Argentine ant (*Linepithema humile*). INSECTES SOCIAUX **54**:363-373.

Thomas, M. L., C. M. Payne-Makrisa, A. V. Suarez, **N. D. Tsutsui**, and D. A. Holway. 2007. Contact between supercolonies elevates aggression in Argentine ants. INSECTES SOCIAUX **54**: 225-233.

Calcaterra, L. A., R. Vander Meer, J. P. Pitts, J. P. Livore, and **N. D. Tsutsui**. 2007. Survey of *Solenopsis* fire ants and their parasitoid flies (Diptera: Phoridae: *Pseudacteon*) in central Chile and central western Argentina. ANNALS OF THE ENTOMOLOGICAL SOCIETY OF AMERICA **100**:512-521.

Thomas, M., C. M. Payne-Makrisa, A. V. Suarez, **N. D. Tsutsui**, and D. A. Holway. 2006. When supercolonies collide: Territorial aggression in an invasive and unicolonial social insect. MOLECULAR ECOLOGY **15**:4303-4315.

Whitfield, C. W., S. K. Behura, S. H. Berlocher, A. G. Clark, S. Johnston, W. S. Sheppard, D. Smith, A. V. Suarez, D. Weaver, and **N. D. Tsutsui**. 2006. Thrice out of Africa: Ancient and recent expansions of the honey bee, *Apis mellifera*. SCIENCE **314**:642-645.

C. Facilities, equipment, and resources

The Tsutsui lab has access to all of the necessary equipment, supplies, and expertise to successfully complete the proposed research.

The Tsutsui lab occupies 1313 square feet of lab and office space in the Valley Life Sciences Building on the campus of UC Berkeley. The lab possesses ample benchtop space with a wet lab, clean areas, and a double-wide fume hood. There are two offices for post-doctoral researchers and graduate students and a separate office for the Principal Investigator. The lab has numerous items for performing molecular biology, genomics, and chemical ecology including: thermal cyclers, gel rigs, power supplies, centrifuges, incubators, hot blocks, stir plates, high capacity and analytical balances, video recording and playback equipment, microscopes, light sources, an Agilent gas chromatograph/mass spectrometer, compressed gas (N_2, CO_2, helium), and numerous other web lab items. The Tsutsui lab has access to insectary rooms with light, temperature, and humidity control in an insectary on the same floor as the main lab, as well as at the Oxford Tract complex, a short walk from the Valley Life Sciences Building. If necessary, at Oxford Tract there is also access to greenhouse space, insect husbandry facilities, and a USDA quarantine facility.

The Environmental Genomics Laboratory, in the same building as the Tsutsui lab, includes staff who offer training in molecular, genetic, and genomic techniques and provides shared equipment and supplies for molecular biology and genetics (electrophoresis rigs and power supplies, gel imaging equipment, thermal cyclers, pipettors, bacterial and yeast culture facilities, an enzyme and reagent stockroom, and much more). Tsutsui is a Faculty Affiliate in the UC Berkeley Essig Museum of Entomology, which contains more than 4,500,000 preserved insects, as well as curatorial and taxonomic expertise. At least two imaging facilities are available nearby: one associated with the Department of Integrative Biology, in the Valley Life Sciences Building and the other affiliated with the Department of Environmental Science, Policy and Management.

UC Berkeley maintains cutting edge facilities and resources for molecular biology, genomics, and bioinformatics. The Tsutsui lab commonly uses several of these for molecular biology,

genomics, and bioinformatic analysis as well as for training of new lab members and consulting on complex projects. Most often, we use the Coates Genome Sequencing Facility for genomic library construction, genome sequencing, and bioinformatic processing and clean-up, and the Computational Genomics Resource Laboratory (CGRL) for computational analyses, including access to high-capacity servers for terabyte-level data storage, intensive analyses, and computational modeling and simulations. The staff of the Coates Facility and the CGRL are experts in their respective fields, and offer a variety of different scenarios for assisting with research projects, including full-service lab work on a recharge basis, hourly consulting for complex projects, or simply access to facilities and resources for more independent researchers. If necessary, the UC Berkeley D-Lab and consulting services in the UC Berkeley Dept. of Statistics offer expertise and consulting for statistical analyses.

UC Berkeley offers a comprehensive suite of computing resources for researchers, including high-performance computing clusters, secure compute environments, virtual machines, and advance consulting. Berkeley Research Computing (BRC) operates **Savio**, the campus Linux HPC cluster, managed by Research IT in collaboration with Lawrence Berkeley Lab. PI Tsutsui is subscribed to the Faculty Computing Allowance, which provides 300,000 service units per year for free, which the Tsutsui lab uses for genomic analysis, data storage, and computational simulations. Computational support from Research IT (RIT) and the BRC program includes free consulting on cloud storage services appropriate for specific research applications from commercial vendors (Amazon Web Services, Azure, etc.) and/or national centers (XSEDE, NERSC, SDSC, etc). There are numerous other computing resources available on campus for a variety of different research and storage needs.

Members of the Tsutsui lab may also request access to many other campus resources including (but not limited to) the Functional Genetics Facility, the GC-MS facility, a proteomics facility, the UC Natural Reserve System, and many others.

D. Demonstrate that the contractor has the organization and staff to perform this work and provide information on the professional qualifications and experience of persons assigned to the project.

The Tsutsui lab is a dynamic, productive, and welcoming environment for researchers from all backgrounds and at all career stages. For the proposed project, Tsutsui will hire a Post-doctoral

researcher to perform the day-to-day research and associated duties (insect collection and husbandry, purchasing of supplies and consumables, data analysis, experimental trouble-shooting, etc.). UC Berkeley is a desirable destination for recent PhD graduates to continue the next stage of their scientific development as Post-doctoral researchers, and Tsutsui has always received a strong cohort of applicants for every Post-doctoral search. Moreover, Tsutsui has an extremely strong record of guiding Post-doctoral researchers through this challenging career stage. Seven of his previous Post-doctoral researchers went on to become Professors themselves, including currently tenured Professors at UC Davis, UC Riverside, Georgia Southern University, and others. There is little doubt that, if this project is funded, Tsutsui will be able to hire an extremely well-qualified Post-doctoral researcher who will be able to perform the proposed research at the highest levels.

As noted above, there are many different professional staff on the campus of UC Berkeley who will be able to assist with the proposed research as needed. These include the professional scientists who are affiliated with the facilities described above, as well as the University staff who handle the myriad aspects of project and research administration, including post-award analysts at the Sponsored Projects Office, research administrators who handle fund management and purchasing, Human Resources specialists who assist with personnel hiring and retention, and many others.

Reduced genetic variation and the success of an invasive species

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Despite the severe ecological and economic damage caused by introduced species, factors that allow invaders to become successful often remain elusive. Of invasive taxa, ants are among the most widespread and harmful. Highly invasive ants are often unicolonial, forming supercolonies in which workers and queens mix freely among physically separate nests. By reducing costs associated with territoriality, unicolonial species can attain high worker densities, allowing them to achieve interspecific dominance. Here we examine the behavior and population genetics of the invasive Argentine ant (*Linepithema humile*) in its native and introduced ranges, and we provide a mechanism to explain its success as an invader. Using microsatellite markers, we show that a population bottleneck has reduced the genetic diversity of introduced populations. This loss is associated with reduced intraspecific aggression among spatially separate nests, and leads to the formation of interspecifically dominant supercolonies. In contrast, native populations are more genetically variable and exhibit pronounced intraspecific aggression. Although reductions in genetic diversity are generally considered detrimental, these findings provide an example of how a genetic bottleneck can lead to widespread ecological success. In addition, these results provide insights into the origin and evolution of unicoloniality, which is often considered a challenge to kin selection theory.

Invasive species are recognized as a leading threat to biodiversity as well as an increasing economic concern (1–3). Despite these problems, the attributes responsible for the establishment and spread of specific invaders are often difficult to pinpoint or are unknown (4–6). In particular, studies that examine the biology of invasive species in both their native and introduced ranges are surprisingly rare, despite the potential insights that can be gained from such comparisons. In many cases, forces external to the biology of the invasive species, such as escape from predators, competitors, and parasites (4, 7) or characteristics of the invaded habitat (4, 6, 8, 9), are implicated as the primary causes of invasion success. When the characteristics of invasive species themselves are considered, it is usually in terms of general demographic or life history traits that typify a broad suite of taxa (4, 6). However, many insights may be gained by examining the specific attributes responsible for success of particular invasive species. Here we examine the behavior and population genetics of the highly invasive Argentine ant (*Linepithema humile*) in its native and introduced ranges, and provide a mechanism to explain its success as an invader.

The Argentine ant is a widespread and damaging invasive species (10). In the United States, it was first detected in New Orleans in 1891, and by 1907, was established in California (11), where it is now common in coastal habitats, riparian woodlands, and irrigated urban and agricultural areas (12, 13). Once established, the Argentine ant displaces most native ants (12–16) and detrimentally affects other non-ant arthropods (17). These direct effects reverberate through communities as other taxa are affected indirectly (18, 19). In contrast, Argentine ants in their native range coexist with other ants in species-rich communities (20, 21). This difference may correspond with variation in colony structure between native and introduced populations of Argentine ants (20). In their native range, Argentine ants appear to defend territories against conspecifics (20), whereas introduced populations are thought to be more unicolonial, forming expansive supercolonies (11, 22–24). Previous work has demonstrated that the absence of intraspecific aggression within supercolonies reduces costs associated with territoriality, and leads to increases in colony size (25). Because individual Argentine ant workers are small, they rely on numerical superiority in interference interactions with larger native species (26). The apparent differences between native and introduced populations in both colony structure and competitive dominance suggest that exploration of the causes of intraspecific aggression will illuminate factors responsible for the Argentine ant's success as an invader.

To elucidate the mechanisms responsible for the success of an invasive species, we compared behavioral and genetic characteristics of *Linepithema humile* at two spatial scales in both its native and introduced ranges. First, we quantified intraspecific aggression among nests at a local scale within seven introduced and eight native populations. Next, we examined the relationship between intraspecific aggression and genetic similarity among nests from sites along 1,000-km transects through California (introduced range) and northern Argentina (native range). Finally, to examine more closely the mechanisms that delineate colony structure, we investigated the relationship between genetic similarity and intraspecific aggression across supercolony boundaries.

Methods

Study Areas. To examine the frequency and degree of intraspecific aggression among nests at a local spatial scale, behavioral data were collected in Argentina at five sites on the Rio Paraná [Reserva Otamendi (8 nests), Reserva Ecologíca Costanera Sur (9 nests), Parque Nacional PreDelta (9 nests), Porto Ocampo (9 nests), and Itá-Ibaté (9 nests)] and at three sites on the Rio Uruguay [Ibicuy (7 nests), Colón (6 nests), and Alvear (7 nests)]. In the introduced range, behavioral data were collected in Bermuda (6 nests) and Chile (12 nests) and from five sites in the United States [New Orleans, LA (6 nests); La Jolla, CA (12 nests); Encinitas, CA (10 nests); Temecula, CA (6 nests); and Palo Alto, CA (7 nests)] (Fig. 1).

We collected workers for large-scale genetic and behavioral analyses from nests at six sites in Argentina: Buenos Aires, Rosario, Parque Nacional PreDelta, Porto Ocampo, Isla de las Cerritas, and Itá-Ibaté. In the United States, workers for these analyses were collected at nine sites within California: Los Angeles, Santa Barbara, Morro Bay, Santa Maria, King City, Salinas, San Jose, Sausalito, and Ukiah (Fig. 1).

To examine more closely the relationship between intraspecific aggression and genetic similarity, we also collected workers

Data deposition: The sequences of the microsatellite loci reported in this paper have been deposited in the GenBank database (accession nos. AF173162, AF173163, AF173164, and AF254979).

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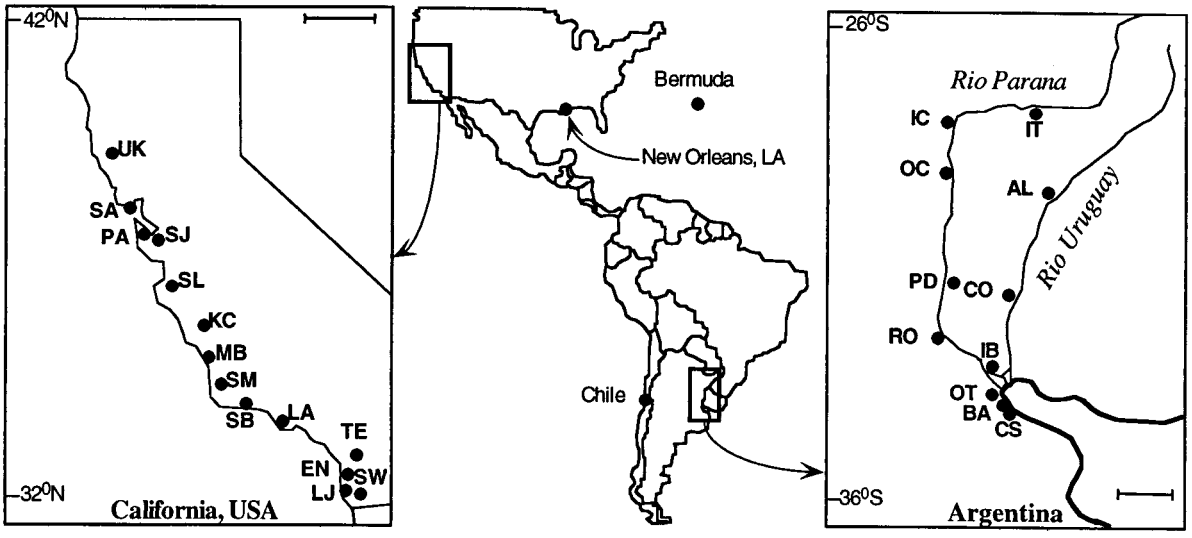

Fig. 1. Sites used in this study. Introduced populations of Argentine ants were sampled in Chile, Bermuda, and the United States. Sampling sites in the United States included New Orleans, Louisiana and the following sites in California: Ukiah (UK), Sausalito (SA), Palo Alto (PA), San Jose (SJ), Salinas (SL), King City (KC), Morro Bay (MB), Santa Maria (SM), Santa Barbara (SB), Los Angeles (LA), Temecula (TE), Encinitas (EN), La Jolla (LJ) and Sweetwater Reservoir (SW). Sites in San Diego County, California not shown: Solana Beach, Lake Hodges and Mission Trails Regional Park. Native populations in Argentina include: Itá-Ibaté (IT), Isla de las Cerritas (IC), Porto Ocampo (OC), Alvear (AL), Parque Nacional PreDelta (PD), Colón (CO), Rosario (RO), Ibicuy (IB), Reserva Nacional Ecológica Otamendi (OT), Buenos Aires (BA), and Reserva Ecológica Costanera Sur (CS). (Bars in the expanded maps represent 100 km.)

from different supercolonies. We defined a supercolony as a group of nests among which intraspecific aggression was absent. In Argentina, workers were collected from eight supercolonies at three sites: Reserva Otamendi (6 nests, 3 colonies), Reserva Ecológica Costanera Sur (9 nests, 4 colonies), and Buenos Aires (2 nests, 1 colony). Because intraspecific aggression is rare in the introduced range, extensive searching was necessary to find different supercolonies in California. In California, workers were collected from seven sites representing six supercolonies: Encinitas (9 nests, 2 supercolonies), La Jolla (8 nests, 1 supercolony), Temecula (4 nests, 2 supercolonies), Solana Beach (1 nest, 1 supercolony), Sweetwater Reservoir (2 nests, 1 supercolony), Lake Hodges (1 nest, 1 supercolony), and Mission Trails Regional Park (1 nest, 1 supercolony) (Fig. 1). One supercolony is represented by nests from multiple sites (Encinitas, La Jolla, Temecula, and Solana Beach).

Behavioral Assays. We measured intraspecific aggression with a standard aggression assay (20, 25). We randomly selected a single worker from each of two nests and placed them together in a vial with Fluon-coated sides for 5–10 min. We scored the behavioral interactions that ensued in order of escalating aggression: touch = 1 (contacts that included prolonged antennation), avoid = 2 (contacts that resulted in one or both ants quickly retreating in opposite directions), aggression = 3 (lunging, biting, and pulling legs or antennae), or fight = 4 (prolonged aggression between individuals). We recorded the highest level of aggression for each trial and then used the mean of 5–10 trials for each nest pairing to calculate an average aggression score. This assay had a high repeatability ($r = 0.881$) (27) and, on average, variance among trials within nest pairings was low ($s^2 = 0.302$).

Molecular Analysis. Four microsatellite loci (Lihu C1.1, Lihu M1, Lihu S3, and Lihu T1) were cloned from a small-insert genomic library (20). Primers for three additional loci (Lhum-11/11B, Lhum-14, and Lhum-33) were designed on the basis of sequences present in GenBank (accession nos. AF093525, AF093520, and AF093517) (51). Ten to 15 individuals from each nest were genotyped at these seven microsatellite loci, and these data were used to calculate unbiased estimates of expected heterozygosity (28) and the percentage of alleles shared by each pair of nests. The percentage of alleles shared is a measure of genetic similarity and refers to the alleles shared at these focal loci, and not the alleles shared overall.

Statistical Analysis. We used the percentage of alleles shared rather than relatedness coefficients (R) (29) as our measure of genetic similarity between nests. Estimates of percent alleles shared were calculated for each nest pair as the number of alleles shared by two nests divided by the sum of alleles possessed by both nests. We did not use R because it is a measure of genetic similarity calculated relative to a reference population (P*) (29). Because introduced populations are very genetically homogeneous, R will be low when calculated with only the introduced population as P*, regardless of the level of absolute genetic similarity. Alternatively, when P* includes both native and introduced populations, R values within introduced populations will be higher (30, 31). However, when two widely divergent groups are combined in this way, the allele frequencies of the reference population will fall between the values of each range and will not accurately represent either. We have therefore chosen to use a more absolute measure, the percentage of alleles shared, to determine levels of genetic similarity between groups.

To examine the relationship between genetic similarity and intraspecific aggression, we used linear regression. Each point in our regressions represents a unique pairing of nests, but some nests were used in more than one pairing. Consequently, these points may not be completely independent of one another. Therefore, we tested significance (one-tailed) by using a Mantel test and 10,000 permutations of the data. Nest pairing for behavioral assays was distributed across nests to prevent overrepresentation by a single or a few sites. In Argentina the mean number of trials per nest was 3.4 ± 0.3 (SE). In California each nest was used for 6.4 ± 0.5 trials. The Student t test was used to

Table 1. The expected heterozygosity (H_{exp}) and number of alleles at seven microsatellite loci for native (Argentina) and introduced (California) populations

	Argentina (n = 255)		California (n = 460)	
Locus	H_{exp}	No. of alleles	H_{exp}	No. of alleles
Lhum-11/11B	0.851	15	0.726	11
Lhum-14	0.430	3	0.058	3
Lhum-33	0.611	9	0.093	3
Lihu C1.1	0.654	5	0.121	2
Lihu M1	0.642	8	0.073	3
Lihu S3	0.465	6	0.160	3
Lihu T1	0.821	13	0.199	5
Mean (SE)	0.639 (0.060)	59	0.204 (0.089)	30

examine differences between the slopes and intercepts of the regressions.

Results

The Argentine ant passed through a genetic bottleneck during its introduction to California. Overall, the number of alleles in the introduced range is half that found in the native range, despite greater sampling in the introduced range (Table 1). Similarly, the average expected heterozygosity in California has decreased by 68% relative to that found in the native range (Table 1).

Within sites, patterns of intraspecific aggression differed between the native and introduced ranges. Intraspecific aggression was typically absent in introduced populations (Fig. 2*A*). In contrast, pronounced intraspecific aggression was common throughout the native range (Fig. 2*B*). These data illustrate the striking disparity in behavior between ants in the two geographic ranges.

At large spatial scales, behavioral and genetic characteristics were also markedly different between the two ranges. In Argentina, distant nest pairs displayed high levels of aggression and low

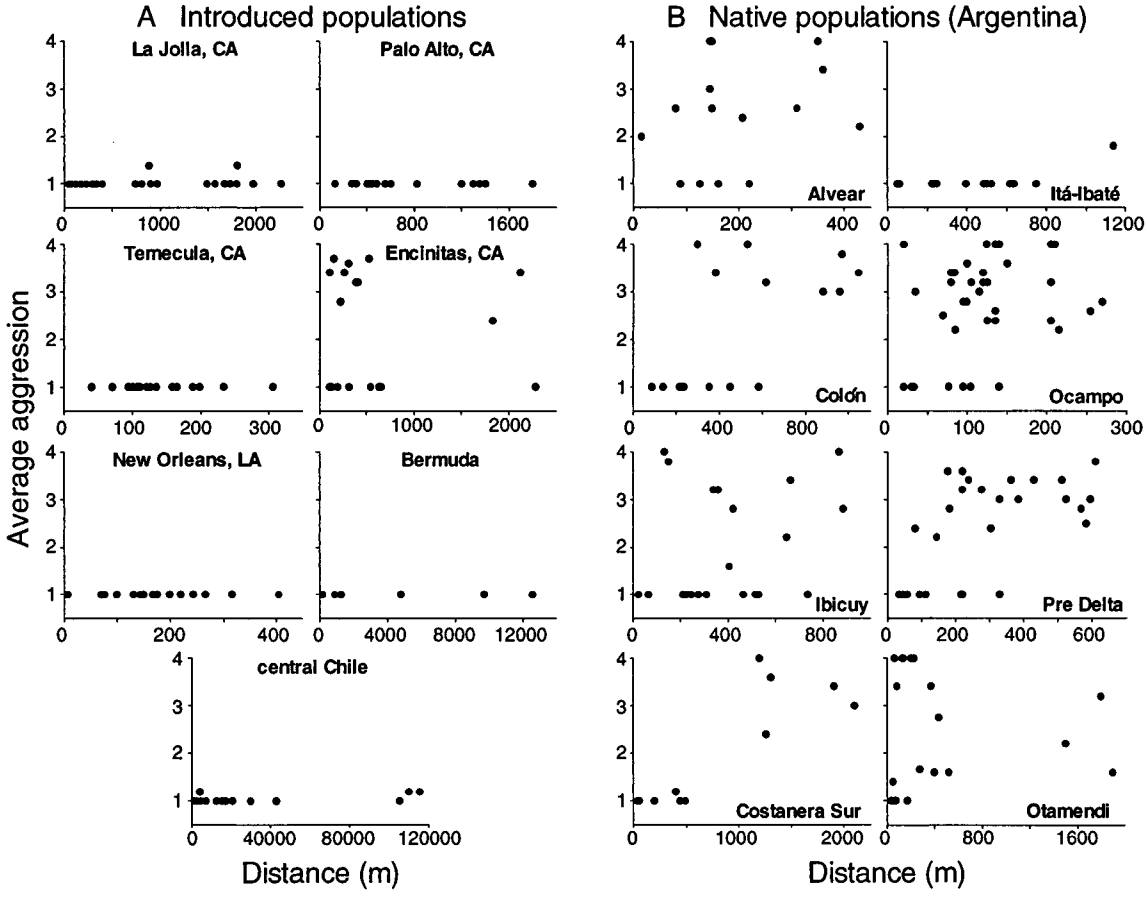

Fig. 2. Relationship between degree of intraspecific aggression and distance between nest pairs at each of 15 sites within native (*A*) and introduced (*B*) ranges of the Argentine ant. Native populations typically exhibited pronounced intraspecific aggression over short distances. In contrast, intraspecific aggression was rare in introduced populations. Aggression between nests was measured by using a behavioral assay ranging from 1 (no aggression) to 4 (high aggression).

