# Grant Application Package

| | |
|---|---|
| **Opportunity Title:** | Biotechnology Risk Assessment Grants Program |
| **Offering Agency:** | National Institute of Food and Agriculture |
| **CFDA Number:** | 10.219 |
| **CFDA Description:** | Biotechnology Risk Assessment Research |
| **Opportunity Number:** | USDA-NIFA-BRAP-005435 |
| **Competition ID:** | |
| **Opportunity Open Date:** | 10/29/2015 |
| **Opportunity Close Date:** | 04/15/2016 |
| **Agency Contact:** | NIFA Help Desk<br>Phone: 202-401-5048<br>electronic@nifa.usda.gov<br>Business hours are M-F, 7:00 am -5:00 pm ET, excluding Federal holidays |

**This opportunity is only open to organizations, applicants who are submitting grant applications on behalf of a company, state, local or tribal government, academia, or other type of organization.**

**Application Filing Name:** CALS_Scott_72156

## Select Forms to Complete

### Mandatory

- SF424 (R & R)
- RR FedNonFed Budget
- Research & Related Personal Data
- Research And Related Other Project Information
- Research and Related Senior/Key Person Profile (Expanded)
- Project/Performance Site Location(s)
- NIFA Supplemental Information

### Optional

- ☐ Research & Related Subaward Budget (Total Fed + Non-Fed) 5 YR 30 ATT

## Instructions

*Show Instructions >>*

**This electronic grants application is intended to be used to apply for the specific Federal funding opportunity referenced here.**

**If the Federal funding opportunity listed is not the opportunity for which you want to apply, close this application package by clicking on the "Cancel" button at the top of this screen. You will then need to locate the correct Federal funding opportunity, download its application and then apply.**

OMB Number: 4040-0001
Expiration Date: 6/30/2016

**APPLICATION FOR FEDERAL ASSISTANCE**
# SF 424 (R&R)

**3. DATE RECEIVED BY STATE:**
**State Application Identifier:**

**1. TYPE OF SUBMISSION**
☐ Pre-application ☒ Application ☐ Changed/Corrected Application

**2. DATE SUBMITTED:**
**Applicant Identifier:**

**4. a. Federal Identifier:**
**b. Agency Routing Identifier:**
**c. Previous Grants.gov Tracking ID:**

**5. APPLICANT INFORMATION**
**Organizational DUNS:** 042092122
Legal Name: North Carolia State University
Department:
Division:
Street1: 2701 Sullivan Drive
Street2: Admin Services III; Box 7514
City: Raleigh
County / Parish: Wake
State: NC: North Carolina
Province:
Country: USA: UNITED STATES
ZIP / Postal Code: 27595-7514

Person to be contacted on matters involving this application
Prefix:
First Name: Sherrie
Middle Name:
Last Name: Settle
Suffix:
Position/Title: Director of Sponsored Programs
Street1: 2701 Sullivan Drive
Street2: Admin Services III; Box 7514
City: Raleigh
County / Parish: Wake
State: NC: North Carolina
Province:
Country: USA: UNITED STATES
ZIP / Postal Code: 27695-7514
Phone Number: 919-515-2444
Fax Number: 919-515-7721
Email: sps@ncsu.edu

**6. EMPLOYER IDENTIFICATION** *(EIN) or (TIN):* 56-6000756

**7. TYPE OF APPLICANT:** H: Public/State Controlled Institution of Higher Education
Other (Specify):
**Small Business Organization Type** ☐ Women Owned ☐ Socially and Economically Disadvantaged

**8. TYPE OF APPLICATION:**
☒ New ☐ Resubmission
☐ Renewal ☐ Continuation ☐ Revision

If Revision, mark appropriate box(es).
☐ A. Increase Award ☐ B. Decrease Award ☐ C. Increase Duration ☐ D. Decrease Duration
☐ E. Other (specify):

Is this application being submitted to other agencies? Yes ☐ No ☒ What other Agencies?

**9. NAME OF FEDERAL AGENCY:**
National Institute of Food and Agriculture

**10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER:** 10.219
TITLE: Biotechnology Risk Assessment Research

**11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:**
Development and evaluation of safeguards for conditional suppressive gene drives for spotted wing Drosophila and the New World screwworm

**12. PROPOSED PROJECT:**
Start Date: 09/01/2016
Ending Date: 08/31/2019

**13. CONGRESSIONAL DISTRICT OF APPLICANT:** NC-004

**14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION**

Prefix: Dr. First Name: Maxwell Middle Name:
Last Name: Scott Suffix:
Position/Title: Associate Professor
Organization Name: North Carolina State University
Department: Entomology Division: CALS
Street1: Thamos Hall 1542B, Box 7613
Street2: NCSU Campus
City: Raleigh County / Parish: Wake
State: NC: North Carolina Province:
Country: USA: UNITED STATES ZIP / Postal Code: 27595-7613
Phone Number: 919-515-0275 Fax Number: 919-515-3355
Email: max_scott@ncsu.edu