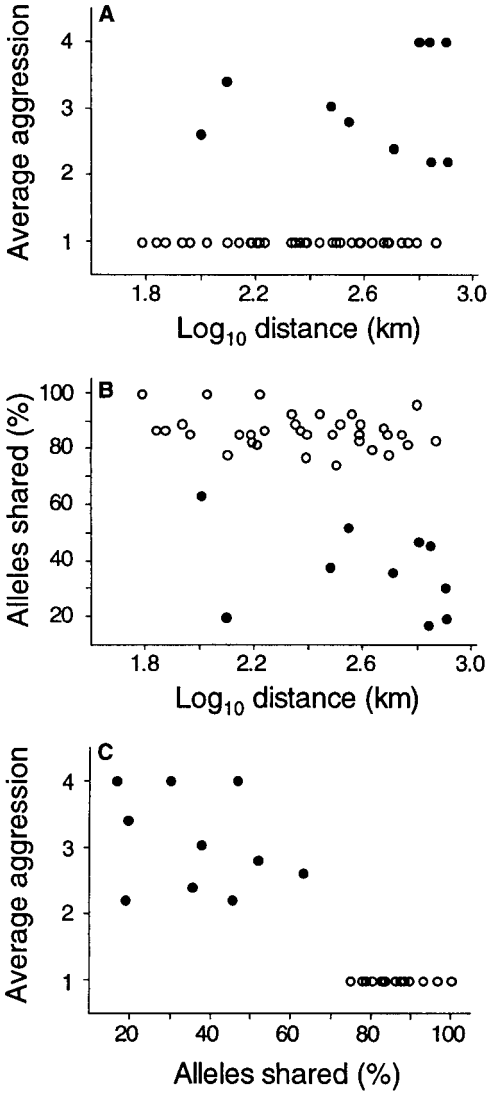

Fig. 3. Relationships among intraspecific aggression, genetic similarity, and distance at large spatial scales (60–800 km) in native (Argentina, ●) and introduced (California, ○) populations. Aggression between nests was measured by using a behavioral assay ranging from 1 (no aggression) to 4 (high aggression). (*A*) Relationship of intraspecific aggression and distance between nests. Aggression scores did not overlap between native and introduced populations. All nest pairs in Argentina displayed high levels of aggression, whereas nest pairs in California did not. (*B*) Genetic similarity between nests versus distance. Nest pairs in California shared at least 75% of alleles. In contrast, 17–63% of alleles were shared between nest pairs in Argentina. (*C*) Relationship between aggression and genetic similarity of nests. In California, aggression was absent and nests were genetically similar, whereas in the native range the opposite pattern held.

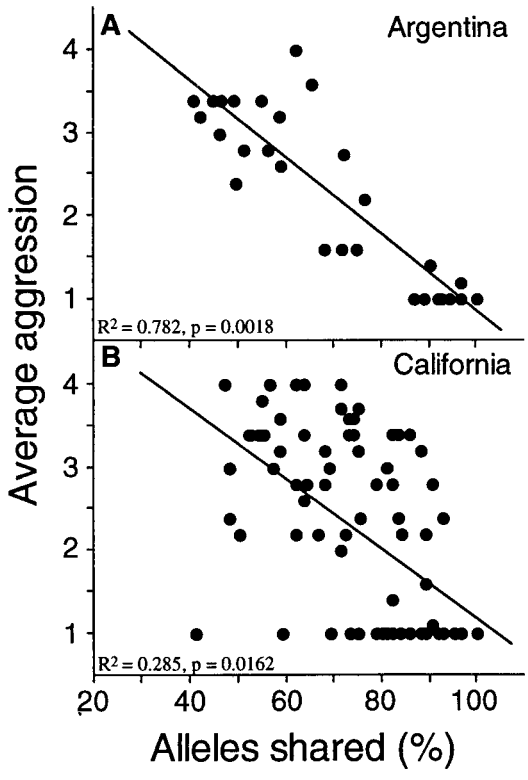

Fig. 4. Relationship of aggression between nests and their genetic similarity. Aggression between nests was measured by using a behavioral assay ranging from 1 (no aggression) to 4 (high aggression). For this analysis, extensive sampling was necessary to locate nest pairs that exhibited intraspecific aggression within California. Average aggression decreased with increasing genetic similarity in both Argentina (*A*; 29 nest pairs, $y = 5.48 - 0.046x$) and California (*B*; 83 nest pairs, $y = 5.37 - 0.042x$). The slopes and intercepts of these regressions did not differ (*t* tests, $P > 0.5$). Additionally, adding the nest pairs from the long distance comparisons (Fig. 3) increases significance.

levels of genetic similarity (Fig. 3). In contrast, equally distant nest pairs in the introduced range were genetically similar, and aggression was not observed (Fig. 3). These data mirror the behavioral differences observed between the native and introduced populations at small spatial scales (Fig. 2).

Once intercolony aggression was detected in the introduced range (e.g., Fig. 2, Encinitas), intensive sampling was initiated to locate other supercolonies and identify their boundaries. The results of these pairings are shown in Fig. 4. Despite the genetic and behavioral differences observed between native and introduced populations, the quantitative relationship between degree of intraspecific aggression and genetic similarity appears similar in both ranges. Specifically, when intraspecific aggression is detected in the introduced range, the degree of aggression among nests decreases with increasing genetic similarity, as in native populations (Fig. 4). In addition, the slopes and intercepts of these regressions did not differ (Fig. 4), suggesting that the mechanism underlying nestmate recognition has been retained in introduced populations. The greater variance about the regression in Fig. 4*B* is largely a consequence of reduced genetic variability of introduced populations. Because the total number of alleles in introduced populations is one-half that observed in native populations, the effect of sampling error (e.g., failure to detect an allele) will be twice as large in the introduced range. This sampling error disproportionately increases the variance around the regression in introduced populations. Additionally, the variation around the regression in both ranges will be increased because microsatellites are indicators of genetic similarity and presumably are not directly involved in the process of nestmate recognition. Therefore, the increased scatter around the regression in Fig. 4*B* is both expected and consistent with predictions based on the loss of genetic diversity experienced by introduced populations. Despite the influence of these factors, the relationship between genetic similarity and intraspecific aggression is highly significant in both ranges (Fig. 4).

Discussion

Our findings demonstrate that, relative to native populations of Argentine ants, introduced populations are less diverse geneti-

cally and display intraspecific aggression less frequently. However, when introduced populations do exhibit intraspecific aggression, the relationship between genetic similarity and the degree of aggression resembles that of native populations. Therefore, the absence of intraspecific aggression in introduced populations has likely resulted from a loss of genetic variability associated with a population bottleneck. Because reduced intraspecific aggression leads to the higher population densities key to the ecological dominance of Argentine ants (25, 26), our results demonstrate how a relatively simple population genetic change can have dramatic ecological and economic consequences. The ecological success of this species is surprising, given that reductions in genetic diversity are generally believed to be harmful (32, 33).

Genetically based cues can be used to identify nestmates because colony members are often related in social insects (34–37). In such systems, selection should set a threshold of genetic similarity necessary for the acceptance of nestmates. In Argentine ants, this threshold will be set relative to the high genetic diversity in native populations. Although introduced populations still possess the ability to recognize genetically different individuals (Fig. 4), a consequence of their widespread genetic similarity is that there is rarely sufficient genetic differentiation to elicit intraspecific aggression. Thus, low levels of genetic differentiation could lead to the formation of expansive supercolonies as reported here (Figs. 2 and 3) and in other introduced populations (11, 23).

Unicoloniality is a recognized problem for kin selection theory because workers appear to bestow altruism upon unrelated individuals (38, 39). However, our findings suggest that unicoloniality may arise through the widespread loss of genetic diversity and the resulting subversion of kin recognition mechanisms. Additional support for this hypothesis comes from the widespread prevalence of unicoloniality among other invasive ant species (such as *Wasmannia auropunctata*, *Pheidole megacephala,* and *Monomorium pharaonis*) (24, 38, 40) many of which undoubtedly experienced a genetic bottleneck during introduction. In contrast, noninvasive unicolonial species, such as mound-building *Formica,* may have achieved unicoloniality via an alternative pathway involving the monopolization of stable resources and long-lived nest sites (38, 41).

One well-studied invasive species is the red imported fire ant (*Solenopsis invicta*), in which differences in colony structure exist between native and introduced populations (42–44). In its introduced range, the more ecologically destructive multiple-queened (polygyne) form of *S. invicta* (45) exhibits reduced intraspecific aggression relative to the single-queened (monogyne) form (46). Introduced populations of *S. invicta* have also passed through a population bottleneck that has reduced genetic variation (43) and affected the sex-determining system (47). It will be of interest to determine whether the occurrence of intraspecific aggression differs between native and introduced populations of other invasive species (such as *S. invicta*) and, if so, to determine whether similar underlying mechanisms are involved. In addition, an examination of native populations of Argentine ants that lack intraspecific aggression over hundreds of meters (e.g., Itá-Ibaté, Fig. 2*B*) will provide further insights into the transition from multicoloniality to unicoloniality.

Although our results suggest that genetic factors play an important role in nestmate recognition in Argentine ants, environmental cues are likely also involved, as they are in other social insects (34, 48). When nestmates share food or nesting material, odors from these sources can be used to assess group membership (34, 48). Three lines of evidence suggest that environmental factors cannot solely explain the occurrence of intraspecific aggression in this system. First, in California, intraspecific aggression was absent across an environmentally heterogeneous 1,000-km transect. Second, in a previous study, intraspecifically aggressive nests of Argentine ants reared on identical diets and under similar lab conditions maintained high levels of aggression over a 3-month period (25). Finally, mate preference studies in the Argentine ant have shown that queens avoid inbreeding by preferentially mating with unrelated males, apparently by using genetically based cues (49).

Despite the apparent short-term success of unicolonial species such as the Argentine ant in its introduced range, there are several reasons why unicoloniality may not persist over evolutionary time. Because many unrelated queens each contribute to the worker caste in unicolonial species, relatedness among nestmates (30, 38, 50), and therefore the heritability of worker behaviors (39), can approach zero. Under these circumstances, adaptive changes in worker behavior could not evolve (39). Alternatively, genetic differentiation among spatially distant populations could lead to increased nestmate recognition and the breakdown of widespread unicoloniality. Additionally, mutations that promote nepotism should be favored and could lead to greater multicoloniality (38). Evidence that unicoloniality may be evolutionarily unstable is provided by its patchy phylogenetic distribution across ant taxa (34, 38).

Our results also suggest a possible control strategy for the Argentine ant. Given the association between genetic variability and intraspecific aggression, the introduction of new alleles into introduced populations could increase genetic differentiation to a level sufficient to trigger intraspecific aggression. An increase in intraspecific competition within introduced populations should decrease the density of Argentine ants, allowing native ant species to compete more effectively, thereby facilitating the recovery of invaded ecosystems. However, a possible risk of this approach is that increasing genetic diversity may undermine future control strategies designed to exploit the genetic homogeneity of introduced populations.

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1. Wilcove, D. S., Rothstein, D., Dubow, J., Phillips, A. & Losos, E. (1998) *Bioscience* **48,** 607–615.
2. Pimentel, D., Lach, L., Zuniga, R. & Morrison, D. (2000) *Bioscience* **50,** 53–65.
3. Vitousek, P. M., D'Antonio, C. M., Loope, L. L. & Westbrooks, R. (1996) *Am. Sci.* **84,** 468–477.
4. Lodge, D. M. (1993) *Trends Ecol. Evol.* **8,** 133–137.
5. Kareiva, P. (1996) *Ecology* **77,** 1651–1697.
6. Simberloff, D. (1989) in *Biological Invasions: A Global Perspective,* eds. Drake, J. A., Mooney, H. A., DiCastri, F., Groves, R. H., Kruger, F. J., Rejmanek, M. & Williamson, M. (Wiley, New York), pp. 61–75.
7. Porter, S. D., Williams, D. F., Patterson, R. S. & Fowler, H. G. (1997) *Env. Entomol.* **26,** 373–384.
8. Orians, G. H. (1986) in *Ecology of Biological Invasions of North America and Hawaii,* eds. Mooney, H. A. & Drake, J. A. (Springer, New York), pp. 133–148.
9. Levine, J. M. & D'Antonio, C. M. (1999) *Oikos* **87,** 15–26.
10. Williams, D. F., ed. (1994) *Exotic Ants: Biology, Impact and Control of Introduced Species* (Westview, Boulder, CO).
11. Newell, W. & Barber, T. C. (1913) *USDA Bur. Entomol. Bull.* **122,** 1–98.
12. Ward, P. S. (1987) *Hilgardia* **55,** 1–16.
13. Suarez, A. V., Bolger, D. T. & Case, T. J. (1998) *Ecology* **79,** 2041–2056.

14. Erickson, J. M. (1971) *Psyche* **78,** 257–266.
15. Human, K. & Gordon, D. M. (1996) *Oecologia* **105,** 405–412.
16. Holway, D. A. (1998) *Oecologia* **116,** 252–258.
17. Cole, F. R., Medeiros, A. C., Loope, L. L. & Zuehlke, W. W. (1992) *Ecology* **73,** 1313–1322.
18. Bond, W. & Slingsby, P. (1984) *Ecology* **65,** 1031–1037.
19. Suarez, A. V., Richmond, J. Q. & Case, T. J. (2000) *Ecol. Appl.* **10**, in press.
20. Suarez, A. V., Tsutsui, N. D., Holway, D. A. & Case, T. J. (1999) *Biol. Invasions* **1,** 43–53.
21. Majer, J. D. (1994) in *Exotic Ants: Biology, Impact and Control of Introduced Species*, ed. Williams, D. F. (Westview, Boulder, CO), pp. 163–173.
22. Markin, G. P. (1968) *J. Kans. Entomol. Soc.* **41,** 511–516.
23. Way, M. J., Cammell, M. E., Paiva, M. R. & Collingwood, C. A. (1997) *Insectes Soc.* **44,** 415–433.
24. Hölldobler, B. & Wilson, E. O. (1977) *Naturwissenschaften* **64,** 8–15.
25. Holway, D. A., Suarez, A. V. & Case, T. J. (1998) *Science* **282,** 949–952.
26. Holway, D. A. (1999) *Ecology* **80,** 238–251.
27. Lessells, C. M. & Boag, P. T. (1987) *Auk* **104,** 116–121.
28. Nei, M. (1987) *Molecular Evolutionary Genetics* (Columbia Univ. Press, New York).
29. Queller, D. C. & Goodnight, K. F. (1989) *Evolution* **43,** 258–275.
30. Crozier, R. H. & Pamilo, P. (1996) *Evolution of Insect Colonies: Sex Allocation and Kin Selection* (Oxford Univ. Press, New York).
31. Pamilo, P. (1989) *Trends Ecol. Evol.* **4,** 353–355.
32. Soulé, M. E. (1986) *Conservation Biology: The Science of Scarcity and Diversity* (Sinauer, Sunderland, MA).
33. Saccheri, I., Kuussaari, M., Kankare, M., Vikman, P., Fortelius, W. & Hanski, I. (1998) *Nature (London)* **392,** 491–494.
34. Hölldobler, B. & Wilson, E. O. (1990) *The Ants* (Harvard Univ. Press, Cambridge, MA).
35. Hölldobler, B. & Michener, C. D. (1980) in *Evolution of Social Behaviour: Hypotheses and Empirical Tests*, ed. Markl, H. (Verlag Chemie, Weinheim, Germany), pp. 35–58.
36. Buckle, G. R. & Greenberg, L. (1981) *Anim. Behav.* **29,** 802–809.
37. Beye, M., Neumann, P., Chapuisat, M., Pamilo, P. & Moritz, R. F. A. (1998) *Behav. Ecol. Sociobiol.* **43,** 67–72.
38. Bourke, A. F. G. & Franks, N. R. (1995) *Social Evolution in Ants* (Princeton Univ. Press, Princeton, NJ).
39. Queller, D. C. & Strassman, J. E. (1998) *Bioscience* **48,** 165–175.
40. Passera, L. (1994) in *Exotic Ants: Biology, Impact and Control of Introduced Species*, ed. Williams, D. F. (Westview, Boulder, CO), pp. 23–43.
41. Chapuisat, M. & Keller, L. (1999) *Behav. Ecol. Sociobiol.* **46,** 405–412.
42. Ross, K. G. & Keller, L. (1995) *Annu. Rev. Ecol. Syst.* **26,** 631–656.
43. Ross, K. G., Vargo, E. L. & Keller, L. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 3021–3025.
44. Tschinkel, W. R. (1998) *Bioscience* **48,** 593–605.
45. Porter, S. D. & Savignano, D. S. (1990) *Ecology* **71,** 2095–2106.
46. Vander Meer, R. K., Obin, M. S. & Morel, L. (1990) in *Applied Myrmecology: A World Perspective*, eds. Vander Meer, R. K., Jaffe, K. & Cedeno, A. (Westview, Boulder, CO), pp. 322–328.
47. Ross, K. G., Vargo, E. L., Keller, L. & Trager, J. C. (1993) *Genetics* **135,** 843–854.
48. Carlin, N. F. (1989) *Neth. J. Zool.* **39,** 86–100.
49. Keller, L. & Passera, L. (1993) *Behav. Ecol. Sociobiol.* **33,** 191–199.
50. Keller, L. (1995) *Trends Ecol. Evol.* **10,** 355–360.
51. Krieger, M. J. B. & Keller, L. (1999) *Mol. Ecol.* **8,** 1075–1092.

Draft genome of the globally widespread and invasive Argentine ant (*Linepithema humile*)

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Ants are some of the most abundant and familiar animals on Earth, and they play vital roles in most terrestrial ecosystems. Although all ants are eusocial, and display a variety of complex and fascinating behaviors, few genomic resources exist for them. Here, we report the draft genome sequence of a particularly widespread and well-studied species, the invasive Argentine ant (*Linepithema humile*), which was accomplished using a combination of 454 (Roche) and Illumina sequencing and community-based funding rather than federal grant support. Manual annotation of >1,000 genes from a variety of different gene families and functional classes reveals unique features of the Argentine ant's biology, as well as similarities to *Apis mellifera* and *Nasonia vitripennis*. Distinctive features of the Argentine ant genome include remarkable expansions of gustatory (116 genes) and odorant receptors (367 genes), an abundance of cytochrome P450 genes (>110), lineage-specific expansions of yellow/major royal jelly proteins and desaturases, and complete CpG DNA methylation and RNAi toolkits. The Argentine ant genome contains fewer immune genes than *Drosophila* and *Tribolium*, which may reflect the prominent role played by behavioral and chemical suppression of pathogens. Analysis of the ratio of observed to expected CpG nucleotides for genes in the reproductive development and apoptosis pathways suggests higher levels of methylation than in the genome overall. The resources provided by this genome sequence will offer an abundance of tools for researchers seeking to illuminate the fascinating biology of this emerging model organism.

Hymenoptera | invasive species | transcriptome | chemoreception | sociality

Ants are pivotal players in the Earth's terrestrial ecosystems. They include >14,000 described species, comprise about half of all insect biomass in the tropics, are the most important predators of insects and other arthropods, and turn and aerate more soil than earthworms (1). The diversity of lifestyles displayed by ants is equally impressive, including minute, acorn-dwelling species (and the ants that parasitize them), fungus-growing leafcutters with complex division of labor, trap-jaw ants that exhibit the fastest animal movements (2), slave-making ants that kidnap and enslave the young of other ants, and the countless teeming multitudes of army ants that prowl the leaf litter and subterranean habitats (1).

Some ant species have been introduced to new geographic ranges by human activities, and a few have emerged as damaging and destructive invasive species (3). The Argentine ant (*Linepithema humile*) is one of the most widely distributed of these invaders and is established in nearly every Mediterranean-type climate in the world (4). Introduced Argentine ants form enormous "supercolonies," often across hundreds or thousand of kilometers (5), and workers from the largest supercolonies on different continents even accept each other as colonymates (6). At a local scale, the absence of aggression among workers within supercolonies allows them to direct resources toward colony growth (7) and attain high population densities. These introduced populations are then able to outcompete and eliminate native ants, which imperils plants and animals that normally interact with the native ants (5). In contrast, Argentine ants in their native South American range display high levels of aggression and genetic structure among colonies across substantially smaller spatial scales (8, 9). Argentine ants are also significant pests and thus are targets of heavy insecticidal control, leading to environmental contamination and harm to nontarget organisms (10).

Despite their importance and prominence, there are essentially no genomic resources for Argentine ants. Here we report the transcriptome (GenBank accession no. 46575) and de novo ge-

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. 46575 (transcriptome), 45799 (genome), and 45805 (genome project)].

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nome sequence of the Argentine ant (GenBank accession no. 45799), generated using a combination of Roche 454 and Illumina sequencing. Project data are archived at the Hymenoptera Genome Database (http://hymenopteragenome.org/linepithema/genome_consortium). This project (GenBank accession no. 45805) represents an organizational advance in emerging model organism genomics (11), as it was not supported by a federal grant or genome sequencing center but, rather, was accomplished using small sums of discretionary funds provided by members of the insect genomics and Argentine ant research communities. This genome sequence provides a powerful resource for future analysis of gene families and phenotypes and candidate SNP markers for future population genetic and association mapping studies.

Results and Discussion

Genomic Features. We sequenced the Argentine ant genome ($2n = 16$) to ~23× coverage using source material from a single nest of the large California supercolony (Table 1 and *SI Appendix S1*, Table S1). The combined assembly of Roche 454 (~9× coverage) and Illumina (~14×) sequencing resulted in 215.6 megabases (Mb) of scaffolded sequence (86% of the 250.8-Mb genome; ref. 12). We also recovered 12,516 bp of the mitochondrial genome in three scaffolds (*SI Appendix S1*, Fig. S1). We assembled the combined 454 and Illumina data using the Roche gsAssembler (454 Life Sciences) and Celera CABOG assemblers (13) (Table 1, *SI Appendix S1*, Table S1). Early 454-only assemblies contained homopolymer errors, but addition of Illumina sequence data produced marked improvements (Table 1) with an overall error rate estimated at 0.012% (*SI Appendix*). Overall, the Celera assembler yielded the best assembly, and addition of the Illumina data dramatically increased contig and scaffold length (Table 1). We also generated a 454 transcriptome sequence from ants of mixed age, caste, and geographic location and used it to train hidden Markov models for a custom MAKER (14) annotation pipeline (*SI Appendix S1*, Table S2).

The assembled genome appears to be relatively complete. First, our genome assembly captured 99% (246 of 248) of the core CEGMA genes (15), and 96% of them (239 of 248) were complete. Second, annotation of the cytoplasmic ribosomal protein genes revealed 83 genes, including the full set of 79 cytoplasmic ribosomal proteins (16, 17), and 4 duplicated genes (*RpS16*, *RpS23*, *RpS28*, and *RpS30*) (*SI Appendix S1*, Table S2). Third, annotation of the 67 nuclear-encoded oxidative phosphorylation genes shows that the *L. humile* genome assembly is only missing *cox7a* (*SI Appendix S1*, Table S2). This gene is also absent from the *Apis mellifera* genome, suggesting that it has been lost from the *L. humile* genome.

We generated a de novo repeat library of 523 elements and performed a whole-genome repeat annotation (Table 2). We found that 41 predictions were classified as retroid transposable elements (TEs) and 42 as DNA transposons, and 424 could not be classified. Although it has been hypothesized that the paucity of TEs in the *A. mellifera* genome is a product of eusociality, their abundance in the *L. humile* genome suggests otherwise. The *L. humile* genome also possesses remnants of viruses and viroids from a variety of families (*SI Appendix S1*, Table S4). The total repeat content was 28.7 Mb (13%), but without de novo repeat libraries, only 3.78% (8.3 Mb) of the *L. humile* assembly was identified as repetitive, with only 1.4% (3 Mb) identified as TEs. The *L. humile* genome was 62% AT, which is intermediate between *A. mellifera* (67%) and *Nasonia vitripennis* (58%). When combined with the 37.7 Mb of missing and ambiguous nucleotides, the total repetitive fraction (~66.4 Mb; 30.8% of the genome) was significantly higher than *A. mellifera* (10%), but lower than *N. vitripennis* (40%).

We described 16,123 genes (16,177 transcripts) in the *L. humile* official gene set (OGS1.1); 8,303 (51%) were supported by EST evidence, which confirmed 70% and 76% of the splice sites and exons for these genes, respectively. Of these genes, 1,364 were shared with *A. mellifera* and *N. vitripennis*, but not *Drosophila melanogaster* (Fig. 1), and 589 were shared by the two aculeate Hymenoptera, *L. humile* and *A. mellifera*, but not with *Nasonia* (the outgroup genome for the Aculeata) or *Drosophila*. Although *L. humile* and *A. mellifera* are both social, Aculeata also contains solitary species. A total of 7,184 genes (45%) were unique to *L. humile* relative to these three other species.

We used Interproscan (18) and KEGG (19) (*SI Appendix S1*, Table S5 and Fig. S2) to identify putative functional domains and compared Gene Ontology (GO; *SI Appendix S1*, Fig. S3) terms for all *L. humile* genes relative to *D. melanogaster*, *A. mellifera*, and *N. vitripennis* (*SI Appendix S1*, Tables S6 and S7) and for the *L. humile*-specific genes. Of 16,123 genes in the OGS1.1, a total of 7,514 (44%) were annotated with at least one GO term (average = 3). Of the 7,184 (16%) genes unique to *L. humile*, 1,174 (Fig. 1 and *SI Appendix S1*, Fig. S3) had ontology terms. In the subset of genes found only in *L. humile*, 99 terms were enriched ($P < 0.05$) relative to all *L. humile* genes (*SI Appendix S1*, Fig. S3). These included odorant receptors, peptidases that may play a role in venom production, genes associated with lipid activities that could be involved in cuticular hydrocarbon (CHC) synthesis or catabolism, and DNA methylation genes, which may play a role in caste development.

Relative to other species, six cellular location terms were enriched, with most associated with the synapse ($P < 1.96E{-}04$) or postsynaptic membrane ($P < 4.74E{-}04$; *SI Appendix S1*, Table S7). Of the 17 genes associated with enriched molecular functions, 6 (all $P < 1.87E{-}08$ for GO categories) included cation binding and may be involved in neurological or other signal transduction ($P < 1.36E{-}04$) processes associated with odorant binding and olfaction, learning, memory, and behavior. Lipid catabolism ($P < 1.59E{-}05$), lipase ($P < 8.88E{-}03$), and phospholipid function (3; all $P < 0.04$) enrichments may include genes for the synthesis of CHCs involved in social communication. Electron transport, heme, and cation binding functions characteristic of cytochrome P450s were also enriched, consistent with the observed expansion of these genes.

The mean GC content of the *L. humile* genome was 37.7%, and the ratio of observed to expected CpG nucleotides [CpG(O/E)] was 1.55 (Fig. 2); both values are within the ranges reported for other Hymenoptera (*A. mellifera* and *N. vitripennis*; refs. 20 and 21). A comparison of GC compositional-domain lengths among

Table 1. Genome and sequencing statistics for three successive assemblies

	Roche gsAssembler	Celera V0.3	Celera V0.4
Total scaffolded sequence, bp*	199,810,258 (80%)	218,892,451 (87%)	215,552,578 (86%)
No. of scaffolds	3,093	3,180	3,030
No. of contigs	48,934	22,086	18,227
N50 contig size, bp	18,503†	28,104	35,858
N50 scaffold size, bp	453,083	1,427,074	1,386,360
Total contig coverage	25×	14.6×	23×
Total reads used in scaffolds	38,902,428	21,970,969	46,779,980
454			
3-kb paired-end	1,613,759	1,276,042	1,255,603
8-kb paired-end	1,789,614	298,804	292,277
Unpaired	5,040,847	4,440,267	4,346,871
Illumina			
3-kb paired-end	NA	12,088,916	1,2265,551
8-kb paired-end	NA	3,866,940	4,047,251
Unpaired	31,222,237	0	24,572,427

NA, not applicable.
*Values in parentheses indicate percent of the total genome size (12).
†Contigs > 500 bp; genome size, 250.8 Mb (0.26 pg).

Table 2. Genes, repeats, and annotated features

	No. of features	Length occupied, bp	% of genome
Genes	16,331	20,828,920	8.30
OGS 1.1 Genes	16,123	ND	ND
OGS 1.1 Transcripts	16,177	20,754,334	8.28
miRNA genes	71	ND	ND
Pseudogenes	58*	ND	ND
snRNA	84	45,847	0.02
rRNA	53	28,739	0.01
Assembled transcriptome contigs[†]	20,070	7,634,808	3.04
Assembled transcriptome isotigs[‡]	5,352	4,845,691	1.93
Repeats	352,005	58,953,684	23.5
Non-interspersed repeats	88,703	5,125,662	2.04
Simple repeats	33,764	1,704,425	0.68
Low complexity	54,932	3,420,628	1.36
Satellite	7	609	0
Microsatellites[§]	30	58,679	0.02
Interspersed repeats	85,324	23,613,393	9.27
LTR	4,021	2,324,263	0.93
LINE	3,068	1,432,386	0.57
DNA	13,307	3,947,143	1.57
SINE	739	152,262	0.06
Helitron	182	117,105	0.05
Novel	67,928	17,057,139	6.8
Predictions	523[¶]	ND	ND
RECON	348[¶]	ND	ND
RepeatScout	132[¶]	ND	ND
PILER-DF	43[¶]	ND	ND

ND, not done.
*Derived from manually curated gustatory, odorant, and ionotropic receptor, ribosomal, and oxidative phosphorylation genes.
[†]Assembled with Roche gsAssembler Version 2.0.
[‡]Assembled with Roche gsAssembler Version 2.3.
[§]Obtained from GenBank.
[¶]Not including predictions screened out as redundant or false positive.

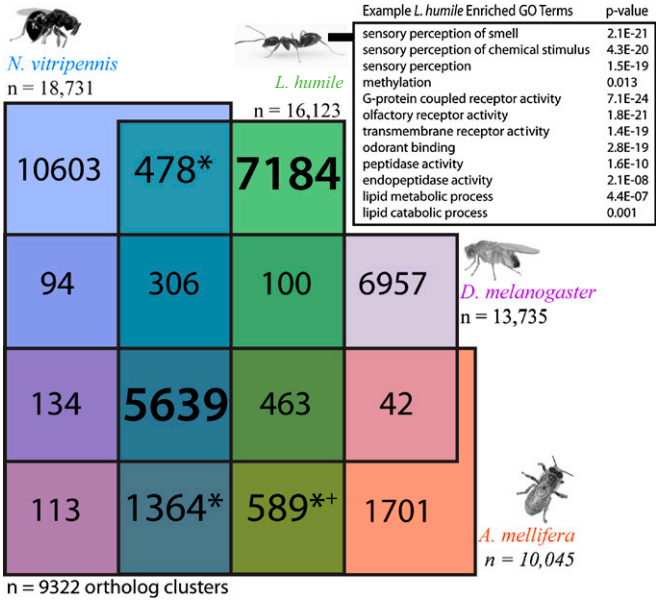

Fig. 1. Venn diagram of predicted orthologs. *L. humile* (green), *A. mellifera* (red), *N. vitripennis* (blue), and *D. melanogaster* (purple) protein sets are shown. (*Inset*) A subset of the 99 GO terms enriched specifically in *L. humile*. Bold numbers represent genes common to all species studies or present in *L. humile* only (green). Predicted orthologs only present in the social hymenoptera tested (+) or in two or three of the hymenoptera tested (*) are indicated.

L. humile, *A. mellifera*, *N. vitripennis*, *Tribolium castaneum*, *Anopheles gambiae*, and *D. melanogaster* shows that the hymenopterans have the smallest proportion (0.1–0.5%) of long compositional domains (>100 kb) and the widest range in GC content (*SI Appendix S1*, Fig. S7 and Table S9). Similar to the other sequenced Hymenoptera, genes in *L. humile* occur in more GC-poor regions (*SI Appendix S1*, Fig. S8). Although the mean CpG(O/E) values of hymenopteran genomes are among the highest known, the distribution of CpG(O/E) within genomes is not consistent among the three hymenopterans (*SI Appendix S1*, Figs. S9 and S10).

Sociality. The sophisticated social structures of ant colonies are regulated by a complex interplay of chemical signaling, perception of those signals, and behavioral responses. Through these interactions, ants coordinate the activities of myriad colony members, allowing division of labor among behavioral and morphological castes. To clarify the genetic and genomic contributions to these aspects of ant sociality, we analyzed genes for the production of chemical signals (desaturases), the reception of chemical signals, gene networks that underlie differential wing and reproductive development between castes, and the yellow/major royal jelly protein gene family (which plays a key role in the development of honey bee queens vs. workers).

As in many social insects, CHCs are colony recognition cues for *L. humile* (22). The number and location of double bonds in unsaturated CHCs are regulated by desaturase genes. We found 16 complete Δ9 desaturase genes in the *L. humile* genome, including 7 with EST support (Fig. 3*D* and *SI Appendix S1*, Fig. S15). We also identified 17 partial or fragmentary Δ9 desaturase genes, including 6 supported by ESTs. Compared with *N. vitripennis* and *A. mellifera*, which possess 16 and 7 Δ9 desaturase genes, respectively, *L. humile* has a large expansion in Δ9 desaturase genes. The details of how and when these genes are expressed and the consequences for ant CHC profiles will provide insights into how these chemicals mediate social interactions within and among colonies.

The detection of chemical signals, such as CHCs, is mediated by a battery of gustatory receptors (Grs), olfactory binding proteins (OBPs), odorant receptors (Ors), and ionotropic receptors (IRs). Our annotation revealed a remarkable proliferation of these chemosensory genes in the Argentine ant. We found and manually annotated 116 *L. humile* Grs (including 20 pseudogenes; *SI Appendix S1*, Table S10), compared with the 11 AmGrs reported in *Apis* (23) and the 58 NvGrs in *Nasonia* (24). Major subfamily gene expansions have occurred in *L. humile* subfamily A, related to the Am/NvGr8/9 genes, and subfamily B, which is related to the NvGr48-50 genes and a subfamily of fragmented pseudogenes in *Apis* (Fig. 3*A*). These two expanded subfamilies may include bitter taste receptors for defensive plant compounds, which may be functionally lost from *A. mellifera* (subfamily B) or unexpanded in *A. mellifera* and *Nasonia* (subfamily A).

The Or family of 7-transmembrane proteins mediates most of insect olfaction, and ants are expected to have a large Or repertoire. We manually annotated 367 Or genes (337 genes and 30 pseudogenes), revealing several ant-specific gene subfamily expansions, as well as losses from ant, bee, and wasp (*SI Appendix S1*, Table S11 and *SI Appendix S2*, Fig. S12). A large 9-exon gene subfamily is highly expanded in *L. humile* and may encode the receptors for the CHCs that are used for colonymate recognition (*SI Appendix*).

The ionotropic glutamate receptor-related chemosensory receptors (IRs) are also likely involved in Argentine ant chemosensory behaviors. In the *L. humile* genome, we found 32 IR genes (Fig. 3*B*, *SI Appendix S1*, Table S12), whereas *A. mellifera* and *N. vitripennis* each possess only 10 (25). Five *L. humile* IRs appear to be orthologous to conserved IRs in other insect genomes that are expressed in olfactory organs (Fig. 3*B*; *IR25a, IR8a, IR93a, IR76b*, and *IR68a*; ref. 26). Other IRs appear to be ant-specific, with low homology to other insect receptors, and may be used for species-specific recognition behaviors (25).

Caste differentiation and division of labor within colonies are key innovations underlying the proliferation of ants, both numerically and taxonomically. Although the processes underlying caste differentiation are not fully understood, CpG DNA methylation is an important process for transcriptional regulation in many animals (27–29) and plays a role in the development of honey bee queens vs. workers (30). *L. humile* possesses a fully intact methylation toolkit with all three *Dnmt* genes (*SI Appendix S1*, Table S2 and Fig. S4). The only other sequenced insect genomes with the de novo methyltransferase *Dnmt3* are the pea aphid, *A. mellifera*, and *N. vitripennis* (20, 31). Interestingly, all three genes occur as single copies in *L. humile*, whereas *A. mellifera* and *N. vitripennis* have two and three copies of *Dnmt1*, respectively. Although inherited and de novo methylation have not yet been tested in *L. humile*, active methylation occurs in at least two other ant subfamilies (32).

To test for a genomic signature of CG methylation, we performed two dinucleotide analyses (*SI Appendix S1*, Figs. S5–S10 and Table S8) that revealed overall CG bias similar to *A. mellifera*, but no indication of exon or intron-specific methylation as seen in *A. mellifera* or *N. vitripennis*, respectively. We also found that dinucleotide transition SNPs associated with DNA methylation (i.e., CG↔TG) were 10-fold more prevalent in the *L. humile* SNP data compared with either of the transversions at this site (i.e., CG↔AG, CG↔GG). We observed 21,623 SNPs at the C position of the CG/TG sites, which should yield an expected 17,428 CG↔TG transition SNPs (80.6%) based on the overall observed genome rate of 4.15 transition mutations per transversion. However, we measured 18,611 CG↔TG transitions (86%), which indicated significant bias of CG↔TG SNPs relative to all other mutations at this site (χ^2 = 80.3, P < <0.001). This bias in dinucleotide CG↔TG mutation relative to all other dinucleotide mutations is consistent with the variable distribution seen for the CG dinucleotide in the genome and suggestive of in vivo methylation (*SI Appendix S1*, Figs. S5, S6, and S10). Genes ranked with the largest numbers of CG↔TG SNPs included *major facilitator superfamily transporters*, male sterility proteins, several classes of zinc finger transcription factors, and several Ig superfamily cell adhesion proteins implicated in neuronal development (*SI Appendix S1*, Table S8).

Wing polyphenism and reproductive division of labor between queens and workers are two important features associated with eusociality in ants. The gene networks that underlie wing and reproductive development have evolved to be differentially expressed between winged reproductive castes and wingless sterile worker castes in response to environmental factors (33, 34). We found that the mean CpG(O/E) (35) for genes involved in reproductive system development (n = 38; mean = 1.21; P << 0.05) and apoptosis (n = 18; mean = 1.22; P << 0.05) were significantly lower than the mean CpG(O/E) (mean = 1.63) for 50 random genes resampled 10,000 times. (Fig. 2 and *SI Appendix*). Genes in the wing polyphenism network were not significantly different from the average gene CpG (O/E) (n = 37; mean = 1.50; P = 0.4865). The mean CpG(O/E) of the *Drosophila* orthologs (coding regions) that underlie wing development (mean = 0.95; P = 0.86), reproduction (mean = 0.98; P = 0.98), and apoptosis (mean = 1.00; P = 0.99) were not significantly different (*SI Appendix S1*, Fig. S11) than average gene CpG(O/E) in *Drosophila*. These results indicate that developmental genes in the reproduction and apoptosis networks have distinct germ-line methylation signatures relative to the rest of the genome, whereas genes in the wing polyphenism network do not.

Yellow genes occur in insects as well as some bacteria and fungi, but they are curiously absent in all noninsect metazoans (36, 37). The initially described *yellow-y* gene (*Y-y*) functions in cuticle pigmentation in *D. melanogaster* (38), but these genes have also been implicated in processes such as male courtship behavior (39), follicle cell function, and egg development (40). Proteins from a gene expansion of the ancestral *yellow-e3* gene in *A. mellifera* [the major royal jelly (*mrjp*) subfamily] are involved in a regulating reproductive division of labor, but also have age-, sex-, caste-, and brain-specific expression (37, 41).

In the *L. humile* assembly we detected 10 *yellow* genes and 10 *major royal jelly protein-like* genes (Fig. 3*C* and *SI Appendix S1*, Fig. S14). The 8 *yellow* genes (*LhumY-y,-b,-c,-e,-e3,-g,-g2, and -h*) are similar to those of *D. melanogaster* and are likely orthologs. The remaining 2 are putative orthologs of the *yellow-x1* and *-x2* genes, which have been found only in the Hymenoptera. Interestingly, the *L. humile* genome contains an independent radiation of *major royal jelly protein-like* (*mrjpl*) genes similar to those in *A. mellifera* and *N. vitripennis*. The *mrjp* and *mrjpl* gene sets of all three focal taxa each form their own strongly supported clade within the monophyletic *mrjp* subfamily (Fig. 3*C* and *SI Appendix S1*, Fig. S14). These independent radiations in different hymenopteran lineages may indicate that the ancestral gene had a tendency to proliferate, allowing *mrjpl*s to take on new functions and to respond quickly to new forms of selection (42).

Invasion Biology. Although Argentine ants are a widespread and familiar invasive species, many details of their invasion biology remain shrouded in mystery. To clarify some of the biological

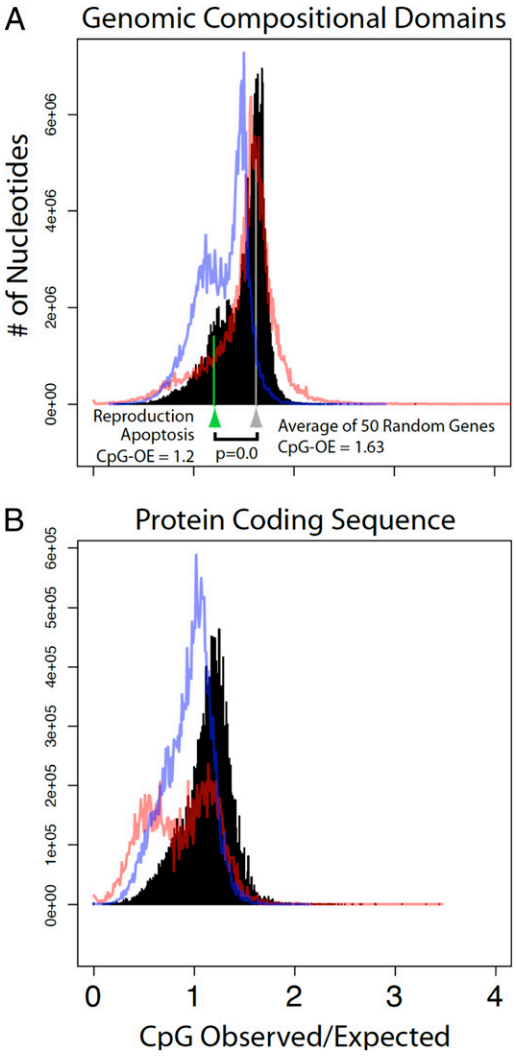

Fig. 2. Distribution of CpG(OE) predictions in genomic compositional domains (*A*) and protein coding regions (*B*) for *L. humile* (black), *A. mellifera* (red line), and *N. vitripennis* (blue line). The combined average CpG(OE) for all genes involved in either reproductive (mean = 1.21) or apoptosis processes (mean = 1.22) (green arrow and line) is significantly lower than the average CpG(OE) for 50 random genes resampled 1,000 times as a control (gray arrow and line; P << 0.05, statistical randomization test).

processes that may be related to their invasive success, we annotated and analyzed genes that are likely to be involved in Argentine ant immune processes, insecticide resistance, and dietary detoxification. We also identified a large number of candidate SNP markers that will be valuable tools for identifying source populations in the Argentine ant's native range, reconstructing the history of introductions around the world, and identifying current routes of transport.

The Argentine ant's unicolonial social structure allows introduced populations to attain enormous population densities in its introduced range. However, it is not clear how Argentine ants control the proliferation of pathogens and parasites in these high-density populations, particularly in light of the extremely low levels of genetic diversity that typify introduced populations (5, 8). We annotated immune genes primarily from the *Toll*, *Imd*, *JNK*, and *JAK*/*STAT* signaling pathways. In total, 90 of 202 immune genes had reciprocal best matches (*SI Appendix S1*, Tables S2 and S16). Of these 202 genes, 152 are present in the *Drosophila* genome and 78 in *Apis*. Thus, *L. humile* appears similar to *Apis* and *Nasonia* in having markedly fewer immune genes than *Drosophila*. Moreover, the many hygienic behaviors and chemical secretions described for the social Hymenoptera may play key roles in controlling pathogens.

Introduced populations of Argentine ants are also frequently targets of heavy insecticidal control, suggesting that genes conferring insecticide resistance may be under strong positive selection. Like many invasive ants, Argentine ants are also extreme dietary generalists, and this plasticity may require an underlying ability to detoxify many different components of their food sources. Cytochrome P450s are a family of heme-thiolate enzymes that catalyze a diverse array of chemical reactions in nearly all organisms (43), and they have been implicated in both insecticide resistance and dietary detoxification.

The *L. humile* genome encodes 111 cytochrome P450s (*SI Appendix S1*, Table S2 and *SI Appendix S2*, Fig. S13), substantially more than *A. mellifera* (46 genes) (44) and *N. vitripennis* (92 genes) (45). It has been hypothesized that the paucity of P450 genes in the genome of *A. mellifera* is a product of its eusocial life history (44), but their abundance in *L. humile* suggests that eusociality alone cannot explain their scarcity in *Apis*. The *L. humile* genome encodes 69 CYP3 clan P450s (*SI Appendix S2*, Fig. S13), the most in any sequenced insect genome. CYP3 P450s are associated with oxidative detoxification of xenobiotics (46), and their abundance in *L. humile* may be an adaptation to a variety of toxins encountered in the diet of this generalist ant, compared with the rather specialized diet of the honey bee.