**15. ESTIMATED PROJECT FUNDING**

| | |
|---|---|
| a. Total Federal Funds Requested | 499,851.00 |
| b. Total Non-Federal Funds | 0.00 |
| c. Total Federal & Non-Federal Funds | 499,851.00 |
| d. Estimated Program Income | 0.00 |

**16. IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?**

a. YES ☐ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:
DATE:

b. NO ☒ PROGRAM IS NOT COVERED BY E.O. 12372; OR
☐ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

**17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious. or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)**

☒ **I agree**

***The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.***

**18. SFLLL (Disclosure of Lobbying Activities) or other Explanatory Documentation**

Add Attachment | Delete Attachment | View Attachment

**19. Authorized Representative**

Prefix: First Name: Roxie Middle Name: B.
Last Name: White Suffix:
Position/Title: Authorized Organizational Representative
Organization: North Carolia State University
Department: Research Adminstration Division: SPARCS
Street1: 2701 Sullivan Drive
Street2: Admin Servicess III; Box 7514
City: Raleigh County / Parish: Wake
State: NC: North Carolina Province:
Country: USA: UNITED STATES ZIP / Postal Code: 27595-7514
Phone Number: 919-515-2444 Fax Number: 919-515-7721
Email: sps@ncsu.edu

**Signature of Authorized Representative**: Roxie White

**Date Signed**: 04/14/2016

**20. Pre-application** Add Attachment | Delete Attachment | View Attachment

**21. Cover Letter Attachment** Add Attachment | Delete Attachment | View Attachment

# RESEARCH & RELATED BUDGET (TOTAL FED + NON-FED) - SECTION A, BUDGET PERIOD 1

OMB Number: 4040-0001
Expiration Date: 6/30/2016

*** ORGANIZATIONAL DUNS:** 0420921220000

*** Budget Type:** [X] Project [ ] Subaward/Consortium

**Enter name of Organization:** North Carolia State University

*** Start Date:** 09/01/2016 *** End Date:** 08/31/2017 *** Budget Period:** 1

**A. Senior/Key Person**

**1.**

| Prefix | * First Name | Middle Name | * Last Name | Suffix |
|---|---|---|---|---|
| Dr. | Maxwell | | Scott | |

* Project Role: PD/PI

| Base Salary ($) | Cal. Months | Acad. Months | Sum. Months | * Req. Salary ($) | * Fringe Ben. ($) | * Total (Sal & FB) (Fed + Non-Fed)($) | * Federal ($) | * Non- Federal ($) |
|---|---|---|---|---|---|---|---|---|
| 142,075.00 | | | 0.90 | 10,656.00 | 3,516.00 | 14,172.00 | 14,172.00 | 0.00 |

**2.**

| Prefix | * First Name | Middle Name | * Last Name | Suffix |
|---|---|---|---|---|
| | | | | |

* Project Role:

| Base Salary ($) | Cal. Months | Acad. Months | Sum. Months | * Req. Salary ($) | * Fringe Ben. ($) | * Total (Sal & FB) (Fed + Non-Fed)($) | * Federal ($) | * Non- Federal ($) |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

**3.**

| Prefix | * First Name | Middle Name | * Last Name | Suffix |
|---|---|---|---|---|
| | | | | |

* Project Role:

| Base Salary ($) | Cal. Months | Acad. Months | Sum. Months | * Req. Salary ($) | * Fringe Ben. ($) | * Total (Sal & FB) (Fed + Non-Fed)($) | * Federal ($) | * Non- Federal ($) |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

**4.**

| Prefix | * First Name | Middle Name | * Last Name | Suffix |
|---|---|---|---|---|
| | | | | |

* Project Role:

| Base Salary ($) | Cal. Months | Acad. Months | Sum. Months | * Req. Salary ($) | * Fringe Ben. ($) | * Total (Sal & FB) (Fed + Non-Fed)($) | * Federal ($) | * Non- Federal ($) |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

# RESEARCH & RELATED BUDGET (TOTAL FED + NON-FED) - SECTION A, BUDGET PERIOD 1

*** ORGANIZATIONAL DUNS:** 0420921220000

*** Budget Type:** [X] Project [ ] Subaward/Consortium

**Enter name of Organization:** North Carolia State University

*** Start Date:** 09/01/2016 *** End Date:** 08/31/2017 *** Budget Period:** 1

**A. Senior/Key Person (continued)**

**5.** Prefix | * First Name | Middle Name | * Last Name | Suffix

* Project Role

| Base Salary ($) | Cal. Months | Acad. Months | Sum. Months | * Req. Salary ($) | * Fringe Ben. ($) | * Total (Sal & FB) (Fed + Non-Fed)($) | * Federal ($) | * Non- Federal ($) |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

**6.** Prefix | * First Name | Middle Name | * Last Name | Suffix

* Project Role

| Base Salary ($) | Cal. Months | Acad. Months | Sum. Months | * Req. Salary ($) | * Fringe Ben. ($) | * Total (Sal & FB) (Fed + Non-Fed)($) | * Federal ($) | * Non- Federal ($) |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

**7.** Prefix | * First Name | Middle Name | * Last Name | Suffix

* Project Role

| Base Salary ($) | Cal. Months | Acad. Months | Sum. Months | * Req. Salary ($) | * Fringe Ben. ($) | * Total (Sal & FB) (Fed + Non-Fed)($) | * Federal ($) | * Non- Federal ($) |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

**8.** Prefix | * First Name | Middle Name | * Last Name | Suffix

* Project Role

| Base Salary ($) | Cal. Months | Acad. Months | Sum. Months | * Req. Salary ($) | * Fringe Ben. ($) | * Total (Sal & FB) (Fed + Non-Fed)($) | * Federal ($) | * Non- Federal ($) |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

| | | | |
|---|---|---|---|
| **9. Total Funds requested for all Senior Key Persons in the attached file** | | | |
| **Total Senior/Key Person** | 14,172.00 | 14,172.00 | 0.00 |

*** Additional Senior Key Persons:** Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED Budget {A-B} (Total Fed + Non-Fed)

## RESEARCH & RELATED BUDGET (TOTAL FED + NON-FED) - SECTION B, BUDGET PERIOD 1

*** ORGANIZATIONAL DUNS:** 0420921220000

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

**Enter name of Organization:** North Carolia State University

*** Start Date:** 09/01/2016 *** End Date:** 08/31/2017 *** Budget Period:** 1

**B. Other Personnel**

| * Number of Personnel | * Project Role | Cal. Months | Acad. Months | Sum. Months | * Req. Salary ($) | * Fringe Ben. ($) | * Total (Sal & FB) (Fed + Non-Fed) ($) | * Federal ($) | * Non- Federal ($) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Post Doctoral Associates | 12.00 | | | 42,840.00 | 8,140.00 | 50,980.00 | 50,980.00 | 0.00 |
| | Graduate Students | | | | | | | | |
| | Undergraduate Students | | | | | | | | |
| | Secretarial/Clerical | | | | | | | | |
| 1 | Researcher | 1.20 | | | 6,356.00 | 2,098.00 | 8,454.00 | 8,454.00 | 0.00 |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| 2 | **Total Number Other Personnel** | | | | **Total Other Personnel** | | 59,434.00 | 59,434.00 | 0.00 |
| | | | | | **Total Salary, Wages and Fringe Benefits (A + B)** | | 73,606.00 | 73,606.00 | 0.00 |

# RESEARCH & RELATED BUDGET (TOTAL FED + NON-FED) - SECTION C, D, & E, BUDGET PERIOD 1

*** ORGANIZATIONAL DUNS:** 0420921220000

*** Budget Type:** [X] Project [ ] Subaward/Consortium

**Enter name of Organization:** North Carolia State University

*** Start Date:** 09/01/2016 *** End Date:** 08/31/2017 *** Budget Period:** 1

**C. Equipment Description**

**List items and dollar amount for each item exceeding $5,000**

| | * Equipment item | * Federal ($) | * Non-Federal ($) | * Total (Fed + Non-Fed) ($) |
|---|---|---|---|---|
| 1. | | | | |
| 2. | | | | |
| 3. | | | | |
| 4. | | | | |
| 5. | | | | |
| 6. | | | | |
| 7. | | | | |
| 8. | | | | |
| 9. | | | | |
| 10. | | | | |
| 11. | Total funds requested for all equipment listed in the attached file | | | |
| | Total Equipment | | | |

*** Additional Equipment:** Add Attachment | Delete Attachment | View Attachment

**D. Travel**

| | | * Federal ($) | * Non-Federal ($) | * Total (Fed + Non-Fed) ($) |
|---|---|---|---|---|
| 1. | Domestic Travel Costs ( Incl. Canada, Mexico and U.S. Possessions) | 2,000.00 | 0.00 | 2,000.00 |
| 2. | Foreign Travel Costs | 11,000.00 | 0.00 | 11,000.00 |
| | Total Travel Costs | 13,000.00 | 0.00 | 13,000.00 |

**E. Participant/Trainee Support Costs**

| | | * Federal ($) | * Non-Federal ($) | * Total (Fed + Non-Fed) ($) |
|---|---|---|---|---|
| 1. | Tuition/Fees/Health Insurance | | | |
| 2. | Stipends | | | |
| 3. | Travel | | | |
| 4. | Subsistence | | | |
| 5. | Other | | | |
| | Number of Participants/Trainees Total Participant/Trainee Support Costs | | | |