To facilitate the development of population genetic markers for future analyses of the origin, history, and movement of Argentine ants, we scanned the genome for SNPs. Because the source material contained a single queen pupa and ~100 workers from a single nest (*SI Appendix S1*, Table S1), we were able to identify predicted polymorphic sites. We discovered 231,248 SNPs (0.9 SNP/kb) that occurred in at least three reads and at least 10% of overlapping reads. A total of 5,734 genes (36%) had at least 1 SNP, with 14,136 SNPs (6%, 0.7 SNP/kb) in exons, 26,720 (12%, 0.9 SNP/kb) in introns, 66,830 (29%, 2.8 SNP/kb) in annotated repeats, and 123,492 (53%, 1.1 SNP/kb) in nonrepeat intergenic regions. Although 77% of exonic SNPs would be nonsynonymous (23% synonymous) if all substitutions were equally likely, we observed that only 54% of SNPs were nonsynonymous and 46% were synonymous ($P < 0.001$). When each type of SNP was normalized to the total number of possible sites for that type, we observed 4.9×10^{-4} SNPs per nonsynonymous site and 1.9×10^{-3} SNPs per synonymous site. As expected, we saw a 3.87-fold excess of synonymous SNPs in the OGS1.1 gene set.

We also manually investigated the genes with the highest number of SNPs per kb of exon sequence (*SI Appendix S1*, Table S3). Interestingly, six *L. humile* genes with Interproscan-predicted functions similar to male sterility and gametogenesis genes in *Drosophila* showed a high degree of polymorphism in *L. humile* (24–38 SNPs/kb). These polymorphic genes may be under sexual selection or diversifying selection and could be useful for studying different patriline lineages in native vs. invasive ants. Cytochrome P450s, lipid metabolism genes, and transcription factors were also ranked highly, with >75 SNPs in the exon and intronic regions.

Conclusion. With the sequencing and annotation of the Argentine ant genome and the development of associated genomic resources, this species is well positioned to become a model organism in which powerful genetic approaches can be coupled with a wealth of natural history and behavioral and ecological knowledge. Given the immense financial and ecological costs associated with introductions of Argentine ants, these tools will likely find widespread application and produce tangible benefits for agriculture, societies, and ecosystems. Finally, the evolutionary forces driving some of the unusual and remarkable genomic patterns reported here

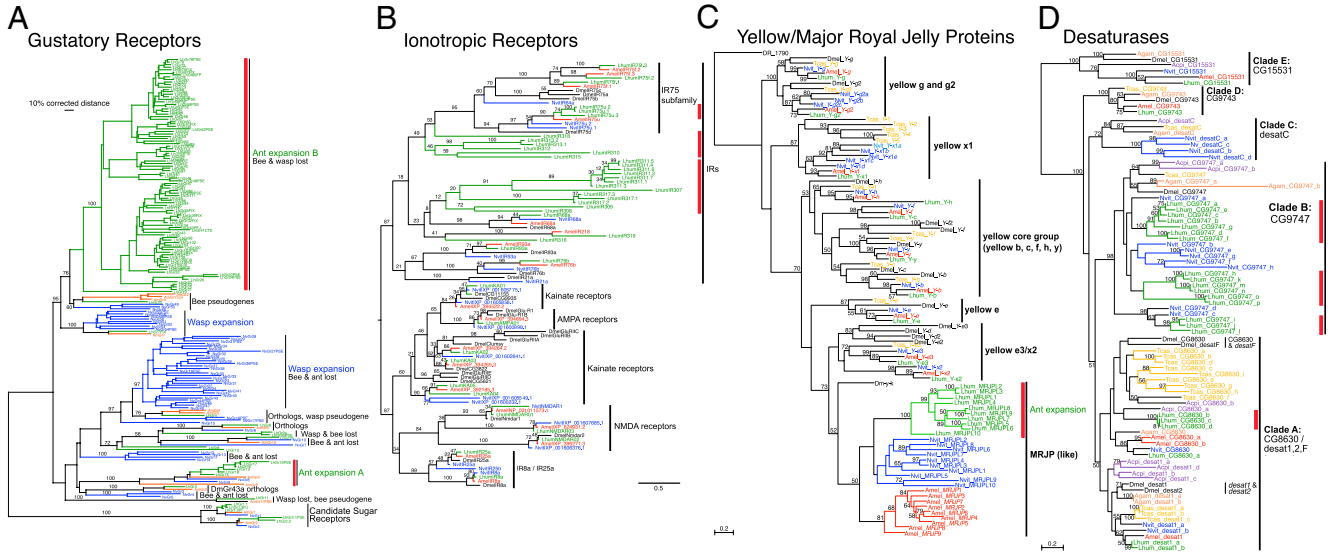

Fig. 3. Gene expansions in the Argentine ant genome. (*A*) Gustatory receptors. (*B*) Ionotropic receptors. (*C*) Yellow/major royal jelly proteins. (*D*) Desaturases. Vertical red bars indicate *L. humile* gene duplications and expansions. The *L. humile* genome also possesses an enormous expansion of odorant receptors (Ors; *SI Appendix S2*, Fig. S12). Green, *L. humile*; red, *A. mellifera*; blue, *N. vitripennis*; black, *D. melanogaster*.

remain unknown and will be productive avenues for future research that explores the basis of eusociality and the causes and consequences of biological invasions.

Materials and Methods

Source Material. We collected an Argentine ant colony fragment from a residential orchard in Santa Clara County, CA. We confirmed that these ants belong to the large supercolony that dominates the introduced range in California using behavioral assays, microsatellite genotyping, and analysis of CHC profiles (*SI Appendix S1*, Table S14 and Fig. S17).

Library Preparation and Sequencing. ***Transcriptome.*** cDNA was generated from mixed source material and sequenced using Roche 454 Genome Sequencer LR70 FLX technology (Table 1 and *SI Appendix S1*, Table S1). This process yielded ~128 Mb of DNA sequence, which was assembled into 20,070 contigs. ***Genomic.*** Genomic DNA was extracted and purified from a single queen pupa (Saratoga) and sequenced using seven runs of 454 FLX Titanium sequencing. Additional worker-derived genomic libraries were constructed and sequenced on the 454 and Illumina platforms.

Assembly. We created several assemblies using Newbler and CABOG assembler (13), as described in *SI Appendix*.

Annotation. We first used the automatic annotation pipeline MAKER (14) to annotate the genome of *L. humile*. We also manually annotated ~1,000 genes (*SI Appendix S1*, Table S2), including a number that are related to fundamental biological processes (oxidative phosphorylation, ribosomal), complex behaviors (learning, memory and aggression), sensory biology (vision, chemoreception), insecticide resistance, immunity, and developmental networks (wings, reproduction).

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1. Hölldobler B, Wilson EO (1990) *The Ants* (Belknap Press, Cambridge, MA), p 733.
2. Patek SN, Baio JE, Fisher BL, Suarez AV (2006) Multifunctionality and mechanical origins: Ballistic jaw propulsion in trap-jaw ants. *Proc Natl Acad Sci USA* 103: 12787–12792.
3. Holway DA, Lach L, Suarez AV, Tsutsui ND, Case TJ (2002) The causes and consequences of ant invasions. *Annu Rev Ecol Syst* 33:181–233.
4. Suarez AV, Holway DA, Case TJ (2001) Patterns of spread in biological invasions dominated by long-distance jump dispersal: Insights from Argentine ants. *Proc Natl Acad Sci USA* 98:1095–1100.
5. Suarez AV, Holway DA, Tsutsui ND (2008) Genetics and behavior of a colonizing species: The invasive Argentine ant. *Am Nat* 172(Suppl 1):S72–S84.
6. van Wilgenburg E, Torres CW, Tsutsui ND (2010) The global expansion of a single ant supercolony. *Evolutionary Applications* 3:136–143.
7. Holway DA, Suarez AV, Case TJ (1998) Loss of intraspecific aggression in the success of a widespread invasive social insect. *Science* 282:949–952.
8. Tsutsui ND, Suarez AV, Holway DA, Case TJ (2000) Reduced genetic variation and the success of an invasive species. *Proc Natl Acad Sci USA* 97:5948–5953.
9. Tsutsui ND, Suarez AV, Holway DA, Case TJ (2001) Relationships among native and introduced populations of the Argentine ant (*Linepithema humile*) and the source of introduced populations. *Mol Ecol* 10:2151–2161.
10. Weston DP, Holmes RW, You J, Lydy MJ (2005) Aquatic toxicity due to residential use of pyrethroid insecticides. *Environ Sci Technol* 39:9778–9784.
11. Smith CR, Dolezal A, Eliyahu D, Holbrook CT, Gadau J (2009) Ants (Formicidae): Models for social complexity. *Cold Spring Harb Protoc* 7:pdb emo125.
12. Tsutsui ND, Suarez AV, Spagna JC, Johnston S (2008) The evolution of genome size in ants. *BMC Evol Biol* 8:64.
13. Miller JR, et al. (2008) Aggressive assembly of pyrosequencing reads with mates. *Bioinformatics* 24:2818–2824.
14. Cantarel BL, et al. (2008) MAKER: An easy-to-use annotation pipeline designed for emerging model organism genomes. *Genome Res* 18:188–196.
15. Parra G, Bradnam K, Korf I (2007) CEGMA: A pipeline to accurately annotate core genes in eukaryotic genomes. *Bioinformatics* 23:1061–1067.
16. Uechi T, Tanaka T, Kenmochi N (2001) A complete map of the human ribosomal protein genes: Assignment of 80 genes to the cytogenetic map and implications for human disorders. *Genomics* 72:223–230.
17. Marygold SJ, et al. (2007) The ribosomal protein genes and minute loci of *Drosophila melanogaster*. *Genome Biol* 8:R216.
18. Quevillon E, et al. (2005) InterProScan: Protein domains identifier. *Nucleic Acids Res* 33(Web Server issue):W116–W120.
19. Moriya Y, Itoh M, Okuda S, Yoshizawa AC, Kanehisa M (2007) KAAS: An automatic genome annotation and pathway reconstruction server. *Nucleic Acids Res* 35(Web Server issue):W182–W185.
20. Werren JH, et al.; Nasonia Genome Working Group (2010) Functional and evolutionary insights from the genomes of three parasitoid *Nasonia* species. *Science* 327:343–348.
21. Weinstock GM, et al.; Honeybee Genome Sequencing Consortium (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* 443:931–949.
22. Torres CW, Brandt M, Tsutsui ND (2007) The role of cuticular hydrocarbons as chemical cues for nestmate recognition in the invasive Argentine ant (*Linepithema humile*). *Insectes Soc* 54:363–373.
23. Robertson HM, Wanner KW (2006) The chemoreceptor superfamily in the honey bee, *Apis mellifera*: Expansion of the odorant, but not gustatory, receptor family. *Genome Res* 16:1395–1403.
24. Robertson HM, Gadau J, Wanner KW (2010) The insect chemoreceptor superfamily of the parasitoid jewel wasp *Nasonia vitripennis*. *Insect Mol Biol* 19(Suppl 1):121–136.
25. Croset V, et al. (2010) Ancient protostome origin of chemosensory ionotropic glutamate receptors and the evolution of insect taste and olfaction. *PLoS Genet* 6:e1001064.
26. Benton R, Vannice KS, Gomez-Diaz C, Vosshall LB (2009) Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. *Cell* 136:149–162.
27. Field LM, Lyko F, Mandrioli M, Prantera G (2004) DNA methylation in insects. *Insect Mol Biol* 13:109–115.
28. Goll MG, Bestor TH (2005) Eukaryotic cytosine methyltransferases. *Annu Rev Biochem* 74:481–514.
29. Jaenisch R, Bird A (2003) Epigenetic regulation of gene expression: How the genome integrates intrinsic and environmental signals. *Nat Genet* 33(Suppl):245–254.
30. Kucharski R, Maleszka J, Foret S, Maleszka R (2008) Nutritional control of reproductive status in honeybees via DNA methylation. *Science* 319:1827–1830.
31. Wang J, et al. (2007) An annotated cDNA library and microarray for large-scale gene-expression studies in the ant *Solenopsis invicta*. *Genome Biol* 8:R9.
32. Kronforst MR, Gilley DC, Strassmann JE, Queller DC (2008) DNA methylation is widespread across social Hymenoptera. *Curr Biol* 18:R287–R288.
33. Abouheif E, Wray GA (2002) Evolution of the gene network underlying wing polyphenism in ants. *Science* 297:249–252.
34. Khila A, Abouheif E (2010) Evaluating the role of reproductive constraints in ant social evolution. *Philos Trans R Soc Lond B Biol Sci* 365:617–630.
35. Elango N, Hunt BG, Goodisman MAD, Yi SV (2009) DNA methylation is widespread and associated with differential gene expression in castes of the honeybee, *Apis mellifera*. *Proc Natl Acad Sci USA* 106:11206–11211.
36. Arakane Y, et al. (2010) Identification, mRNA expression and functional analysis of several yellow family genes in *Tribolium castaneum*. *Insect Biochem Mol Biol* 40: 259–266.
37. Drapeau MD, Albert S, Kucharski R, Prusko C, Maleszka R (2006) Evolution of the *Yellow/Major Royal Jelly Protein* family and the emergence of social behavior in honey bees. *Genome Res* 16:1385–1394.
38. Wittkopp PJ, True JR, Carroll SB (2002) Reciprocal functions of the *Drosophila* yellow and ebony proteins in the development and evolution of pigment patterns. *Development* 129:1849–1858.
39. Drapeau MD, Radovic A, Wittkopp PJ, Long AD (2003) A gene necessary for normal male courtship, *yellow*, acts downstream of *fruitless* in the *Drosophila melanogaster* larval brain. *J Neurobiol* 55:53–72.
40. Claycomb JM, Benasutti M, Bosco G, Fenger DD, Orr-Weaver TL (2004) Gene amplification as a developmental strategy: Isolation of two developmental amplicons in *Drosophila*. *Dev Cell* 6:145–155.
41. Schmitzová J, et al. (1998) A family of major royal jelly proteins of the honeybee *Apis mellifera* L. *Cell Mol Life Sci* 54:1020–1030.
42. Johnson BR, Linksvayer TA (2010) Deconstructing the superorganism: Social physiology, groundplans, and sociogenomics. *Q Rev Biol* 85:57–79.
43. Isin EM, Guengerich FP (2007) Complex reactions catalyzed by cytochrome P450 enzymes. *Biochim Biophys Acta* 1770:314–329.
44. Claudianos C, et al. (2006) A deficit of detoxification enzymes: Pesticide sensitivity and environmental response in the honeybee. *Insect Mol Biol* 15:615–636.
45. Oakeshott JG, et al. (2010) Metabolic enzymes associated with xenobiotic and chemosensory responses in *Nasonia vitripennis*. *Insect Mol Biol* 19(Suppl 1):147–163.
46. Li XC, Schuler MA, Berenbaum MR (2007) Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu Rev Entomol* 52:231–253.

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Social Organization Is Associated With Relaxed Selection on Worker Genes in Highly Polygyne Ants

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ABSTRACT

Ants display a diversity of social structures reflected by differences in caste, nest, and colony organization. Previous research has shown that highly eusocial insects (Hymenoptera) exhibit genome-wide signatures of relaxed selection due to their smaller effective population sizes. However, it is unknown how the colony structure itself may shape the evolution of eusocial species through its effects on the worker caste. Worker ants are typically sterile or produce only male offspring, so traits affecting their behavior evolve mainly through kin selection. Kin selection is predicted to be strongest when relatedness within the colony is high, as in species with a single queen per colony (monogyne). In these cases, the reproductive individuals who are the recipients of worker helping behavior are more likely to carry the same allele for that behavior, with probability proportional to relatedness between worker and reproductive. In contrast, in species with multiple queens and lower relatedness, like weakly or highly polygyne ants, there is a higher chance that altruistic behavior benefits non-relatives. These colony structures are predicted to weaken kin selection, leading to more relaxed selection on worker-biased genes. We find some evidence in highly polygyne species that genes with worker-biased expression experience more relaxed selection compared to queen-biased or non-differentially expressed genes. However, this pattern does not appear to hold consistently across species with lower or more variable queen numbers, where the degree of relaxed selection in worker genes shows no clear association with average queen number per nest. This may point to possible compensatory mechanisms present in these contexts to counteract relaxed selection in workers or that these predicted patterns are too subtle to be detected with current methods, highlighting areas of future study.

1 | Introduction

Ants, and other eusocial species in the order Hymenoptera, exhibit a wide diversity of social complexity (Hölldobler and Wilson 1990). The colony structures present in many of these species are rare in the animal kingdom, as some members of a given social group take on obligate altruistic life history strategies to serve the colony as a whole, and the reproductive individuals within it, instead of solely expending energy and resources in a selfish manner to maximize their chances of reproduction. This strategy is evident in the division of labor into distinct worker and queen castes that is seen in the eusocial Hymenoptera (Wilson 1971; Hölldobler and Wilson 1990; Bourke 1995). Some ant species have worker castes that have pushed this to an extreme, exhibited by complete worker sterility, to maximally allocate available colony resources into the nursing of young and other colony tasks (Wheeler 1991; Smith et al. 2008; Strassmann and Queller 2007). Ant colonies can have a single queen in a nest that produces all workers and reproductive brood, a structure called monogyny. In other cases, colonies can be polygyne,

possessing several or many reproductive queens per nest, each producing workers and new reproductives for the nest.

Such differences in nest structure also produce a wide range of kin structures within the nest. In a monogyne setting, all daughters produced from a singly-mated queen share roughly 75% of their genomes due to haplodiploid reproduction, thus creating high levels of relatedness among workers of such a nest (Hamilton 1964). As the number of queens per nest rises, average relatedness between nestmates predictably begins to drop. In some species, the average number of queens per nest can vary widely, ranging from a single-queen structure to many queens per nest (Rissing and Pollock 2019), such as in the fire ant, *Solenopsis invicta*. These differences in this species are genetically determined by the presence of distinct alleles of a single supergene (Ross et al. 1996; Wang et al. 2013). Other socially polymorphic species, such as *Formica selysi*, *Acromyrmex echinatior*, and *Temnothorax longispinosus*, exhibit facultative polygyny, where nests may accept more queens in environments that might favor polygynous organization over monogynous (Bernasconi and Strassmann 1999; Hughes and Boomsma 2007; Nehring et al. 2018).

Additionally, ant colonies can be highly polygyne with many queens per nest. This phenomenon is well-documented in unicolonial ants, including *Linepithema humile*, *Monomorium pharaonis*, *Wasmannia auropunctata*, *Nylanderia fulva*, and *Pheidole megacephala* (Holldobler and Wilson 2009; Tsutsui and Suarez 2003; Wetterer 2010; Lowe et al. 2000; LeBrun et al. 2014; Dejean et al. 2007), many of which have been of great recent interest due to their highly invasive status. What most differentiates this type of colony structure is its scale. While many ant colonies have cooperative kin structures of between one and several nests, unicolonial colonies are typically composed of a network of cooperative nests that can be spread as far as across continents, as is the case for the single large Argentine ant supercolony in California (Van Wilgenburg et al. 2010). Thus, workers from even distal edges of a supercolony spanning thousands of miles will consider each other as colonymates and will not exhibit aggressive behavior toward each other. While this type of social organization can be seen in the introduced range of numerous species, other non-invasive ants can also exhibit a unicolonial colony structure, including several *Formica* species, such as *F. lugubris, F. exsecta*, and *F. polyctena* (Fortelius et al. 1993; Gris and Cherix 1977; Cherix 1980). Unicolonial colonies typically have very high numbers of queens per nest (i.e., from twenty to several hundred) and higher levels of worker dispersal between nests, and therefore have much lower average within-nest relatedness (Tsutsui and Case 2001).

Kin structures, or the average levels of relatedness among nestmates, may have direct implications for the evolution of the worker caste. Workers in most ant species do not reproduce sexually and produce only male offspring through arrhenotokous parthenogenesis (Goudie and Oldroyd 2018), or are completely sterile, as is the case in many unicolonial species (Crozier and Pamilo 1996; Ronai et al. 2016). Given that workers typically adopt an altruistic life history strategy, forgoing direct sexual reproduction to allocate more energy toward nest tasks such as brood care, foraging, nest defense, etc., this caste is predicted to evolve largely through kin selection. As described in Hamilton's Rule, an altruistic trait is favored by natural selection only if the benefit to the (reproductive) recipient, scaled by the relatedness of the altruist to the recipient, is greater than the cost incurred by the altruist to perform the altruistic act (Bourke 1995; Hamilton 1964). Therefore, the more closely related a worker is to the brood it cares for, the more likely it is that they share genes, including, crucially, genes with biased expression in the worker caste that control altruistic behavior. Workers possessing beneficial mutations, for example, those that make them better nurses, will ensure the survival of those mutations by directly ensuring the survival of their close kin, who will eventually reproduce sexually and pass those traits on to new reproductives and workers. Following the same example, workers with maladaptive mutations naturally impede the survival of their beneficiaries through inadequate nursing practices. Closed kin structures (as in a monogyne setting) will likewise ensure that these mutations are purged through this differential survival of close kin (Liao et al. 2015; Crozier and Pamilo 1996). On the other hand, in more diffuse kin structures characterized by very low intra-nest relatedness, altruistic worker behavior becomes decoupled from their close kin, weakening kin selection in workers.

We used the average number of queens per nest as a practical proxy for intra-nest relatedness to test predictions from kin selection theory about the strength of selection on worker-associated traits. Queen number is widely reported, relatively consistently measured across studies, and is a straightforward way to classify social organization across many ant species. Importantly, queen number has a direct impact on relatedness. In monogyne species with a single queen per nest, workers are highly related to their nestmates, creating the ideal conditions for worker traits to evolve via kin selection. In contrast, highly polygyne species, including those classified here as unicolonial, have many queens per nest and often extensive nest interconnectivity, producing much lower intra-nest relatedness and reducing the probability that altruistic worker behavior benefits individuals carrying the same allele. Thus, kin selection is expected to operate most effectively in monogyne systems, have reduced effectiveness in socially polymorphic or weakly polygyne systems, and be least effective in highly polygyne species.

Patterns consistent with reduced selection on worker-associated genes in highly polygyne contexts have been previously uncovered in *M. pharaonis*, a unicolonial desert ant. Authors of this study reported significantly lower levels of positive selection in worker-associated genes compared to reproductive-associated genes (Warner et al. 2017). Additionally, models of social trait evolution have similarly predicted that such traits exposed to indirect selection should experience slower rates of molecular evolution (Linksvayer and Wade 2009, 2016). While several studies have linked sociality or social traits to patterns of natural selection in termites (Roux et al. 2024) and ants (Barkdull and Moreau 2023), to our knowledge, no empirical work has directly tested whether within-nest relatedness influences the strength of selection acting on worker or altruistic traits. This study aims to directly test the prediction generated from kin selection theory that average within-nest relatedness should be inversely proportional to the strength of selection on worker traits. More broadly, we aim to characterize whether different types of social organization leave distinct selection signatures in the genomes of different ant species. Such questions can highlight patterns

of evolution in these systems that relate to, or are driven by, colony and nest organization. This can also shed light on how these systems come to evolve, and perhaps even their long-term evolutionary fate.