# RESEARCH & RELATED BUDGET (TOTAL FED + NON-FED) - SECTION F-G, BUDGET PERIOD 1

*** ORGANIZATIONAL DUNS:** 0420921220000

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

**Enter name of Organization:** North Carolia State University

*** Start Date:** 09/01/2016 *** End Date:** 08/31/2017 *** Budget Period:** 1

**F. Other Direct Costs**

| | | * Federal ($) | * Non-Federal ($) | * Total (Fed + Non-Fed) ($) |
|---|---|---|---|---|
| 1. | Materials and Supplies | 22,000.00 | 0.00 | 22,000.00 |
| 2. | Publication Costs | 2,000.00 | 0.00 | 2,000.00 |
| 3. | Consultant Services | | | |
| 4. | ADP/Computer Services | | | |
| 5. | Subawards/Consortium/Contractual Costs | | | |
| 6. | Equipment or Facility Rental/User Fees | | | |
| 7. | Alterations and Renovations | | | |
| 8. | Repairs and Maintenance/Shipping | 1,000.00 | 0.00 | 1,000.00 |
| 9. | Off Campus Lab Analysis | 1,000.00 | 0.00 | 1,000.00 |
| 10. | On Campus Lab Analysis | 1,000.00 | 0.00 | 1,000.00 |
| | **Total Other Direct Costs** | 27,000.00 | 0.00 | 27,000.00 |

**G. Direct Costs**

| | * Federal ($) | * Non-Federal ($) | * Total (Fed + Non-Fed) ($) |
|---|---|---|---|
| **Total Direct Costs (A thru F)** | 113,606.00 | 0.00 | 113,606.00 |

# RESEARCH & RELATED BUDGET (TOTAL FED + NON-FED) - SECTION H-K, BUDGET PERIOD 1

*** ORGANIZATIONAL DUNS:** 0420921220000

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

**Enter name of Organization:** North Carolia State University

*** Start Date:** 09/01/2016 *** End Date:** 08/31/2017 *** Budget Period:** 1

**H. Indirect Costs**

| | * Indirect Cost Type | Indirect Cost Rate (%) | Indirect Cost Base ($) | * Federal ($) | * Non-Federal ($) | * Total (Fed + Non-Fed) ($) |
|---|---|---|---|---|---|---|
| 1. | Total Federal Funds Awarded | 30.00 | 162,294.00 | 48,688.00 | 0.00 | 48,688.00 |
| 2. | | | | | | |
| 3. | | | | | | |
| 4. | | | | | | |
| | **Total Indirect Costs** | | | 48,688.00 | 0.00 | 48,688.00 |

**Cognizant Agency** DHHS, Steven Zuraf, 202-401-2808 (Agency Name, POC Name, and Phone Number)

**I. Total Direct and Indirect Costs**

| | * Federal ($) | * Non-Federal ($) | * Total (Fed + Non-Fed) ($) |
|---|---|---|---|
| **Total Direct and Indirect Costs (G + H)** | 162,294.00 | 0.00 | 162,294.00 |

**J. Fee**

| Federal ($) |
|---|
| |

**K. * Budget Justification** BudgetJustification.pdf Add Attachment Delete Attachment View Attachment (Only attach one file.)

## RESEARCH & RELATED BUDGET (TOTAL FED + NON-FED) - Cumulative Budget

| | Total Federal ($) | Total Non-Federal ($) | Totals ($) |
|---|---|---|---|
| **Section A, Senior/Key Person** | 42,516.00 | 0.00 | 42,516.00 |
| **Section B, Other Personnel** | 181,380.00 | 0.00 | 181,380.00 |
| Total Number Other Personnel | | | 6 |
| **Total Salary, Wages and Fringe Benefits (A + B)** | 223,896.00 | 0.00 | 223,896.00 |
| **Section C, Equipment** | | | |
| **Section D, Travel** | 43,000.00 | 0.00 | 43,000.00 |
| **1.** Domestic | 6,000.00 | 0.00 | 6,000.00 |
| **2.** Foreign | 37,000.00 | 0.00 | 37,000.00 |
| **Section E, Participant/Trainee Support Costs** | | | |
| **1.** Tuition/Fees/Health Insurance | | | |
| **2.** Stipends | | | |
| **3.** Travel | | | |
| **4.** Subsistence | | | |
| **5.** Other | | | |
| **6.** Number of Participants/Trainees | | | |
| **Section F, Other Direct Costs** | 83,000.00 | 0.00 | 83,000.00 |
| **1.** Materials and Supplies | 66,000.00 | 0.00 | 66,000.00 |
| **2.** Publication Costs | 8,000.00 | 0.00 | 8,000.00 |
| **3.** Consultant Services | | | |
| **4.** ADP/Computer Services | | | |
| **5.** Subawards/Consortium/Contractual Costs | | | |
| **6.** Equipment or Facility Rental/User Fees | | | |
| **7.** Alterations and Renovations | | | |
| **8.** Other 1 | 3,000.00 | 0.00 | 3,000.00 |
| **9.** Other 2 | 3,000.00 | 0.00 | 3,000.00 |
| **10.** Other 3 | 3,000.00 | 0.00 | 3,000.00 |
| **Section G, Direct Costs (A thru F)** | 349,896.00 | 0.00 | 349,896.00 |
| **Section H, Indirect Costs** | 149,955.00 | 0.00 | 149,955.00 |
| **Section I, Total Direct and Indirect Costs (G + H)** | 499,851.00 | 0.00 | 499,851.00 |
| **Section J, Fee** | | | |

OMB Number: 4040-0001
Expiration Date: 6/30/2016

# RESEARCH & RELATED PERSONAL DATA
## Project Director/Principal Investigator and Co-Project Director(s)/Co-Principal Investigator(s)

The Federal Government has a continuing commitment to monitor the operation of its review and award processes to identify and address any inequities based on gender, race, ethnicity, or disability of its proposed PDs/PIs and co-PDs/PIs. To gather information needed for this important task, the applicant should submit the requested information for each identified PD/PI and co-PDs/PIs with each proposal. Submission of the requested information is voluntary and is not a precondition of award. However, information not submitted will seriously undermine the statistical validity, and therefore the usefulness, of information received from others. Any individual not wishing to submit some or all the information should check the box provided for this purpose. Upon receipt of the application, this form will be separated from the application. This form will not be duplicated, and it will not be a part of the review process. Data will be confidential.

**Project Director/Principal Investigator**

| **Prefix:** | *** First Name:** | **Middle Name:** |
|---|---|---|
| Dr. | Maxwell | |

| *** Last Name:** | **Suffix:** |
|---|---|
| Scott | |

**Gender:**

**Race (check all that apply):**

- [ ] American Indian or Alaska Native
- [ ] Asian
- [ ] Black or African American
- [ ] Native Hawaiian or Other Pacific Islander
- [ ] White
- [x] Do Not Wish to Provide

**Ethnicity:**

**Disability Status (check all that apply):**

- [ ] Hearing
- [ ] Visual
- [ ] Mobility/Orthopedic Impairment
- [ ] Other
- [ ] None
- [x] Do Not Wish to Provide

**Citizenship:**

Do Not Wish to Provide

# RESEARCH & RELATED Other Project Information

OMB Number: 4040-0001
Expiration Date: 6/30/2016

1. Are Human Subjects Involved? ☐ Yes ☒ No

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ☐ Yes ☐ No

If yes, check appropriate exemption number. ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6

If no, is the IRB review Pending? ☐ Yes ☐ No

IRB Approval Date:

Human Subject Assurance Number:

2. Are Vertebrate Animals Used? ☐ Yes ☒ No

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ☐ Yes ☐ No

IACUC Approval Date:

Animal Welfare Assurance Number:

3. Is proprietary/privileged information included in the application? ☐ Yes ☒ No

4.a. Does this Project Have an Actual or Potential Impact - positive or negative - on the environment? ☐ Yes ☒ No

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ☐ Yes ☐ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place? ☐ Yes ☒ No

5.a. If yes, please explain:

6. Does this project involve activities outside of the United States or partnerships with international collaborators? ☒ Yes ☐ No

6.a. If yes, identify countries: Panama

6.b. Optional Explanation:

**7. Project Summary/Abstract** project_summary.pdf Add Attachment | Delete Attachment | View Attachment

**8. Project Narrative** Project Narrative.pdf Add Attachment | Delete Attachment | View Attachment

**9. Bibliography & References Cited** Bibliography and References Cited.pdf Add Attachment | Delete Attachment | View Attachment

**10. Facilities & Other Resources** FacilitiesOtherResources.pdf Add Attachment | Delete Attachment | View Attachment

**11. Equipment** Equipment.pdf Add Attachment | Delete Attachment | View Attachment

**12. Other Attachments** Add Attachments | Delete Attachments | View Attachments ☒

OMB Number: 4040-0001
Expiration Date: 6/30/2016

# RESEARCH & RELATED Senior/Key Person Profile (Expanded)

## PROFILE - Project Director/Principal Investigator

Prefix: Dr. * First Name: Maxwell Middle Name:
* Last Name: Scott Suffix:
Position/Title: Associate Professor Department: Entomology
Organization Name: North Carolina State University Division: CALS
* Street1: Thamos Hall 1542B, Box 7613
Street2: NCSU Campus
* City: Raleigh County/ Parish: Wake
* State: NC: North Carolina Province:
* Country: USA: UNITED STATES * Zip / Postal Code: 27595-7613
* Phone Number: 919-515-0275 Fax Number: 919-515-3355
* E-Mail: max_scott@ncsu.edu

Credential, e.g., agency login:

*** Project Role:** PD/PI **Other Project Role Category:**

**Degree Type:** Ph.D.
**Degree Year:** 1986

| | | | | |
|---|---|---|---|---|
| ***Attach Biographical Sketch** | biographical_sketch_Scott.pdf | Add Attachment | Delete Attachment | View Attachment |
| **Attach Current & Pending Support** | current_pending_Scott.pdf | Add Attachment | Delete Attachment | View Attachment |

## PROFILE - Senior/Key Person 1

Prefix: * First Name: Middle Name:
* Last Name: Suffix:
Position/Title: Department:
Organization Name: Division:
* Street1:
Street2:
* City: County/ Parish:
* State: Province:
* Country: USA: UNITED STATES * Zip / Postal Code:
* Phone Number: Fax Number:
* E-Mail:

Credential, e.g., agency login:

*** Project Role:** **Other Project Role Category:**

**Degree Type:**
**Degree Year:**

| | | | | |
|---|---|---|---|---|
| **Attach Biographical Sketch** | | Add Attachment | Delete Attachment | View Attachment |
| **Attach Current & Pending Support** | | Add Attachment | Delete Attachment | View Attachment |

Delete Entry Next Person

To ensure proper performance of this form; after adding 20 additional Senior/ Key Persons; please save your application, close the Adobe Reader, and reopen it.