2 | Materials and Methods

2.1 | Species Categorization

This study investigates the impact of social organization on the evolution of the worker caste, and specifically, patterns of selection on worker-biased genes. As explained above, we categorized different colony structures based on the average number of queens per nest, as this functions as a simple numerical threshold for different categorizations and is a strong proxy for within-nest relatedness. We began by first finding all publicly available reference genomes with confirmed species identity in *Formicidae* that were associated with either contig, scaffold, or chromosome-level whole-genome assemblies. We defined "monogyne" species as those with one (or sometimes two) queens per nest, and "weakly polygyne" as having an average between three and 20 queens per nest. "Socially polymorphic" species were defined as those with appreciable numbers of both monogyne and polygyne nests within the same range, and "highly polygyne" species with 20 to several hundred queens per nest. See 'Supporting Information' for more information.

Our 'total' species set was composed of 66 species in the family *Formicidae* fulfilling the aforementioned criteria for inclusion. We analyzed all 66 species in the General Descriptive model (see Methods: RELAX hypothesis tests), but only 49 species with documented reports of queen number or type of social organization were used in our hypothesis tests. As the RELAX method requires that species vary according to some trait of interest, we could only confidently verify queen number for this subset. Our 'hypothesis test' species set consisted of 23 monogyne, 15 weakly polygyne, four highly polygyne (unicolonial), and seven socially polymorphic species. The remaining 17 species (*Ooceraea biroi, Cataglyphis hispanica, Pogonomyrmex barbatus, Polyergus mexicanus, Acromyrmex charruanus, Acromyrmex insinuator, Pseudoatta argentina*, *Temnothorax americanus, Temnothorax ravouxi, Harpagoxenus sublaevis, Aphaenogaster miamiana, Aphaenogaster fulva, Aphaenogaster floridana, Aphaenogaster picea, Solenopsis fugax, Tetramorium parvispinum*, and *Trachymyrmex cornetzi*) were categorized as 'outliers'. These species either possessed life history strategies that did not align with our categories (such as obligate social parasitism or unusual, non-canonical forms of reproduction), or we could not find reliable counts of queen number for them in the literature. See Table S1 for accession numbers and queen count information.

2.2 | Download Gene Panels

Query gene panels were obtained from an RNAseq study of the desert ant *M. pharaonis* (Warner et al. 2017), which tested for differential gene expression in the worker and queen castes across multiple life stages and tissue types. Genes were selected if they had a reported overall average effect of caste-biased expression across all samples and tissues, filtering for only those with the tightest associations to worker or reproductive traits (Warner et al. 2017). While these genes show strong expression biases within a particular caste, they are still likely to exhibit some baseline expression across all individuals and castes. Since genes with truly caste-exclusive expression are rare or yet to be identified, these genes represent the most practical candidates we could find for detecting differential selection between castes (See the Discussion section for a more detailed examination of this issue). We chose to include queen-biased genes to provide a comparison gene set where selection should be more penetrant than in worker genes, given that queens reproduce sexually and thus their genes are directly exposed to various selective forces. We also selected non-differentially expressed (NDE) genes from the Warner et al. (2017) dataset, specifically all genes with inconsistent or no significant differences in expression between castes. This gene set serves as a control in which selection intensity should be intermediate between the other two panels, as these genes are exposed to some level of indirect selection in non-reproductive workers. Our gene sets before filtering consisted of 1756 worker-biased, 3295 queen-biased, and 1618 non-differentially expressed (NDE) genes. We then extracted the longest isoform of the corresponding protein sequence for each gene using CD-Hit v4.8.1 (Fu et al. 2012; Li and Godzik 2006).

2.3 | Gene Prediction With AUGUSTUS

Thirty-seven of 66 reference genomes did not have corresponding gene annotations on NCBI. To create annotations in these genomes, we performed gene prediction with AUGUSTUS v3.3, using a model trained on genes in *Drosophila melanogaster* and '-alternatives-from-sampling = true' (Stanke et al. 2008).

2.4 | Using BLAST to Find *M. pharaonis* Gene Orthologs

A blast database was first constructed for each reference genome for a subsequent BLAST search. We then performed a BLAST search for each query *M. pharaonis* protein using the 'tblastn' command (blast v2.15.0+) (Camacho et al. 2009). The top significant hit ($p < 0.05$) for each protein, if found, was retained for each reference, and query isoforms were removed. Finally, nucleotide coding sequences were extracted for each BLAST hit using the corresponding gene annotation, adjusting for reading frame. Genes that did not have a significant BLAST hit in all 66 species were removed from further analysis.

A reviewer noted that this BLAST strategy could select for paralogous genes, ie, gene duplications of an ancestral gene, in our reference species, since paralogs would have high sequence similarity to the target gene of interest. These paralogs may not have similar functions or expression patterns between castes as the target gene, and thus may not be exposed to the same selective forces. To address this, we also performed 'reciprocal best BLAST' between the query *M. pharaonis* gene and the matching reference gene. The original best matching gene in the reference genome was BLAST-ed back against the *M. pharaonis* genome, and kept in this 'reciprocal best BLAST' gene set only if the gene pairs were each other's best match in the other's genome. This

produced gene set sizes of 64 worker genes, 132 queen genes, and 84 NDE genes.

2.5 | Post-Processing and Multiple Sequence Alignment

To prepare data for input into RELAX, the authors made available several additional tools to generate a codon-aware multiple sequence alignment (https://github.com/veg/hyphy-analyses/tree/master/codon-msa). This pipeline begins with 'pre-msa.bf', a script that ensures correct reading frame by removing potential frameshift mutations, and then translates each corrected nucleotide sequence into a protein sequence. We ran this script with '--E 0.0005', a flag that permits low homology in alignments, to ensure that no species was filtered out in each alignment. We then generated multiple sequence alignments (MSAs) of these protein sequences for each gene using MAFFT v7.505 (Katoh and Standley 2013). We ran the final step in this pipeline, 'post-msa.bf', to generate a final nucleotide MSA by back-translating the aligned protein sequences using the input nucleotide sequences from the first step. Our final 'total' dataset included 287 worker-expressed genes, 455 queen-expressed genes, and 251 NDE genes.

2.6 | Phylogenetic Trees

RELAX requires a gene-based phylogenetic tree with branch labels assigned to the 'Test', 'Reference', or undefined/unlabeled branch groupings. We inferred a best-scoring maximum likelihood tree for each gene using RAxML v.8.2.12, with the GTRCAT nucleotide model, 100 rapid bootstrap searches, and 20 ML searches (Stamatakis 2014). We then labeled each branch according to the social organization group of that species, where the 'Test' branches contain either weakly polygyne, socially polymorphic, or highly polygyne species, and 'Reference' branches contain all monogyne species, for a total of three separate label sets per tree for each gene. Internal nodes in the tree were not labeled before the RELAX tests.

2.7 | RELAX Hypothesis Tests and General Descriptive Model

To investigate how selection intensity varies across caste-biased gene expression categories and social structures, we used the RELAX hypothesis test (implemented in HYPHY v.2.5.61(MP); Wertheim et al. 2015), which detects shifts in the strength of natural selection by comparing dN/dS (ω) distributions across phylogenetic branches. RELAX fits a discrete distribution of ω values composed of three site classes, corresponding to purifying ($\omega<1$), neutral ($\omega\approx1$), and positive selection ($\omega>1$). It first fits these distributions for a set of Reference branches, which are defined a priori based on the presence or absence of a biological trait of interest, relative to a set of 'Test' branches with the opposite trait state. Rather than estimating entirely separate ω distributions for the sets of Test and Reference branches, RELAX models the Test distribution as a transformation of the Reference distribution via a selection intensity parameter k, such that each ω value in the Test distribution is defined as $\omega_T=\omega_R^k$. This formulation allows the model to test whether selection on the Test branches is relaxed ($k<1$), intensified ($k>1$), or unchanged ($k=1$) relative to the Reference branches. Importantly, separating the ω distribution into distinct selection categories enables RELAX to distinguish between genuine relaxation and intensification; under relaxed selection, purifying sites shift toward neutrality (ω increases toward 1), while positively selected sites decrease in intensity (ω decreases toward 1); under intensification, the opposite occurs. If a single dN/dS distribution was inferred jointly across sites for all mutation types at once, these opposing shifts could cancel out, masking real differences in selection pressure. RELAX formally tests whether k significantly differs from 1 using a likelihood ratio test comparing a null model ($k=1$) to an alternative model in which k is estimated freely. This allows inference of whether selection patterns have changed in a given species group and also the direction and magnitude of that change.

We ran this test to infer a single k value for each gene in each set of Test branches (weakly polygyne, socially polymorphic, and highly polygyne species) with default parameters and the -srv flag, allowing for synonymous rate variation for nonsynonymous and synonymous substitutions. We also used the '--starting-points 5' and '--grid-size 500' flags to expand the initial search for starting points in cases where the model has higher sensitivity to initial starting conditions, which may otherwise result in convergence issues. p-values generated from the model output were corrected for multiple tests using the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995).

We then wanted to estimate how selection might vary across our entire phylogeny. To do this, we ran the RELAX General Descriptive model for all genes in each gene set. This model is not intended for hypothesis testing, but rather provides an overall description of how selection is fluctuating across the entire tree. First, an omega distribution is fit to the entire tree to estimate a shared selection regime across all branches, without regard to branch-specific Test and Reference labels. Then, the 'overall' omega distribution is tuned for each branch in turn, producing an estimate k_b representing the extent to which selection on the focal branch is relaxed or intensified relative to the entire tree. An average kb value per branch was calculated by estimating the median kb value across all genes for that branch.

2.8 | Significance Testing

To assess whether selection patterns (k values) differed among caste-biased genes across social contexts, we fit a generalized linear mixed model (GLMM) using the glmmTMB package in R (Brooks et al. 2017; McGillycuddy et al. 2025). We first applied a $\log_2$ transformation to the k values to facilitate the interpretation of selection strength. This centers the distribution around zero and more fairly weights the scale between relaxed and intensified k values, such that values like $k=0.1$ and $k=10$ indicate equally strong signals of relaxed and intensified selection, respectively. K values indicating relaxed selection can fall in the range of 0–1, whereas intensified values can take values anywhere from 1 to 50, thus necessitating this rescaling to account for the broader range of intensified values. Fixed effects for the GLMM included caste bias (worker, queen, or NDE), social

comparison (weakly polygyne vs. monogyne, highly polygyne vs. monogyne, and socially polymorphic vs. monogyne), and their interaction. We used a random intercept for gene to prevent pseudoreplication, as each gene appears in all comparisons (i.e., 3 separate times). To accommodate residual heteroskedasticity, we modeled dispersion as a function of social comparison category. Given moderate deviations from normality in residuals, we specified a Student's *t* family function, which provided a more robust fit than a Gaussian alternative. Estimated marginal means and pairwise contrasts were computed using the emmeans package (Lenth 2025), allowing us to test whether $\log_2(k)$ differed significantly between caste groups within each social category, while accounting for gene-level variability.

We conducted this GLMM analysis on both the full gene set (hereafter 'total' gene set) and a subset of genes identified by RELAX as having experienced significantly intensified or relaxed selection (hereafter 'significant' gene set). These datasets address complementary questions. The 'total' gene set allows us to detect average shifts in k magnitude across groups, including genes with $k \approx 1$ that are likely evolving under similar selection pressures across the Test and Reference branches. The 'significant' gene set focuses specifically on genes where RELAX detected a shift in selection intensity between the Test and Reference branches. We analyzed this subset separately to better isolate cases where changes in selection regime had occurred and to evaluate whether such shifts were disproportionately associated with specific caste or social contexts. This approach allowed us to more precisely examine patterns in the direction of selection change, such as caste-level biases toward relaxation or intensification, rather than relying solely on differences in the magnitude of k values across broader, mixed gene sets.

We assessed whether certain gene sets contained disproportionately more or fewer significantly relaxed or intensified genes than expected by chance using permutation tests. For each gene set and social comparison category, we first calculated the observed proportion of genes classified as significantly relaxed or intensified. To generate null expectations, we pooled all $\log_2(k)$ values from the relevant RELAX runs within a given social comparison (Test group), then performed 10,000 permutations. In each permutation, we randomly shuffled the caste bias labels across all genes and sampled gene sets of matching sizes for worker-, queen-, and NDE-associated genes. We then calculated the pairwise differences in the proportion of relaxed (or intensified) genes between gene sets (worker vs. queen, worker vs. NDE, and queen vs. NDE), and added each difference to a separate null distribution.

We performed one-tailed tests for the worker vs. queen and worker vs. NDE comparisons to compute *p*-values, as we had a priori expectations (based on patterns observed in the 'relaxed-only' $\log_2(k)$ distributions, Figure 3) that worker genes would exhibit a higher proportion of relaxed genes and a lower proportion of intensified genes relative to the other groups. For these cases, *p*-values were calculated as the proportion of permuted differences greater than or equal to the observed difference (in the expected direction). For the queen vs. NDE comparison, we had no strong directional expectation, so we used a two-tailed test. We calculated the absolute value of the observed difference and compared it to the distribution of absolute differences from the permutations. *p*-values reflect the proportion of permuted values with a difference greater than or equal to the observed one. Multiple testing corrections across comparisons were applied using the Benjamini-Hochberg procedure to control the false discovery rate (Benjamini and Hochberg 1995).

3 | Results

3.1 | RELAX Hypothesis Tests

In our analysis of the 'total' gene set, we found that worker-biased genes in highly polygyne species had slightly lower median $\log_2(k)$ values (−0.06) compared to queen-biased (0.08) and non-differentially expressed (NDE) genes (−0.004), though all medians remained close to zero (Figure 1A). In contrast, analysis of the subset of genes with significantly relaxed or intensified selection ('significant' gene set) revealed more pronounced caste-based differences (Figure 1B). In the highly polygyne comparison, where the greatest effects were anticipated, worker-biased genes exhibited much lower median $\log_2(k)$ value (−0.47) relative to queen-biased (0.42) and NDE genes (0.34). Surprisingly, in the socially polymorphic and weakly polygyne comparisons, worker-biased genes showed positive $\log_2(k)$ medians (0.28 and 0.41, respectively), suggesting intensified rather than relaxed selection (Figure 1B). These patterns were partially recapitulated in the 'reciprocal best BLAST' gene subset, particularly in highly polygyne species, where worker-biased genes again showed lower $\log_2(k)$ values compared to other gene sets (Figure S1).

Generalized linear mixed model (GLMM) analysis of $\log_2(k)$ values supported these findings (Figure 2). In the 'total' dataset, estimated marginal means for worker-biased genes were lower than those for queen and NDE genes in both highly and weakly polygyne comparisons, though the contrasts were not statistically significant (Figure 2A). In the 'significant' gene subset, worker-biased genes in highly polygyne species had strongly negative mean estimates for $\log_2(k)$ values (Figure 2B), and pairwise GLMM contrasts in this Test group revealed a significant difference in mean $\log_2(k)$ estimates between worker- and queen-biased genes ($p < 0.05$). Additionally, while worker-biased genes never had the highest estimated mean $\log_2(k)$ values in any Test case, we did not observe a consistent pattern of greater relaxation in worker genes in the weakly polygyne or socially polymorphic species, suggesting that this effect may be specific to or only detectable in highly polygyne species (Figure 2A,B). In the 'reciprocal best BLAST' subset results, we only observed patterns consistent with increased worker gene relaxation in highly polygyne species in our 'significant' subset, though neither pairwise comparison, ie, worker vs. queen nor worker vs. NDE, was statistically significant (likely due to limited power from smaller sample sizes) (Figure S2B). Pairwise contrasts for all datasets are located in Table S2.

3.2 | Conditioning on Relaxed or Intensified Genes

To determine whether the lower $\log_2(k)$ values observed in worker-biased genes from highly polygyne species were driven by more extreme relaxation of selection or simply by a greater frequency of relaxed genes, we analyzed only those

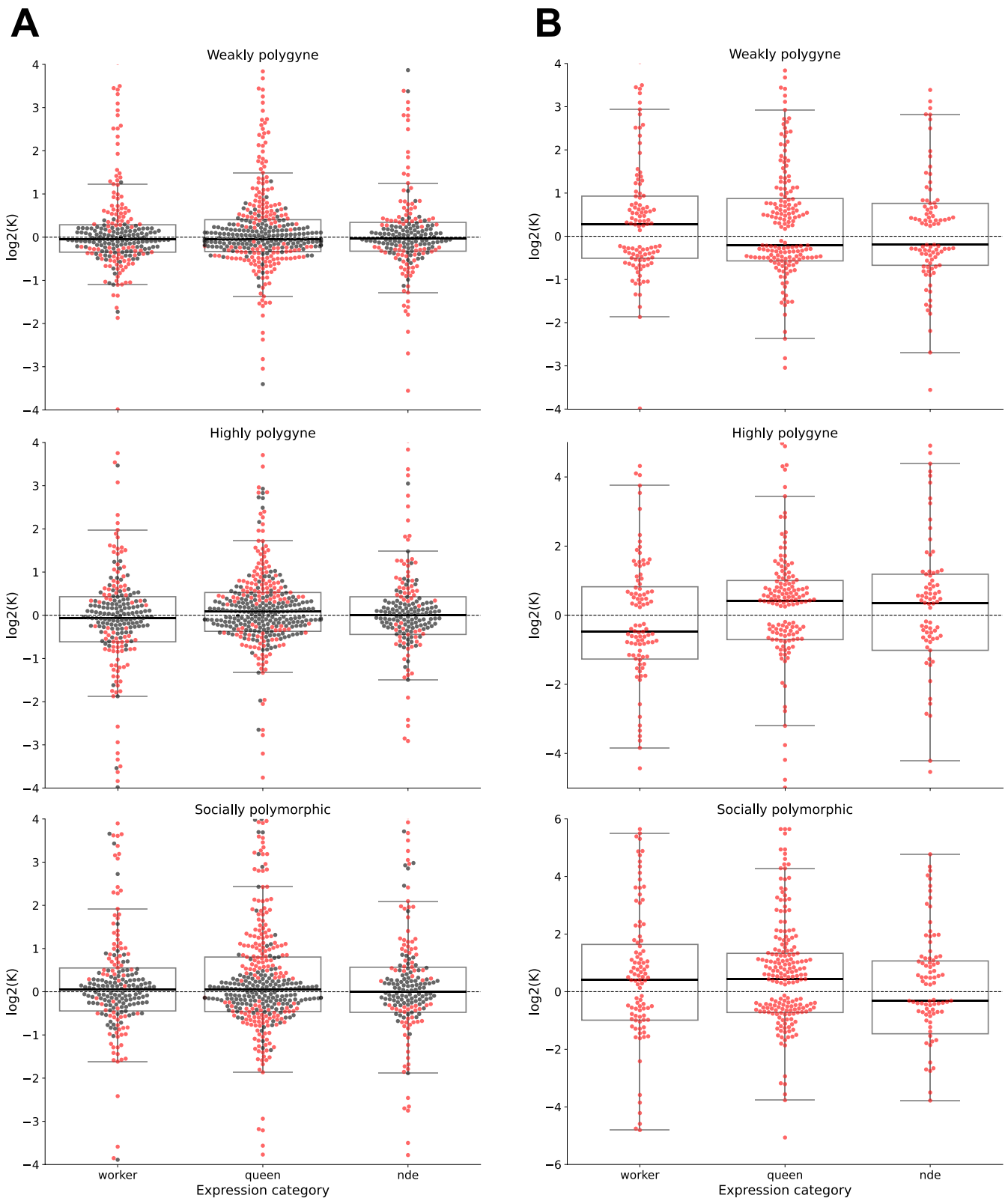

FIGURE 1 | RELAX hypothesis test k values per gene for each type of social organization and gene set. Results from RELAX hypothesis tests for each Test group, including weakly polygyne species, highly polygyne species, and socially polymorphic species for (A) 'total' gene and (B) 'significant' gene sets. Each data point represents the $\log_2$-transformed k value for each gene in a particular gene set, and is colored red if the hypothesis test found significant ($p < 0.05$) relaxation or intensification of selection, and black if results were not significant (k not significantly different from 1). Horizontal black lines represent the median $\log_2(k)$ value for a particular gene set.

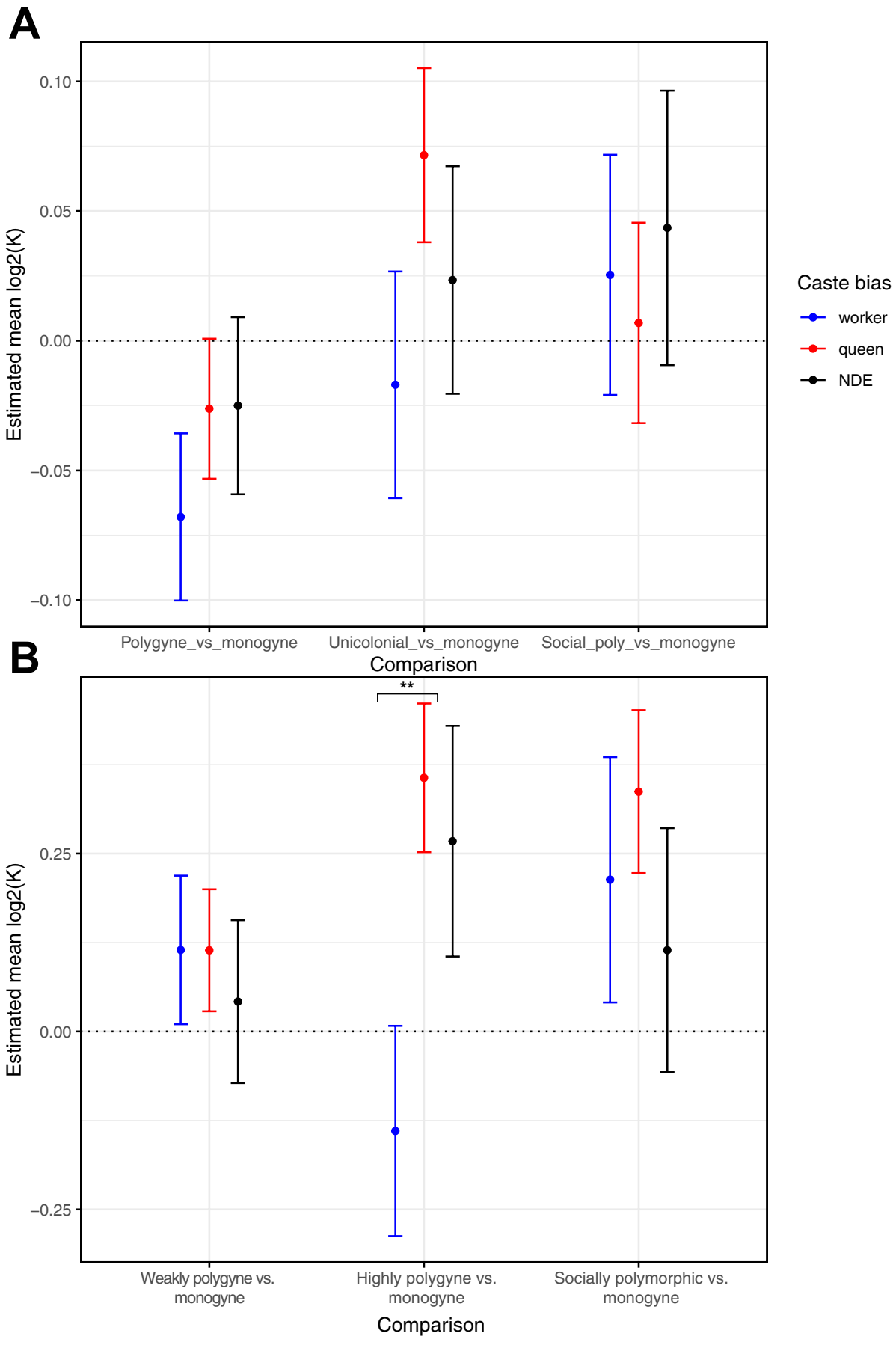

FIGURE 2 | Legend on next page.