OMB Number: 4040-0010
Expiration Date: 9/30/2016

# Project/Performance Site Location(s)

**Project/Performance Site Primary Location**

☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: North Carolina

DUNS Number: 0420921220000

* Street1: Thomas Hall 1542B, Box 7613

Street2: NCSU Campus

* City: Raleigh

County: Wake

* State: NC: North Carolina

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: 27695-7613

* Project/ Performance Site Congressional District: NC-004

**Project/Performance Site Location** 1

☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: USDA-ARS Screwworm Research Unit

DUNS Number:

* Street1: Instalaciones del Antiguo Ingenio de Felipillo, COPEG

Street2: Planta de Pacora

* City: Pacora

County:

* State:

Province:

* Country: PAN: PANAMA

* ZIP / Postal Code:

* Project/ Performance Site Congressional District:

**Additional Location(s)**

Add Attachment | Delete Attachment | View Attachment

# Supplemental Information Form

OMB Number: 0524-0039
Expiration Date: 10/31/2018

Please complete this form in conjunction with the SF-424 Application for Federal Financial Assistance.

**1. Funding Opportunity**

Funding Opportunity Name

Biotechnology Risk Assessment Grants Program

Funding Opportunity Number

USDA-NIFA-BRAP-005435

**2. Program to which you are applying**

Program Code Name

Biotechnology Risk Assessment Research

Program Code

HX

**3. Type of Applicant**

H: Public/State Controlled Institution of Higher Education

**4. Additional Applicant Types**

1862 Land-Grant University

**5. Supplemental Applicant Types** *(Check all that apply)*

- [ ] Alaska Native-Serving Institution
- [x] Cooperative Extension Service
- [ ] Hispanic-Serving Institution
- [ ] Historically Black College or University (other than 1890)
- [ ] Minority-Serving Institution
- [ ] Native Hawaiian-Serving Institution
- [ ] Public Nonprofit Junior or Community College
- [ ] Public Secondary School
- [ ] School of Forestry
- [x] State Agricultural Experiment Station
- [ ] Tribal College (other than 1994)
- [ ] Veterinary School or College

**6. ASAP Recipient Information**

Does the legal applicant have an active Automated Standard Application for Payments (ASAP) Recipient Identification Number for NIFA awards?

- [x] Yes
- [ ] No

What is the ASAP Recipient ID (which corresponds with this applications's DUNS and EIN) to be used in the event of an award?

3769001

**7. Key Words**

sterile insect technique, screwworm, spotted wing drosophila, gene drive

**8. Conflict of Interest List**

conflict_of_interest_Scott.pdf

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# PROJECT SUMMARY

**Instructions:**
The summary is limited to 250 words. The names and affiliated organizations of all Project Directors/Principal Investigators (PD/PI) should be listed in addition to the title of the project. The summary should be a self-contained, specific description of the activity to be undertaken and should focus on: overall project goal(s) and supporting objectives; plans to accomplish project goal(s); and relevance of the project to the goals of the program. The importance of a concise, informative Project Summary cannot be overemphasized.

**Title:** Development And Evaluation Of Safeguards For Conditional Suppressive Gene Drives For Spotted Wing Drosophila And The New World Screwworm


| | |
|---|---|
| **PD:** PD/PI Name (Scott, Maxwell, J) | **Institution:** North Carolina State University |
| **CO-PD:** PD/PI 2 Name (Last, First, MI) | **Institution:** |
| **CO-PD:** PD/PI 3 Name (Last, First, MI) | **Institution:** |
| **CO-PD:** PD/PI 4 Name (Last, First, MI) | **Institution:** |
| **CO-PD:** PD/PI 5 Name (Last, First, MI) | **Institution:** |
| **CO-PD:** PD/PI 6 Name (Last, First, MI) | **Institution:** |
| **CO-PD:** PD/PI 7 Name (Last, First, MI) | **Institution:** |



CRISPR/Cas9-mediated gene drives offer a potentially very efficient genetic mechanism for suppressing populations of insect pests. However, they pose a challenge for regulators as, by their very nature, they are designed to not be contained. The overall goal is to engineer conditional contained gene drives and evaluate efficacy and safeguards in spotted wing Drosophila (D. suzukii) and the New World screwworm (Cochliomyia hominivorax). These dipteran species were selected as they are major agricultural pests and we have extensive experience engineering conditional expression systems in these species. Flies will be contained using physical barriers and molecular strategies. For the latter, Cas9 expression will be controlled using the reverse tetracycline transactivator (rtTA) that is only active if tetracycline is added to the diet. Additionally, the U6 promoter driven guide RNA (U6-gRNA) gene will be on a separate chromosome to the targeted gene. Genes required for female development or reproduction will be targeted by flanking the Cas9/rtTA gene cassette with left and right homology arms. We will evaluate the potential for suppression of a population carrying the U6-gRNA transgene and the efficacy of stopping a drive by releasing flies with a recoded target gene. This research will provide information for regulators on the use of conditional control of Cas9 expression and split systems for containment of population suppressive gene drives in the laboratory. Replacing rtTA with tTA would provide a conditional CRISPR/Cas9 gene drive that could be suppressed in containment but would drive in the field.



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## PROJECT NARRATIVE: INTRODUCTION

**Overall hypothesis or goal:** To engineer conditional gene drives and evaluate safeguards in spotted wing *Drosophila* (*D. suzukii*) and the New World screwworm (*Cochliomyia hominivorax*). These dipteran species were selected as they are major agricultural pests and we have extensive experience engineering conditional expression systems in these species.

## BACKGROUND

In his seminal paper in 2003, Austin Burt described how gene drive systems based on a homing endonuclease gene (HEG) could be an efficient means for population suppression of pest insects (1). Homing endonucleases typically have long, very specific recognition sequences (usually 20-30 bp) (2). The homing endonuclease cuts both strands of DNA within the recognition sequence and is often copied across as a consequence of homology-directed DNA repair (HDR). It is the homing property that leads to non-Mendelian inheritance of the HEG and is the basis for the gene drive mechanism (1). To be useful for population suppression, the HEG recognition sequence should be within a gene essential for viability of the pest insect. Modeling showed that suppression is particularly efficient if the HEG is targeted to a gene essential for females but not males or a gene required for germ-cell development or reproduction in one sex (1, 3). However, implementation of Burt's ideas was hampered by the high specificity of HEGs as few, if any, essential genes in pest insects contained a recognition sequence. Further, it has proved difficult to modify HEG specificity to recognize a site within a specific gene (4). In contrast, it is relatively easy to target the CRISPR/Cas9 nuclease to a specific gene of interest in insect genomes (5, 6). The gene drive mechanism would be the same as for HEGs and thus Burt's ideas of optimal target genes also apply to Cas9-mediated drives (7). Thus, we will initially describe the relevant background on HEG and Cas9-mediated gene drives and then briefly introduce the two pest species before describing our results to date on engineering *D. suzukii* and *C. hominivorax* that are relevant to this proposal.

### Gene drives based on HEGs

To illustrate a gene drive system based on a HEG, consider the situation of a release of a few GM insects that carry a dominant fluorescent protein marker gene (Fig. 1A). All of the offspring from mating between GM insects and wild type will be fluorescent. Most likely these insects will mate with wild type insects as they are in vast excess. From these matings, only half of the offspring will show fluorescence as a consequence of normal Mendelian inheritance. Now consider a release of a few insects carrying the fluorescent protein marker gene linked to a HEG that is expressed in the germline (Fig. 1B). The HEG and marker gene are both within the recognition sequence for the HEG. As for the first release, all of the offspring from matings with wild type insects will be fluorescent. In the germline of the first generation, expression of the HEG will cause cleavage of its recognition site in the chromosome that does not contain the HEG (Fig. 1C). Insertion of the HEG and marker gene will occur as a consequence of HDR. If this process is 100% efficient, all of the gametes will contain a chromosome with the HEG and linked marker gene. Thus, when the first generation insects mate with wild type insects, all of the offspring will be fluorescent. Further generations will lead to the marker gene being driven into the population. Assuming the HEG and marker gene have no fitness effects, a 1% release can lead to 99% of the population carrying the marker gene after just 9 generations (1, 3).