FIGURE 2 | GLMM results. Estimated marginal means of $\log_2(k)$ values from the GLMM analysis for worker (blue), queen (red), and NDE (black) gene sets across Test groups for (A) 'total' gene and (B) 'significant' gene sets. Standard errors are plotted with vertical lines extending from each point. '**' indicates pairwise contrast found to be significant ($p < 0.05$).

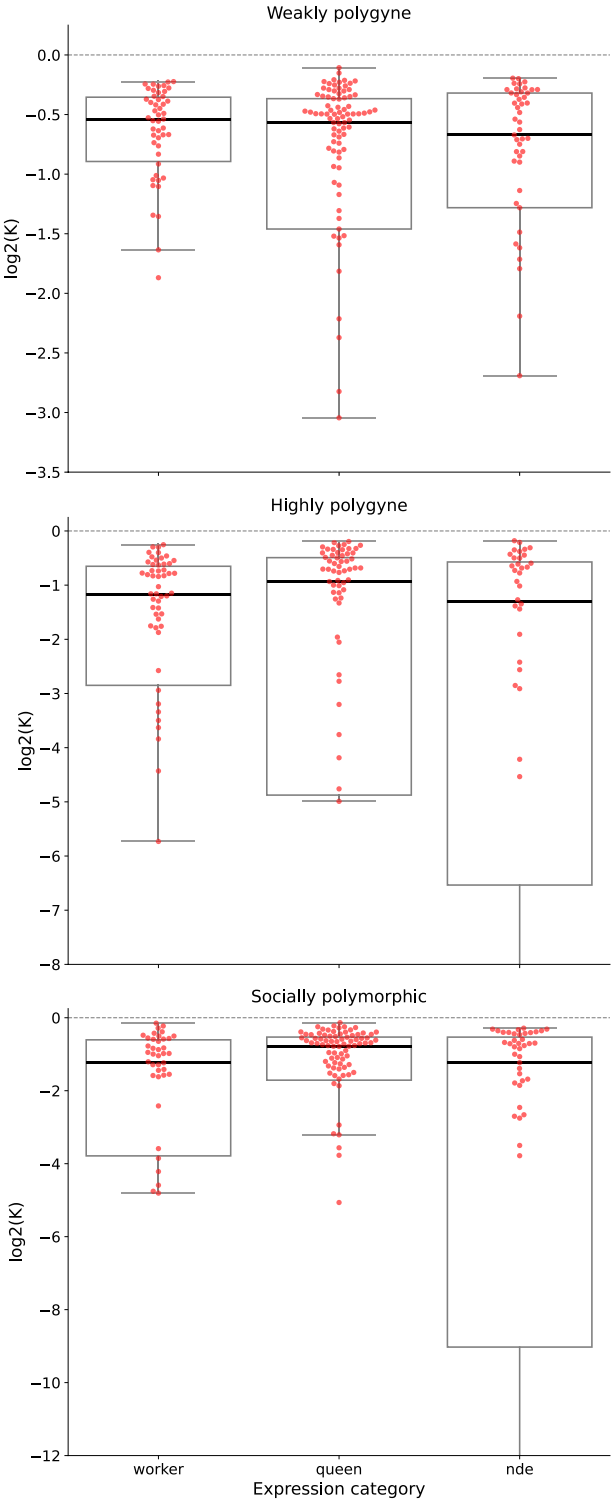

FIGURE 3 | RELAX hypothesis test k values per gene, conditioned on relaxed selection. $\text{Log}_2(k)$ values from RELAX hypothesis tests for the subset of genes found to be significantly relaxed ($p < 0.05$, $k < 1$) for each gene set in weakly polygyne, highly polygyne, and socially polymorphic species. Horizontal black lines represent the median $\log_2(k)$ value for a particular gene set.

genes classified as significantly relaxed by RELAX (Figure 3). If worker-biased genes are exposed to, on average, more extreme relaxation than other genes, they would still exhibit lower $\log_2(k)$ values than the other groups in this comparison. However, if the observed pattern was instead due to disproportionately more genes in the 'relaxed' category but roughly similar magnitudes of relaxation, we would expect worker $\log_2(k)$ values to resemble those of other gene sets. Our results supported the latter; in the relaxed-only gene subset, average worker $\log_2(k)$ values in highly polygyne were intermediate between those of queen- and NDE-biased genes, indicating that the earlier patterns from Figure 1B are likely driven by a greater number of relaxed worker genes rather than stronger average relaxation per gene (Figure 3). This was corroborated by calculating relaxed and intensified gene proportions in each comparison (Figure 4), which revealed significantly more relaxed worker-biased genes in this Test case compared to queen-biased genes ($p = 0.02$, permutation test), and a non-significant trend toward higher proportions compared to NDE genes ($p > 0.05$). These patterns were mirrored in the 'reciprocal best BLAST' dataset, though none of the pairwise differences reached significance (Figure S3).

3.3 | General Descriptive Model

We fit the general descriptive model to each gene for all gene sets in order to get a better understanding of how selection intensity might fluctuate across different clades in our phylogenetic tree. For each of the three gene sets, an overall distribution (across all genes) of k values per branch was also produced to assess the extent of variance in selection intensity for that particular branch (Figure 5). We used each median value (green) to compare average (for each gene set) selection intensification/relaxation on each branch relative to the overall tree-wide mean values. Within the highly polygyne group, the *Formica* species appear to have more relaxed selection in all gene sets compared to overall mean values across the tree, and to the other two invasive unicolonial species, indicated by the '-' symbols next to each bar. Interestingly, selection appears to actually be intensified in worker genes in *L. humile* and *M. pharaonis* relative to the tree-wide average, suggesting that the relaxed signal we see from the hypothesis tests is likely driven by the two *Formica* species in this group. This is a particularly interesting finding, in light of findings from Warner et al. (2017). This study found that NDE and worker-expressed genes share similarly low rates of adaptive substitution in their 'overall' expression comparison, with reproductive-associated genes exhibiting significantly more intensified selection. We find the opposite pattern, with the highest intensification signals in worker and NDE genes, and lowest in queen-biased genes (Figure 5).

The general descriptive model also reveals that, perhaps counterintuitively, worker genes exhibit the highest selection intensities relative to the other gene sets in roughly half of the species across

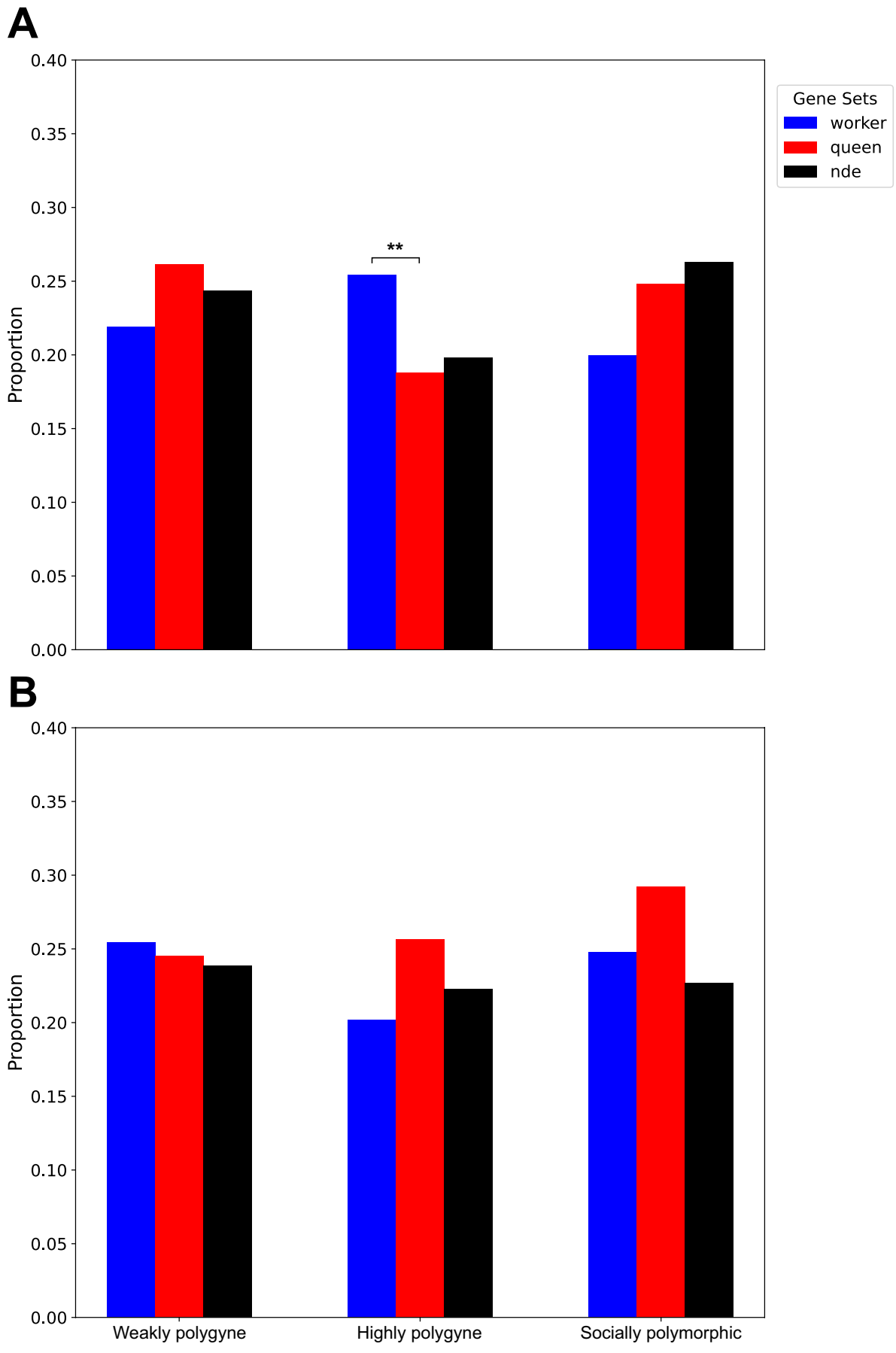

FIGURE 4 | Legend on next page.

FIGURE 4 | Proportions of relaxed and intensified genes. Proportions of worker (blue), queen (red), and NDE (black) genes for each species Test set found to be (A) significantly relaxed, or (B) significantly intensified, out of all genes in each set (regardless of significance). '**' indicates significant pairwise differences in proportions ($p < 0.05$, permutation tests).

the tree (Figure 5). We also find that, strikingly, species in the *Pseudoatta*, *Acromyrmex*, and *Aphaenogaster* clades have consistently relaxed selection across all gene sets, though this pattern is not present in all Myrmicinae species. Overall, the distributions of kb values per species in the general descriptive model appear to be more consistent within-genus than within social organization type. In other words, the median and variance of kb value distributions across gene sets appear to have roughly similar magnitudes among more highly related species, most notably in *Formica*, *Trachymyrmex*, *Pseudoatta*, *Acromyrmex*, *Aphaenogaster*, and *Temnothorax*, though most of these genera represent a mix of at least two types of social organization.

4 | Discussion

We tested whether social organization, measured by queen number and nest structure, influences selection on worker-biased genes, as predicted by kin selection theory. We expected signatures of kin selection to be most robust in monogyne species (with high intra-nest relatedness) and weakest in highly polygyne species (with diffuse kin structures). We predict that relaxation of selection may also be potentially observable in species with multiple but relatively few queens on average. Our results partially support these predictions, given our observations of more relaxed selection on worker-biased genes in highly polygyne species when focusing on genes with significant selection shifts, but not across all genes. No consistent patterns emerged in non-monogyne species with lower queen numbers (i.e., weakly polygyne and socially polymorphic species), suggesting that only extreme reductions in relatedness may measurably relax selection on worker traits.

4.1 | RELAX Hypothesis Tests

Our RELAX hypothesis tests and GLMM results provide some support for the prediction that low relatedness within nests of highly polygynous species can weaken kin selection in workers. In the full dataset ('total' gene set), we find patterns of more relaxed selection on worker-biased genes in highly polygyne species, though caste-based differences in k estimates were not significant. This dataset includes many genes that did not show significant differences in selection intensity ($k \approx 1$, $p > 0.05$) between the Reference (monogyne) and Test groups, which is likely obscuring intensity differences present in other genes. Accordingly, when focusing only on genes with strong selection intensity differences in the 'significant' gene subset, we see clearer patterns consistent with caste-specific differences in selection, particularly between worker- and queen-biased genes (Figure 2B). Importantly, using a linear mixed-effects model that includes both caste bias and social category (Test species group) as fixed effects, along with their interaction, allows us to directly test whether the effect of caste bias on selection strength varies depending on social structure. The model's estimated marginal means and pairwise contrasts allow us to compare k values across castes within each social context, providing evidence that reduced relatedness in highly polygyne species may indeed influence the strength and direction of selection on worker-expressed genes.

We found that this pattern was likely driven by workers having disproportionately more genes with significantly relaxed selection than intensified selection, but we did not find any evidence that the magnitude of relaxed selection on these genes was more extreme (significantly lower k values on average) than genes with relaxed selection in other expression categories (Figure 3). One explanation is that our study design may enrich for more conserved genes across ant taxa, potentially removing genes with high allelic variation and extremely low or extremely high k values, and leaving only those with comparable levels of relaxation or intensification to other gene categories. In other words, genes from older phylostrata may be less likely to have large differences in selection intensity or relaxation compared to genes driving diversification of more recently evolved ant subfamilies such as *Dorylinae* or *Pseudomyrmecinae* (Moreau et al. 2006), or genes controlling traits that have deteriorated in certain species. Evolution in more highly conserved genes is still crucially important for broadly conserved phenotypes like worker behavior and queen reproduction, and thus our study provides a complementary perspective to those that focus on more recent taxonomically limited and/or newly arisen genes. Both types of approaches are necessary to gain a more complete understanding of social evolution.

This observed pattern in our highly polygyne group appears to be mostly driven by the two unicolonial *Formica* species, as worker genes in the other two species (*M. pharaonis* and *L. humile*) in this group exhibited increased selective intensity relative to the rest of the tree in the general descriptive model (Figure 5). One possibility is that the native unicolonial structure present in some *Formica* may have been more stable over evolutionary time compared to the other species. Because these *Formica* species have maintained this social structure over longer evolutionary time scales (thousands of generations), the signal of relaxed selection may have had more time to accumulate. The introduced unicolonial species, on the other hand, have likely acquired this extreme colony structure within the past 100–150 generations. In *L. humile*, although some native populations of this introduced species possess large colonies (hundreds of meters long with likely thousands of queens), none are of the scale that we see in the introduced ranges (hundreds of kilometers long, millions of queens) (Tsutsui and Case 2001). Thus, worker genes in the native range may be experiencing selection pressures more typical of the 'weakly polygyne' category, and only more recently developed the unicolonial colony structure. A follow-up simulation study of these populations may help to generate more accurate predictions of weakly or highly polygyne selection dynamics over different timescales, and the power necessary to detect drift or selection processes within this

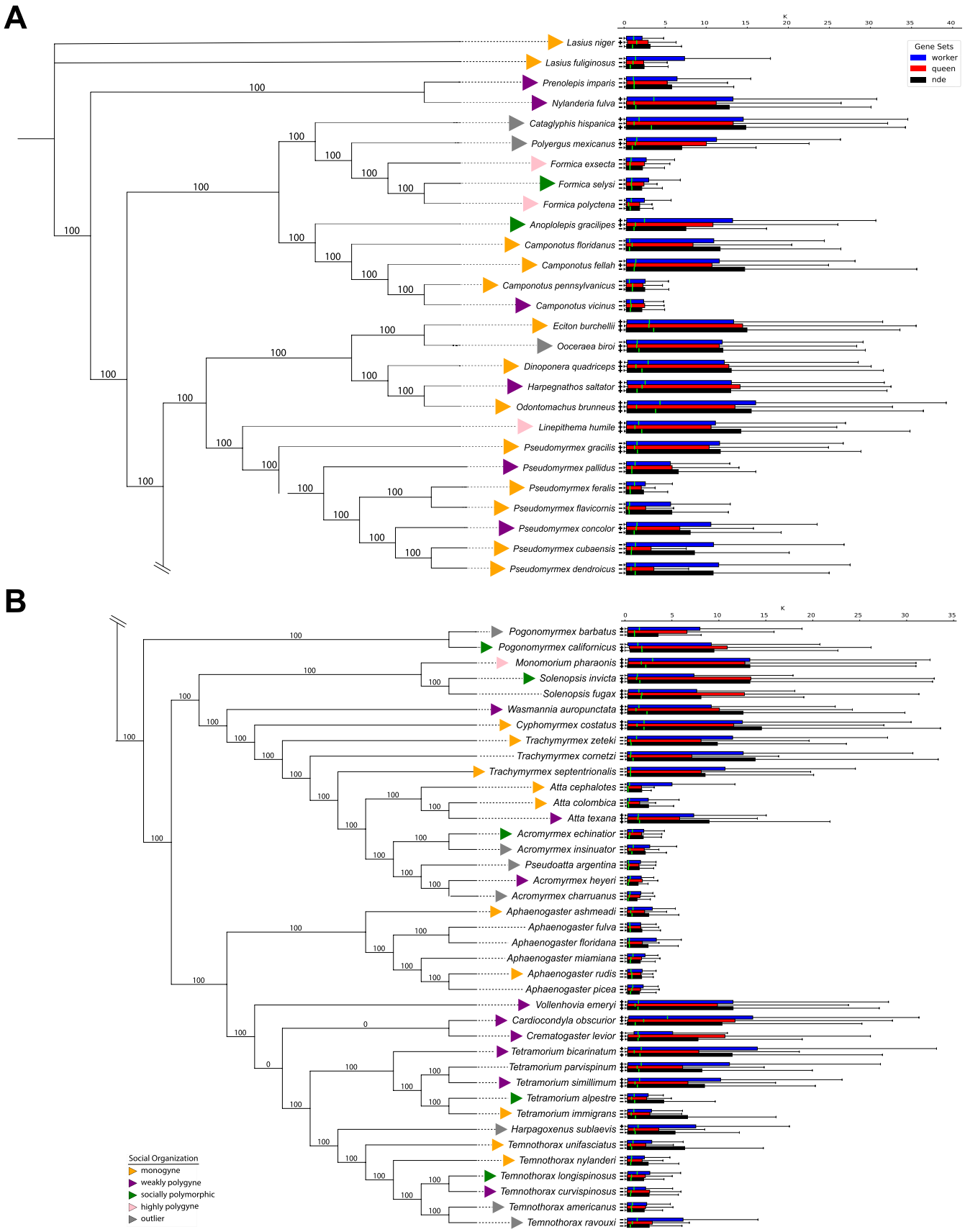

FIGURE 5 | Legend on next page.

FIGURE 5 | Phylogeny of Formicidae and general descriptive model results. Phylogeny generated with RAxML v.8.2.12 using a concatenated alignment of all NDE genes, with support values as percentages out of 100 shown for each node. The phylogenetic tree is divided into (A) all non-myrmicine and (B) myrmicine species to aid visual clarity. Horizontal blue, red, and black bar graphs show the distribution of branch-specific k_b values from the General Descriptive model for the worker, queen, and NDE gene sets, respectively, where each *k* represents the deviation of a particular branch from the overall tree-wide ω distribution for a particular gene. Vertical green lines within each bar represent the median k_b value for a particular gene set. Lower and upper whiskers extend to the most extreme values within Q—1.5×IQR (interquartile range) for the Q1 and Q3 quartiles, respectively, as per the default behavior in matplotlib: Boxplot. The plus (+) or minus (–) to the left of each bar plot indicates if the median k_b value for a particular branch and gene set is greater than or less than the tree-wide mean k_b value, calculated as the mean value of all medians for that gene set. Trait states, ie, our Test groups are indicated with colored triangles to the left of each species name, with yellow = monogyne, purple = weakly polygyne, green = socially polymorphic, pink = highly polygyne, and gray = outlier species.

introduction timeframe (100–150 generations). Additional sampling of other unicolonial/highly polygyne invasive species, ie, multiple independent transitions to this type of colony structure, would also help to clarify the impact of kin selection in recently introduced vs. more established species.

As we expect intra-nest relatedness to be lower in weakly polygyne and socially polymorphic species compared to monogyne settings, we predicted that these test groups should also exhibit some relaxation signals in worker genes. However, we did not observe this to be the case overall. In both settings, worker genes did not exhibit significantly more relaxed selection compared to other gene types. We also did not find significant differences in the proportions of genes in each selection category (relaxed or intensified) in these two groups, providing more supporting evidence that selection intensity either does not vary according to caste-biased gene expression or is not detectable in species with variable but relatively low queen numbers. This pattern could be the result of large differences in the scale of each social structure; while queen numbers were reported to reach hundreds per nest in our highly polygyne species, they are much lower (< 20/nest) in most weakly polygyne/socially polymorphic species. Most of the invasive species, in particular, have relatively few (< 5) queens per nest in their native range, which may provide a more accurate representation of the evolutionary history of these species than the unicolonial structure seen in their introduced ranges. This pattern could also reflect some inherent threshold in queen numbers per nest, under which kin selection operates similarly to a monogyne setting and does not leave an observable signal in the worker genome.

4.2 | General Descriptive Model

Selection patterns across the entire phylogeny, inferred from the general descriptive model, reveal several surprising findings. Most broadly, selection on worker genes appears to be more intensified than on other genes in roughly half of all species (regardless of social organization), which is the opposite pattern expected from our predictions. Though we predict that non-monogyny species should show at least some evidence of relaxation in worker genes, this pattern may also indicate that worker traits evolve rapidly and play a large role in the evolution of eusocial species.

Several studies in honeybees have found evidence of strong intensification of selection in worker-associated genes, with these genes implicated as drivers of eusocial evolution in *Apis mellifera* (Harpur et al. 2014; Johnson and Tsutsui 2011). Further, caste-biased or plastic genes have been shown to be rapidly evolving in some ant species, such as in *Solenopsis invicta* (Hunt et al. 2011). While our genes may have a bias toward older phylostrata, this bias does not exclude the possibility of detecting biologically meaningful patterns. Although younger genes are often more plastic and frequently associated with caste-specific expression, particularly in workers, there is evidence that genes with social functions have experienced both ancient and recent positive selection in various eusocial insects (Privman et al. 2018; Dang et al. 2019; Helanterä 2022). Thus, while our dataset may underrepresent fast-evolving, lineage-specific genes, it still captures a subset of genes that have likely played important roles both in the ancient origins and ongoing evolution of eusociality in *Formicidae*.

There is also evidence in *Formicidae* that worker reproduction, especially worker production of new reproductives (the most penetrant selection scenario), impacts the selection landscape in worker-associated genes, as these genes are more directly exposed to selection in species with this trait. Barkdull and Moreau (2023) found that some genes with plastic expression between castes exhibited intensified selection in species with reproductive workers, which could also contribute to the patterns we see in worker genes, although worker production of queens is rare in ants. While it is clear that novel worker traits play an important role in eusocial evolution, further work is needed to elucidate how many sociality-associated factors together shape the adaptation landscape in worker genomes.

Another interesting pattern revealed in the general descriptive model is the extensive relaxation of selection in various myrmicine clades, including *Pseudoatta*, *Acromyrmex*, *Aphaenogaster*, and *Temnothorax* (Figure 5). While little is currently known about the life history or social organization in most *Aphaenogaster* species used in this study, we did find suggestive traits in the other three clades. *Pseudoatta argentina, Acromyrmex charruanus, Acromyrmex insinuator, Temnothorax americanus*, and *Temnothorax ravouxi* are obligately or facultatively socially parasitic, meaning they typically rely on a closely-related host species to perform worker tasks in the nest (Rabeling et al. 2015; Schultz et al. 1998; Schrader et al. 2021; Gstöttl et al. 2020; Seifert 2018; Latreille 1798; Báthori et al. 2024). Social parasitism has commonly been cited as a mechanism for the erosion of worker traits in eusocial species, usually through the partial or complete suppression of the worker caste, and direct evidence for genome-wide erosion of selection has previously been found in three out of five of these species (Aron et al. 1999; Sumner,

Hughes, and Boomsma 2003; Schrader et al. 2021). Our results provide direct support for findings that social parasitism relaxes selection in worker traits. Some *Temnothorax* species are not socially parasitic but still exhibit relaxation across gene sets, which could be evidence of a recent switch in life history strategy, and/or that a common ancestor to *Temnothorax* was similarly exposed to weaker selective forces. Exceptions to the patterns observed for socially parasitic species include *Polyergus mexicanus* and *Harpagoxenus sublaevis*, which have roughly average selection intensity in all gene sets (Figure 5).

4.3 | Queen Life History Traits

One aspect of our study design likely to impact results is how we define types of social organization. Using queen number per nest, while simple and effective for representing intranest relatedness, might obscure some underlying differences within groups. It is possible that several species we classified as weakly polygyne are actually functionally monogyne, meaning that they may have several queens per nest, but only have 1–2 queens reproducing at any given time. Additionally, some socially polymorphic species may not have distinct social morphs everywhere, or may not have had a stable social polymorphism over long periods of evolutionary time. As variable social structures and/or evolutionary histories could differentially impact the selection landscape in these species, this type of heterogeneity may add noise to our observed signal. This category already serves as a 'catch-all' in some sense, since the ratios of monogyne: polygyne nests in each species vary widely within this category.

Ant species can also have varying levels of polyandry (i.e., multiple mating by queens), which can substantially influence relatedness between workers and reproductive offspring. However, we chose not to incorporate this trait into our species categorizations because polyandry is generally rare across ants and other eusocial Hymenoptera (Strassmann 2001). Additionally, measuring actual polyandry levels reliably is difficult, as this usually involves dissecting queen spermathecae, and observed mating frequencies tend to overestimate actual effective insemination rates (Boomsma and Ratnieks 1996). Although some polyandry has been documented, particularly in the genus *Atta* (Fjerdingstad and Boomsma 1998), the average rate across various ant species was found by one study to barely exceed one (1.16; Boomsma and Ratnieks 1996). Thus, while rare instances of polyandry may slightly reduce within-nest relatedness in some species, we do not expect it to substantially impact relatedness estimates or bias our overall conclusions regarding caste-based selection patterns.

4.4 | Choice of Species and Gene Sets

While this study provides important insights into the interplay between colony organization and social evolution, it would benefit from increased and more even sampling across the ant phylogeny and across different social structures as more reference genomes become available. As most of our species are members of the *Myrmicinae* and *Formicinae* subfamilies, future work incorporating species from other subfamilies would highlight whether these patterns are consistent even in species with divergent evolutionary histories or caste-specific life history traits. A larger and more even sample of species would also create more balanced social organization groups, as currently our highly polygyne Test group (four species) and monogyne Reference group (23 species) are unbalanced. RELAX can encounter convergence issues when Test or Reference species are patchily distributed across the phylogeny. Thus, future genomic data from more highly polygyne species such as those in *Tetramorium* (Astruc et al. 2001), *Myrmica* (Huszár et al. 2014), *Polyrhachis* (Van Zweden et al. 2007), *Formica* (Borowiec et al. 2021; Rosengren et al. 1993), or *Pseudomyrmex* (Janzen 1973) would likely also increase the amount of viable genes (those without convergence problems) in our study, and potentially provide more support for our findings of relaxed selection in highly polygyne species.

As mentioned previously, our data-processing pipeline necessarily filters genes to include only orthologs found universally in all species in our dataset, thereby likely enriching for 'older' or more conserved genes. This filtering may bias our results by excluding younger genes, some of which have been found to exhibit more plastic or caste-specific expression in various eusocial species (Simola et al. 2013). These younger, omitted genes may be disproportionately worker-biased and thus exposed to more relaxed selection, meaning our analysis may underrepresent the full extent of relaxed selection acting on worker-expressed genes. However, the *M. pharaonis* study by Warner et al. (2017) found that many caste-associated genes belonged to older phylostrata, and still exhibited strong signals of differential selection. More published RNAseq datasets would additionally help to confirm caste-biased expression patterns in each of our species, as worker- and queen-biased expression in our gene sets has only been confirmed in *M. pharaonis* (Warner et al. 2017). At the time of this analysis, this dataset of worker/queen differentially expressed genes was the most comprehensive published dataset of this type we could find. Additionally, targeting genes in a highly polygyne species ensures accurate differential expression patterns between workers and queens for at least one out of four of species in this Test group. This helps to maximize accuracy in our inferences of different selection regimes between castes in the Test group (highly polygyne), where we predict the most robust differences will occur.

Further, while we selected only genes in the original *M. pharaonis* dataset that Warner et al. (2017) found to exhibit a robust and significant caste-biased expression pattern across samples and life stages, it is likely that many of these genes are still expressed in both castes to some degree. A main assumption of our study is that genes in our 'worker-biased' and 'queen-biased' datasets are only expressed in and therefore exposed to selective forces in their respective castes. However, if worker-biased genes are also variably expressed in the queen caste of certain species, this could reduce our power to observe true signatures of kin selection. Specifically, this could potentially reduce the observable signal of relaxed selection in these genes, given that they would be under direct selection when expressed in queens. Similarly, we may observe more relaxation/less intensification in queen-biased genes than what we expect from the strict assumption of caste-exclusive expression due to low levels of expression in workers.

This issue is reflected in recent comparative transcriptomic studies, which suggest that caste-biased expression patterns are often not conserved across ant species. While there is some evidence that orthologs with high sequence conservation across mammalian and non-mammalian species often show conserved expression patterns (Zemke et al. 2023; Chan et al. 2009), other studies (Feldmeyer et al. 2014; Morandin et al. 2015, 2016, 2017) consistently find limited overlap in caste-biased gene sets across ant species, even among more conserved genes. While Morandin et al. (2016) did find evidence of correlated constraints on sequence and expression evolution in genes related to metabolic and developmental processes. Overall, they observed that only a small subset of genes exhibited both conserved sequence and caste-biased expression across species. Moreover, Morandin et al. (2015) found that only 56% of caste-biased genes shared between *Formica exsecta* and other ants exhibited bias in the same direction. This raises the possibility that genes identified as strongly caste-biased in *M. pharaonis* could have more balanced expression across castes or even opposite expression bias patterns in some of the other species in our study. While we still find significant differences in average *k* estimates between the two distinct castes in our 'significant' gene subset, this variability in the extent of caste-biased expression may limit our power to infer strong signals of kin selection in other worker-biased genes.

4.5 | Worker Life History Strategies and Nesting Strategy

While brood care is a worker trait with obvious implications for kin selection within a colony, as it involves direct interactions between altruist and beneficiary, other worker traits may be subject to different selective forces as well. Worker tasks such as cooperative foraging, nest defense, or nest building typically involve less direct kinship interactions than brood care, and may therefore be subject to even weaker selection on average, as most nest members act as beneficiaries of these behaviors (Reeves and Moreau 2019). Future work involving the evolution of specific types of worker traits may reveal interesting selection dynamics within the worker caste that may or may not be strongly influenced by colony structure.

Additionally, while this study uses queen number as a predictor of within-nest kin structures, nesting strategy may similarly affect relatedness between workers and beneficiaries. This is due to the impact of nesting strategy on worker dispersal and, therefore, kinship associations on a local scale. Ant colonies are typically either polydomous, with several or many spatially separated but socially connected nests within a colony, or monodomous, with only a single nest per colony (Debout et al. 2007). Both monogyne and polygyne species can be either polydomous or monodomous, but unicolonial and many highly polygyne species in general exhibit a highly polydomous structure (Robinson 2014; Debout et al. 2007). As polydomy allows workers to move freely between neighboring nests, this nesting strategy would serve to decouple kinship associations between brood and caretaker, in the same way that monodomy would reinforce these associations within a nest. Thus, in a polygyne setting, we would expect polydomy to reinforce patterns of weakened selection on worker traits present due to decreased intra-nest relatedness, but monodomy to counter these forces, and vice versa for monogyne species. Some have suggested that local viscosity is present within some highly polygyne colonies, and that this may help to maintain kin selection through the preservation of local kin structures (Helanterä 2022). While there is evidence of viscosity present on local scales in unicolonial species such as *Linepithema humile* (Tsutsui and Case 2001; Ingram and Gordon 2003), the extent to which viscosity can 'rescue' workers from trait degradation still lacks empirical support.

4.6 | Eusociality and Effective Population Size

Because queens produce their own reproductive offspring, we expect queen-biased genes to evolve under stronger direct selection than worker-biased genes, which are shaped by more variable kin selection. However, this prediction is complicated by differences in effective population size (N_e) between castes. Queens are usually much fewer in number than workers, meaning genes expressed primarily in queens may experience reduced N_e, weakening the efficacy of selection in these genes and potentially offsetting the signal of strong direct selection. This effect has been noted more broadly in eusocial taxa, where elevated dN/dS ratios have been linked to relaxed selection associated with reduced N_e (Romiguier et al. 2014; Chak et al. 2021; Weyna and Romiguier 2021; Roux et al. 2024). As a result, any apparent similarity in selection strength between queen- and worker-biased genes in our GLMM contrasts may not reflect equal selective pressures, but could instead arise from reduced selection efficacy in queen-biased genes due to lower effective population size. In other words, this effect may partially obscure true caste-based differences in selection intensity.

5 | Conclusion

Our study across the ant family *Formicidae* reveals complex dynamics related to worker evolution in different social contexts. We predicted that kin structures within a nest should impact worker trait evolution due to principles outlined in kin selection theory, and we found this prediction to be supported for some social contexts. Primarily, we found that in extreme cases of low intra-nest relatedness and high worker dispersal (highly polygyne species), some worker-biased genes exhibit a signal of relaxed selection, consistent with lower efficacy of kin selection. Contrary to our initial predictions, we did not find consistent evidence of worker-biased relaxed selection in species with multiple but low numbers of queens per nest on average (weakly polygyne and socially polymorphic species). This could result from a variety of factors relating to worker or queen life history traits, the evolutionary history of a species, or that weakly polygyne settings cause weakened selection in workers, but the effect is too small to observe with current methods. While our results provide valuable insights into the interplay between intra-nest relatedness and worker caste evolution, broader sampling across ant subfamilies and social structures would help to better elucidate the evolutionary dynamics of worker genes in eusocial insects.

Author Contributions

Kailey Ferger: conceptualization (equal), data curation (lead), formal analysis (lead), funding acquisition (supporting), investigation (lead), methodology (lead), project administration (equal), resources (lead), software (lead), supervision (equal), validation (lead), visualization (lead), writing – original draft (lead), writing – review and editing (lead). **Neil D. Tsutsui:** conceptualization (equal), data curation (supporting), formal analysis (supporting), funding acquisition (lead), investigation (supporting), methodology (supporting), project administration (equal), resources (supporting), software (supporting), supervision (equal), validation (supporting), visualization (supporting), writing – original draft (supporting), writing – review and editing (equal).

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All reference genomes used are publicly available on www.ncbi.nlm.nih.gov (see Table S1 for accession numbers). Complete pipeline and associated scripts can be found at https://github.com/kferger320/GenomicKinSelection.

References

*Akre, R. D., L. D. Hansen, and E. A. Myhre. 1994. "Colony Size and Polygyny in Carpenter Ants (Hymenoptera: Formicidae)." *Journal of the Kansas Entomological Society* 67: 1–9.

*Alloway, T. M., R. Stuart, C. Thomas, A. Buschinger, and M. Talbot. 1982. "Polygyny and Polydomy in Three North American Species of the Ant Genus *Leptothorax* Mayr (Hymenoptera: Formicidae)." *Psyche: A Journal of Entomology* 89: 249–274.

Aron, S., L. Passera, and L. Keller. 1999. "Evolution of Social Parasitism in Ants: Size of Sexuals, Sex Ratio and Mechanisms of Caste Determination." *Proceedings of the Royal Society of London, Series B: Biological Sciences* 266, no. 1415: 173–177.

Astruc, C., C. Malosse, and C. Errard. 2001. "Lack of Intraspecific Aggression in the Ant *Tetramorium bicarinatum*: A Chemical Hypothesis." *Journal of Chemical Ecology* 27: 1229–1248.

Barkdull, M., and C. S. Moreau. 2023. "Worker Reproduction and Caste Polymorphism Impact Genome Evolution and Social Genes Across the Ants." *Genome Biology and Evolution* 15, no. 6: evad095.

Báthori, F., J. Heinze, A. Trindl, B. Seifert, G. Herczeg, and S. Csősz. 2024. "Host-Switching Events Are Not Always the Driver of Speciation in Social Parasites: A Case Study in *Temnothorax* (*Myrmoxenus*) Ants (Hymenoptera, Formicidae)." *Journal of Zoology* 322, no. 3: 221–231.

*Bekkevold, D., J. Frydenberg, and J. J. Boomsma. 1999. "Multiple Mating and Facultative Polygyny in the Panamanian Leafcutter Ant *Acromyrmex echinatior*." *Behavioral Ecology and Sociobiology* 46: 103–109.

Benjamini, Y., and Y. Hochberg. 1995. "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing." *Journal of the Royal Statistical Society. Series B, Statistical Methodology* 57, no. 1: 289–300.

Bernasconi, G., and J. E. Strassmann. 1999. "Cooperation Among Unrelated Individuals: The Ant Foundress Case." *Trends in Ecology & Evolution* 14, no. 12: 477–482.

Boomsma, J. J., and F. L. W. Ratnieks. 1996. "Paternity in Eusocial Hymenoptera." *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 351: 947–975.

Borowiec, M. L., S. P. Cover, and C. Rabeling. 2021. "The Evolution of Social Parasitism in *Formica* Ants Revealed by a Global Phylogeny." *Proceedings of the National Academy of Sciences of the United States of America* 118, no. 38: e2026029118.

*Boulay, R., M. Quagebeur, E. J. Godzinska, and A. Lenoir. 1999. "Social Isolation in Ants: Evidence of Its Impact on Survivorship and Behavior in *Camponotus fellah* (Hymenoptera, Formicidae)." *Sociobiology* 33, no. 2: 111–124.

Bourke, A. F. 1995. *Social Evolution in Ants*. Princeton University Press.

Brooks, M. E., K. Kristensen, K. J. van Benthem, et al. 2017. "glmmTMB Balances Speed and Flexibility Among Packages for Zero-Inflated Generalized Linear Mixed Modeling." *R Journal* 9, no. 2: 378–400. https://doi.org/10.32614/RJ-2017-066.

*Bruder, K. W., and A. P. Gupta. 1972. "Biology of the Pavement Ant, *Tetramorium caespitum* (Hymenoptera: Formicidae)." *Annals of the Entomological Society of America* 65, no. 2: 358–367.

Camacho, C., G. Coulouris, V. Avagyan, et al. 2009. "BLAST+: Architecture and Applications." *BMC Bioinformatics* 10: 1–9.

Chak, S. T., J. A. Baeza, and P. Barden. 2021. "Eusociality Shapes Convergent Patterns of Molecular Evolution Across Mitochondrial Genomes of Snapping Shrimps." *Molecular Biology and Evolution* 38, no. 4: 1372–1383.

Chan, E. T., G. T. Quon, G. Chua, et al. 2009. "Conservation of Core Gene Expression in Vertebrate Tissues." *Journal of Biology* 8: 1–17.

*Chapuisat, M., S. Bocherens, and H. Rosset. 2004. "Variable Queen Number in Ant Colonies: No Impact on Queen Turnover, Inbreeding, and Population Genetic Differentiation in the Ant *Formica selysi*." *Evolution* 58, no. 5: 1064–1072.

Cherix, D. 1980. "Note preliminaire sur la structure la phenologie et le regime alimentaire d'une super-colonie de *Formica lugubris* Zett." *Insectes Sociaux* 27: 226–236.

*Clark, R. M., K. E. Anderson, J. Gadau, and J. H. Fewell. 2006. "Behavioral Regulation of Genetic Caste Determination in a *Pogonomyrmex* Population With Dependent Lineages." *Ecology* 87, no. 9: 2201–2206.

*Cordonnier, M., G. Escarguel, A. Dumet, and B. Kaufmann. 2020. "Multiple Mating in the Context of Interspecific Hybridization Between Two *Tetramorium* Ant Species." *Heredity* 124, no. 5: 675–684.

Crozier, R. H., and P. Pamilo. 1996. *Evolution of Social Insect Colonies: Sex Allocation and Kin Selection*. Oxford University Press.

*Dacquin, P., F. Degueldre, and R. E. Vicente. 2021. "Relative Colony Size of Parabiotic Species Demonstrates Inversion With Growth." *Insectes Sociaux* 68, no. 1: 77–80.

Dang, V. D., A. B. Cohanim, S. Fontana, E. Privman, and J. Wang. 2019. "Has Gene Expression Neofunctionalization in the Fire Ant Antennae Contributed to Queen Discrimination Behavior?" *Ecology and Evolution* 9, no. 22: 12754–12766.

Debout, G., B. Schatz, M. Elias, and D. Mckey. 2007. "Polydomy in Ants: What We Know, What We Think We Know, and What Remains to Be Done." *Biological Journal of the Linnean Society* 90, no. 2: 319–348.

Dejean, A., M. Kenne, and C. S. Moreau. 2007. "Predatory Abilities Favour the Success of the Invasive Ant *Pheidole megacephala* in an Introduced Area." *Journal of Applied Entomology* 131, no. 9–10: 625–629.

*Dewes, E. 1993. "Die Kerbameise (*Formica* (*Coptoformica*) *Exsecta* Nyl.) im Naturpark Saar-Hunsruck (Saarlandischer Teil)." *Ameisenschutz Aktuell* 7: 5–8.

*Diehi, E., A. M. De Araújo, and S. Cavalli-Molina. 2001. "Genetic Variability and Social Structure of Colonies in *Acromyrmex heyeri* and *A. striatus* (Hymenoptera: Formicidae)." *Brazilian Journal of Biology* 61: 667–678.

*Eyer, P. A., B. McDowell, L. N. Johnson, et al. 2018. "Supercolonial Structure of Invasive Populations of the Tawny Crazy Ant *Nylanderia fulva* in the US." *BMC Evolutionary Biology* 18: 1–14.

Feldmeyer, B., D. Elsner, and S. Foitzik. 2014. "Gene Expression Patterns Associated With Caste and Reproductive Status in Ants: Worker-Specific Genes Are More Derived Than Queen-Specific Ones." *Molecular Ecology* 23, no. 1: 151–161.

Fjerdingstad, E. J., and J. J. Boomsma. 1998. "Multiple Mating Increases the Sperm Stores of *Atta colombica* Leafcutter Ant Queens." *Behavioral Ecology and Sociobiology* 42: 257–261.

*Fonseca, C. R. 1993. "Nesting Space Limits Colony Size of the Plant-Ant *Pseudomyrmex concolor*." *Oikos* 67: 473–482.

Fortelius, W., R. Rosengren, D. Cherix, and D. Chautems. 1993. "Queen Recruitment in a Highly Polygynous Supercolony of *Formica lugubris* (Hymenoptera, Formicidae)." *Oikos* 67: 193–200.

*Foucaud, J., D. Fournier, J. Orivel, et al. 2007. "Sex and Clonality in the Little Fire Ant." *Molecular Biology and Evolution* 24, no. 11: 2465–2473.

*Foucaud, J., J. Orivel, D. Fournier, et al. 2009. "Reproductive System, Social Organization, Human Disturbance and Ecological Dominance in Native Populations of the Little Fire Ant, *Wasmannia auropunctata*." *Molecular Ecology* 18, no. 24: 5059–5073.

*Fournier, D., and L. Keller. 2001. "Partitioning of Reproduction Among Queens in the Argentine Ant, *Linepithema humile*." *Animal Behaviour* 62, no. 6: 1039–1045.

*Frumhoff, P. C., and P. S. Ward. 1992. "Individual-Level Selection, Colony-Level Selection, and the Association Between Polygyny and Worker Monomorphism in Ants." *American Naturalist* 139, no. 3: 559–590.

Fu, L., B. Niu, Z. Zhu, S. Wu, and W. Li. 2012. "CD-HIT: Accelerated for Clustering the Next-Generation Sequencing Data." *Bioinformatics* 28, no. 23: 3150–3152.

*Gadau, J., J. Heinze, B. Hölldobler, and M. Schmid. 1996. "Population and Colony Structure of the Carpenter Ant *Camponotus floridanus*." *Molecular Ecology* 5, no. 6: 785–792.

*Gallardo, Á. 1916. "Notes systématiques et éthologiques sur les fourmis attines de la République Argentine." *Anales del Museo Nacional de Historia Natural de Buenos Aires* 28: 317–344.

*Glancey, B. M., C. H. Craig, C. E. Stringer, and P. M. Bishop. 1973. "Multiple Fertile Queens in Colonies of the Imported Fire Ant, *Solenopsis invicta*." *Journal of Georgia Entomological Society* 8: 237–238.

Goudie, F., and B. P. Oldroyd. 2018. "The Distribution of Thelytoky, Arrhenotoky and Androgenesis Among Castes in the Eusocial Hymenoptera." *Insectes Sociaux* 65, no. 1: 5–16.

Gris, G., and D. Cherix. 1977. "Les grandes colonies de fourmis des bois du Jura (groupe *Formica rufa*)." *Mitteilungen Der Schweizerischen Entomologischen Gesellschaft* 50: 249–250.

Gstöttl, C., M. Stoldt, E. Jongepier, et al. 2020. "Comparative Analyses of Caste, Sex, and Developmental Stage-Specific Transcriptomes in Two *Temnothorax* Ants." *Ecology and Evolution* 10, no. 10: 4193–4203.

*Gunawardana, D., and Dennis. 2022. *Anoplolepis gracilipes (Yellow Crazy Ant)*, 5575. CABI Compendium, CABI International.

*Hagan, H. R., and T. C. Schneirla. 1954. "The Reproductive System of the Army-Ant Queen, *Eciton* (Eciton) Part 3, the Oöcyte Cycle." *American Museum Novitates* 1665: 1-20.

Hamilton, W. D. 1964. "The Genetical Evolution of Social Behaviour. I." *Journal of Theoretical Biology* 7, no. 1: 1–16.

Harpur, B. A., C. F. Kent, D. Molodtsova, et al. 2014. "Population Genomics of the Honey Bee Reveals Strong Signatures of Positive Selection on Worker Traits." *Proceedings of the National Academy of Sciences of the United States of America* 111, no. 7: 2614–2619.

*Hart, L. M. 2009. "Brief Natural History on the Ant *Odontomachus brunneus* (Patton)." Master's Thesis, Florida State University). *Florida State University Libraries*.

*Heinze, J., and J. Delabie. 2005. "Population Structure of the Male-Polymorphic Ant *Cardiocondyla obscurior*." *Studies on Neotropical Fauna and Environment* 40, no. 3: 187–190.

*Heinze, J., B. Hölldobler, and C. Peters. 1994. "Conflict and Cooperation in Ant Societies." *Naturwissenschaften* 81, no. 11: 489–497.

*Heinze, J., D. Ortius, M. Kaib, and B. Hölldobler. 1994. "Interspecific Aggression in Colonies of the Slave-Making Ant *Harpagoxenus sublaevis*." *Behavioral Ecology and Sociobiology* 35: 75–83.

*Heinze, J., W. Puchinger, and B. Hölldobler. 1997. "Worker Reproduction and Social Hierarchies in *Leptothorax* Ants." *Animal Behaviour* 54, no. 4: 849–864.

Helanterä, H. 2022. "Supercolonies of Ants (Hymenoptera: Formicidae): Ecological Patterns, Behavioural Processes and Their Implications for Social Evolution." *Myrmecological News* 32: 1–22.

*Herbers, J. M., and S. Grieco. 1994. "Population Structure of *Leptothorax ambiguus*, a Facultatively Polygynous and Polydomous Ant Species." *Journal of Evolutionary Biology* 7, no. 5: 581–598.

Hölldobler, B., and E. O. Wilson. 1990. *The Ants*. Harvard University Press.

Holldobler, B., and E. O. Wilson. 2009. *The Superorganism: The Beauty Elegance and Strangeness of Insect Societies*. WW Norton & Company.

Hughes, W. O., and J. J. Boomsma. 2007. "Genetic Polymorphism in Leaf-Cutting Ants Is Phenotypically Plastic." *Proceedings of the Royal Society B: Biological Sciences* 274, no. 1618: 1625–1630.

Hunt, B. G., L. Ometto, Y. Wurm, et al. 2011. "Relaxed Selection Is a Precursor to the Evolution of Phenotypic Plasticity." *Proceedings of the National Academy of Sciences of the United States of America* 108, no. 38: 15936–15941.

Huszár, D. B., R. S. Larsen, S. Carlsen, J. J. Boomsma, and J. S. Pedersen. 2014. "Convergent Development of Ecological, Genetic, and Morphological Traits in Native Supercolonies of the Red Ant *Myrmica rubra*." *Behavioral Ecology and Sociobiology* 68: 1859–1870.

*Ingram, K. K. 2002. "Plasticity in Queen Number and Social Structure in the Invasive Argentine Ant (*Linepithema humile*)." *Evolution* 56, no. 10: 2008–2016.

Ingram, K. K., and D. M. Gordon. 2003. "Genetic Analysis of Dispersal Dynamics in an Invading Population of Argentine Ants." *Ecology* 84, no. 11: 2832–2842.

*Jacquier, L., M. Molet, and C. Doums. 2023. "Urban Colonies Are Less Aggressive but Forage More Than Their Forest Counterparts in the Ant *Temnothorax nylanderi*." *Animal Behaviour* 199: 11–21.

Janzen, D. H. 1973. "Evolution of Polygynous Obligate Acacia-Ants in Western Mexico." *Journal of Animal Ecology* 42: 727–750.

Johnson, B. R., and N. D. Tsutsui. 2011. "Taxonomically Restricted Genes are Associated With the Evolution of Sociality in the Honey Bee." *BMC Genomics* 12: 1–10.

Katoh, K., and D. M. Standley. 2013. "MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability." *Molecular Biology and Evolution* 30, no. 4: 772–780.

*Krapf, P., L. Russo, W. Arthofer, M. Möst, F. M. Steiner, and B. C. Schlick-Steiner. 2018. "An Alpine Ant's Behavioural Polymorphism:

Monogyny With and Without Internest Aggression in *Tetramorium alpestre*." *Ethology Ecology & Evolution* 30, no. 3: 220–234.

*Kümmerli, R., and L. Keller. 2007. "Contrasting Population Genetic Structure for Workers and Queens in the Putatively Unicolonial Ant *Formica exsecta*." *Molecular Ecology* 16, no. 21: 4493–4503.

Latreille, P. A. 1798. *Essai sur l'histoire des fourmis de la France*. Slatkine.

LeBrun, E. G., N. T. Jones, and L. E. Gilbert. 2014. "Chemical Warfare Among Invaders: A Detoxification Interaction Facilitates an Ant Invasion." *Science* 343, no. 6174: 1014–1017.

*LeBrun, E. G., R. M. Plowes, P. J. Folgarait, M. Bollazzi, and L. E. Gilbert. 2019. "Ritualized Aggressive Behavior Reveals Distinct Social Structures in Native and Introduced Range Tawny Crazy Ants." *PLoS One* 14, no. 11: e0225597.

*Leniaud, L., H. Darras, R. Boulay, and S. Aron. 2012. "Social Hybridogenesis in the Clonal Ant *Cataglyphis hispanica*." *Current Biology* 22, no. 13: 1188–1193.

Lenth, R. 2025. "emmeans: Estimated Marginal Means, Aka Least-Squares Means (R Package Version 1.11.1-00001)." https://rvlenth.github.io/emmeans/.

Li, W., and A. Godzik. 2006. "Cd-Hit: A Fast Program for Clustering and Comparing Large Sets of Protein or Nucleotide Sequences." *Bioinformatics* 22, no. 13: 1658–1659.

Liao, X., S. Rong, and D. C. Queller. 2015. "Relatedness, Conflict, and the Evolution of Eusociality." *PLoS Biology* 13, no. 3: e1002098.

Linksvayer, T. A., and M. J. Wade. 2009. "Genes With Social Effects Are Expected to Harbor More Sequence Variation Within and Between Species." *Evolution* 63, no. 7: 1685–1696.

Linksvayer, T. A., and M. J. Wade. 2016. "Theoretical Predictions for Sociogenomic Data: The Effects of Kin Selection and Sex-Limited Expression on the Evolution of Social Insect Genomes." *Frontiers in Ecology and Evolution* 4: 65.

*Longino, J. T. 2003. "The *Crematogaster* (Hymenoptera, Formicidae, Myrmicinae) of Costa Rica." *Zootaxa* 151, no. 1: 1–150.

Lowe, S., M. Browne, S. Boudjelas, and M. De Poorter. 2000. *100 of the World's Worst Invasive Alien Species: A Selection From the Global Invasive Species Database*. Vol. 12, 12. Invasive Species Specialist Group.

*Mankowski, M. E., and J. J. Morrell. 2011. "Role of Relative Humidity in Colony Founding and Queen Survivorship in Two Carpenter Ant Species." *Journal of Economic Entomology* 104, no. 3: 740–744.

McGillycuddy, M., D. I. Warton, G. Popovic, and B. M. Bolker. 2025. "Parsimoniously Fitting Large Multivariate Random Effects in glmmTMB." *Journal of Statistical Software* 112, no. 1: 1–19. https://doi.org/10.18637/jss.v112.i01.

*Mintzer, A. 1990. "Foundress Female Weight and Cooperative Foundation in *Atta* Leaf-Cutting Ants." In *Applied Myrmecology: A World Perspective*, edited by R. K. Vander Meer, K. Jaffe, and A. Cedeno, 180–183. Westview Press.

*Mintzer, A., and S. B. Vinson. 1985. "Cooperative Colony Foundation by Females of the Leafcutting Ant *Atta texana* in the Laboratory." *Journal of the New York Entomological Society* 93, no. 5: 1047–1051.

*Monnin, T., and C. Peeters. 1997. "Cannibalism of Subordinates' Eggs in the Monogynous Queenless Ant *Dinoponera quadriceps*." *Naturwissenschaften* 84: 499–502.

Morandin, C., K. Dhaygude, J. Paviala, K. Trontti, C. Wheat, and H. Helanterä. 2015. "Caste-Biases in Gene Expression Are Specific to Developmental Stage in the Ant *Formica exsecta*." *Journal of Evolutionary Biology* 28, no. 9: 1705–1718.

Morandin, C., A. S. Mikheyev, J. S. Pedersen, and H. Helanterä. 2017. "Evolutionary Constraints Shape Caste-Specific Gene Expression Across 15 Ant Species." *Evolution* 71, no. 5: 1273–1284.

Morandin, C., M. M. Y. Tin, S. Abril, et al. 2016. "Comparative Transcriptomics Reveals the Conserved Building Blocks Involved in Parallel Evolution of Diverse Phenotypic Traits in Ants." *Genome Biology* 17: 1–19.

Moreau, C. S., C. D. Bell, R. Vila, S. B. Archibald, and N. E. Pierce. 2006. "Phylogeny of the Ants: Diversification in the Age of Angiosperms." *Science* 312, no. 5770: 101–104.

*Muñoz-Valencia, V., K. Kähkönen, J. Montoya-Lerma, and F. Díaz. 2020. "Characterization of a New Set of Microsatellite Markers Suggests Polygyny and Polyandry in *Atta cephalotes* (Hymenoptera: Formicidae)." *Journal of Economic Entomology* 113, no. 6: 3021–3027.

Nehring, V., M. Dijkstra, S. Sumner, W. Hughes, and K. Boosmsma. 2018. "Reconstructing the Relatedness of Cooperatively Breeding Queens in the Panamanian Leaf-Cutting Ant *Acromyrmex echinatior* (Hymenoptera: Formicidae)." *Myrmecological News* 27: 33–45.

*Oettler, J., and A. Schrempf. 2016. "Fitness and Aging in *Cardiocondyla obscurior* Ant Queens." *Current Opinion in Insect Science* 16: 58–63.

*Ohkawara, K. 2002. "Queen Polymorphism and Reproductive Behaviour in Myrmicine Ant *Vollenhovia emeryi*." In *Proceedings of the XIV International Congress of the International Union for the Study of Social Insects, Sapporo, Japan, 27 July–3 August 3 2002*, vol. 206. Hokkaido University Press.

Overson, R., J. Fewell, and J. Gadau. 2016. "Distribution and Origin of Intraspecific Social Variation in the California Harvester Ant *Pogonomyrmex californicus*." *Insectes Sociaux* 63: 531–541.

*Pamilo, P., and R. Rosengren. 1984. "Evolution of Nesting Strategies of Ants: Genetic Evidence From Different Population Types of *Formica* Ants." *Biological Journal of the Linnean Society* 21, no. 3: 331–348.

*Peacock, A. D., J. H. Sudd, and A. T. Baxter. 1955. "Studies in Pharaoh's Ant, *Monomorium pharaonis* (L.). 11. Colony Foundation." *Entomologist's Monthly Magazine* 91: 125–129.

*Peeters, C., J. Liebig, and B. Hölldobler. 2000. "Sexual Reproduction by Both Queens and Workers in the Ponerine Ant *Harpegnathos saltator*." *Insectes Sociaux* 47: 325–332.

*Pérez-Ortega, B., H. Fernández-Marín, M. S. Loiácono, P. Galgani, and W. T. Wcislo. 2010. "Biological Notes on a Fungus-Growing Ant, *Trachymyrmex cf. zeteki* (Hymenoptera, Formicidae, Attini) Attacked by a Diverse Community of Parasitoid Wasps (Hymenoptera, Diapriidae)." *Insectes Sociaux* 57: 317–322.

Privman, E., P. Cohen, A. B. Cohanim, O. Riba-Grognuz, D. Shoemaker, and L. Keller. 2018. "Positive Selection on Sociobiological Traits in Invasive Fire Ants." *Molecular Ecology* 27, no. 15: 3116–3130.

Rabeling, C., T. R. Schultz, M. Bacci, and M. Bollazzi. 2015. "*Acromyrmex charruanus*: A New Inquiline Social Parasite Species of Leaf-Cutting Ants." *Insectes Sociaux* 62: 335–349.

Reeves, D., and C. Moreau. 2019. "The Evolution of Foraging Behavior in Ants (Hymenoptera: Formicidae)." *Arthropod Systematics & Phylogeny* 77: 351–363.

Rissing, S. W., and G. B. Pollock. 2019. "Pleometrosis and Polygyny in Ants." In *Interindividual Behavioral Variability in Social Insects*, 179–222. CRC Press.

Robinson, E. J. 2014. "Polydomy: The Organisation and Adaptive Function of Complex Nest Systems in Ants." *Current Opinion in Insect Science* 5: 37–43.

Romiguier, J., J. Lourenco, P. Gayral, et al. 2014. "Population Genomics of Eusocial Insects: The Costs of a Vertebrate-Like Effective Population Size." *Journal of Evolutionary Biology* 27, no. 3: 593–603.

Ronai, I., V. Vergoz, and B. P. Oldroyd. 2016. "The Mechanistic, Genetic, and Evolutionary Basis of Worker Sterility in the Social Hymenoptera." In *Advances in the Study of Behavior*, vol. 48, 251–317. Academic Press.

Rosengren, R., L. Sundström, and W. Fortelius. 1993. "Monogyny and Polygyny in *Formica* Ants: The Result of Alternative Dispersal Tactics." In *Queen Number and Sociality in Insects*, 308–333. Oxford University Press.

Ross, K. G., E. L. Vargo, and L. Keller. 1996. "Simple Genetic Basis for Important Social Traits in the Fire Ant *Solenopsis invicta*." *Evolution* 50, no. 6: 2387–2399.

Roux, C., A. Weyna, M. Lodé, and J. Romiguier. 2024. "Social Evolution in Termites Reduces Natural Selection Efficacy." *bioRxiv*. https://doi.org/10.1101/2024.04.26.591327.

*Schmid, V. S., M. Kaltenpoth, E. Strohm, and J. Heinze. 2013. "Worker Self-Restraint and Policing Maintain the Queen's Reproductive Monopoly in a *Pseudomyrmecine* Ant." *Behavioral Ecology and Sociobiology* 67: 571–581.

Schrader, L., H. Pan, M. Bollazzi, et al. 2021. "Relaxed Selection Underlies Genome Erosion in Socially Parasitic Ant Species." *Nature Communications* 12, no. 1: 2918.

*Schrempf, A., S. Cremer, and J. Heinze. 2011. "Social Influence on Age and Reproduction: Reduced Lifespan and Fecundity in Multi-Queen Ant Colonies." *Journal of Evolutionary Biology* 24, no. 7: 1455–1461.

Schultz, T. R., D. Bekkevold, and J. J. Boomsma. 1998. "*Acromyrmex insinuator* New Species: An Incipient Social Parasite of Fungus-Growing Ants." *Insectes Sociaux* 45: 457–471.

Seifert, B. 2018. *The Ants of Central and North Europe*, 408. Lutra Verlags-Und Vertriebsgesellschaft.

*Seppä, P., N. Gyllenstrand, J. Corander, and P. Pamilo. 2004. "Coexistence of the Social Types: Genetic Population Structure in the: Ant *Formica Exsecta*." *Evolution* 58, no. 11: 2462–2471.

Simola, D. F., L. Wissler, G. Donahue, et al. 2013. "Social Insect Genomes Exhibit Dramatic Evolution in Gene Composition and Regulation While Preserving Regulatory Features Linked to Sociality." *Genome Research* 23, no. 8: 1235–1247.

Smith, C. R., A. L. Toth, A. V. Suarez, and G. E. Robinson. 2008. "Genetic and Genomic Analyses of the Division of Labour in Insect Societies." *Nature Reviews Genetics* 9, no. 10: 735–748.

Stamatakis, A. 2014. "RAxML Version 8: A Tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies." *Bioinformatics* 30, no. 9: 1312–1313.

Stanke, M., M. Diekhans, R. Baertsch, and D. Haussler. 2008. "Using Native and Syntenically Mapped cDNA Alignments to Improve de Novo Gene Finding." *Bioinformatics* 24, no. 5: 637–644.

Strassmann, J. 2001. "The Rarity of Multiple Mating by Females in the Social Hymenoptera." *Insectes Sociaux* 48: 1–13.

Strassmann, J. E., and D. C. Queller. 2007. "Insect Societies as Divided Organisms: The Complexities of Purpose and Cross-Purpose." *National Academy of Sciences of the United States of America* 104: 8619–8626.

*Stuart, R. J., L. Gresham-Bissett, and T. M. Alloway. 1993. "Queen Adoption in the Polygynous and Polydomous Ant, *Leptothorax curvispinosus*." *Behavioral Ecology* 4, no. 3: 276–281.

Sumner, S., W. O. Hughes, and J. J. Boomsma. 2003. "Evidence for Differential Selection and Potential Adaptive Evolution in the Worker Caste of an Inquiline Social Parasite." *Behavioral Ecology and Sociobiology* 54: 256–263.

*Sumner, S., D. R. Nash, and J. J. Boomsma. 2003. "The Adaptive Significance of Inquiline Parasite Workers." *Proceedings of the Royal Society of London, Series B: Biological Sciences* 270, no. 1521: 1315–1322.

*Trager, J. C. 2013. "Global Revision of the Dulotic Ant Genus *Polyergus* (Hymenoptera: Formicidae, Formicinae, Formicini)." *Zootaxa* 3722: 501–548.

*Tschinkel, W. R. 1987. "Seasonal Life History and Nest Architecture of a Winter-Active Ant, *Prenolepis imparis*." *Insectes Sociaux* 34, no. 3: 143–164.

*Tschinkel, W. R. 1998. "An Experimental Study of Pleometrotic Colony Founding in the Fire Ant, *Solenopsis invicta*: What Is the Basis for Association?" *Behavioral Ecology and Sociobiology* 43: 247–257.

*Tschinkel, W. R. 2011. "The Nest Architecture of Three Species of North Florida *Aphaenogaster* Ants." *Journal of Insect Science* 11, no. 1: 105.

*Tschinkel, W. R., and D. F. Howard. 1983. "Colony Founding by Pleometrosis in the Fire Ant, *Solenopsis invicta*." *Behavioral Ecology and Sociobiology* 12: 103–113.

Tsutsui, N. D., and T. J. Case. 2001. "Population Genetics and Colony Structure of the Argentine Ant (*Linepithema humile*) in Its Native and Introduced Ranges." *Evolution* 55, no. 5: 976–985.

Tsutsui, N. D., and A. V. Suarez. 2003. "The Colony Structure and Population Biology of Invasive Ants." *Conservation Biology* 17, no. 1: 48–58.

*Van Elst, T., and J. Gadau. 2018. "Temporal Variation in Social Structure and Worker Reproduction in the Temporary Social Parasite *Lasius fuliginosus* (Hymenoptera: Formicidae)." *Myrmecological News* 27: 75–85.

Van Wilgenburg, E., C. W. Torres, and N. D. Tsutsui. 2010. "The Global Expansion of a Single Ant Supercolony." *Evolutionary Applications* 3, no. 2: 136–143.

Van Zweden, J. S., M. E. Carew, M. T. Henshaw, S. K. A. Robson, and R. H. Crozier. 2007. "Social and Genetic Structure of a Supercolonial Weaver Ant, *Polyrhachis robsoni*, With Dimorphic Queens." *Insectes Sociaux* 54: 34–41.

Wang, J., Y. Wurm, M. Nipitwattanaphon, et al. 2013. "A Y-Like Social Chromosome Causes Alternative Colony Organization in Fire Ants." *Nature* 493, no. 7434: 664–668.

*Ward, P. S. 1993. "Systematic Studies on *Pseudomyrmex* Acacia-Ants (Hymenoptera: Formicidae: Pseudomyrmecinae)." *Journal of Hymenoptera Research* 2, no. 1: 117–168.

Warner, M. R., A. S. Mikheyev, and T. A. Linksvayer. 2017. "Genomic Signature of Kin Selection in an Ant With Obligately Sterile Workers." *Molecular Biology and Evolution* 34, no. 7: 1780–1787.

*Weber, N. A. 1957. "Fungus-Growing Ants and Their Fungi: *Cyphomyrmex costatus*." *Ecology* 38, no. 3: 480–494.

Wertheim, J. O., B. Murrell, M. D. Smith, S. L. Kosakovsky Pond, and K. Scheffler. 2015. "RELAX: Detecting Relaxed Selection in a Phylogenetic Framework." *Molecular Biology and Evolution* 32, no. 3: 820–832.

Wetterer, J. K. 2010. "Worldwide Spread of the Pharaoh Ant, *Monomorium pharaonis* (Hymenoptera: For-Micidae)." *Myrmecological News* 13: 115–129.

Weyna, A., and J. Romiguier. 2021. "Relaxation of Purifying Selection Suggests Low Effective Population Size in Eusocial Hymenoptera and Solitary Pollinating Bees." *Peer Community Journal* 1: e2.

Wheeler, D. 1991. "The Developmental Basis of Worker Caste Polymorphism in Ants." *American Naturalist* 138: 1218–1238. https://doi.org/10.1086/285279.

*Whelden, R. M. 1963. "Anatomy of Adult Queen and Workers of Army Ants *Eciton burchelli* Westw. and *E. hamatum* Fabr.(Hymenoptera: Formicidae)." *Journal of the New York Entomological Society* 71, no. 1: 14–30.

Wilson, E. O. 1971. *The Insect Societies*. Harvard University Press.

*Yamauchi, K. 1981. "Sociobiological Studies of the Polygynic Ant *Lasius sakagamii*. I. General Features of Its Polydomous System." *Insectes Sociaux* 28: 279–296.

Zemke, N. R., E. J. Armand, W. Wang, et al. 2023. "Conserved and Divergent Gene Regulatory Programs of the Mammalian Neocortex." *Nature* 624, no. 7991: 390–402.

*Zenner-Polania, I. 2019. "Biological Aspects of the "Hormiga Loca," *Paratrechina (Nylanderia) fulva* (Mayr), in Colombia." In *Applied Myrmecology*, 290–297. CRC Press.

* cited in Table S1 in Supporting Information.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.