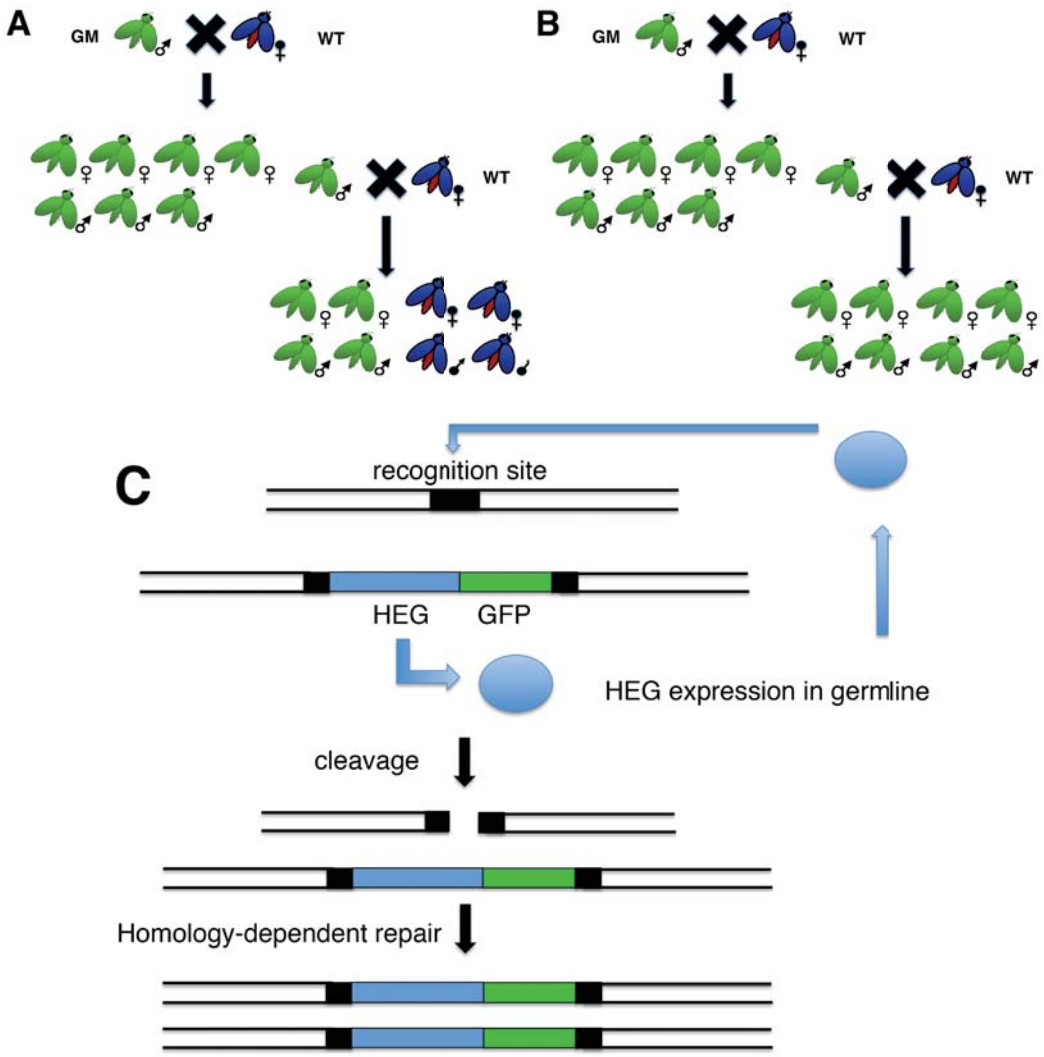



**Figure 1. HEG-mediated gene drive of a fluorescent protein marker gene.** Release of transgenic males carrying a GFP marker gene without (A) or with (B) a gene drive mechanism. The GFP gene is physically linked to a HEG. Both HEG and GFP are copied to the wild type chromosome as a consequence of HDR of the HEG-mediated DSB in the recognition site.

For population suppression, the HEG would be targeted to an essential gene (1). A greater load on the population could be achieved if the HEG targeted a gene essential for females but not males (1). Further modeling shows that if homing is restricted to the male germline and the targeted gene is essential for female viability or fertility, then the load on the population is reduced compared to expression of the HEG in the germline of both sexes (3). HEG-mediated gene knockouts of genes essential for reproduction would also be effective, if they have no influence on mating success (1). Deredec *et al.*, suggest targeting recessive genes essential for female or male fertility.

In the example shown in Figure 1, HDR of the HEG-induced double stranded break is clearly critical for homing. Most cells have an alternative pathway for repairing double-stranded breaks (DSBs) known as non homologous end joining (NHEJ) (8). With NHEJ, the broken ends are ligated together without regard for homology. Errors during the NHEJ repair process can lead to products that contain small deletions or insertions. In many cell types NHEJ repair events can

outnumber HDR events. A NHEJ-mediated mutation of the HEG recognition site would suppress homing (1). If the HEG recognition site is within a critical region of a gene essential for viability or reproduction, then any mutation due to an error in the NHEJ-mediated repair would be as deleterious as an insertion of the HEG due to HDR. Nevertheless, to achieve a high homing frequency it would be advantageous to express the HEG in cells that preferentially use HDR to repair DSBs than NHEJ.

Proof-of-principle gene drive experiments were performed with the I-SceI HEG in the malaria vector *Anopheles gambiae* and in *Drosophila melanogaster*. As a first step, transgenic lines were made that carried a single I-SceI recognition site by standard germ-line transformation. In *An. gambiae*, I-SceI expression was driven by the *An. gambiae* testes-specific β2 tubulin promoter (9). As the I-SceI site was within a *gfp* gene, digestion by I-SceI and homing or NHEJ repair error would produce non-fluorescent mosquitoes. However, molecular analysis found that in 97% of the mosquitoes, loss of *gfp* expression was due to homing. The authors went on to show that the I-SceI gene spread through caged populations of *An. gambiae* largely as predicted by deterministic and stochastic models. In contrast to the ease with which efficient homing was achieved in *An. gambiae*, developing an effective homing system in *D. melanogaster* was more difficult (10, 11). This appears to reflect a fundamental difference in spermatogenesis, which proceeds via an achiasmate mechanism in *Drosophila* and an absence of recombination in the male germline (11). Consequently HDR appears to be restricted to the mitotic spermatogonial stage. Thus, to obtain efficient homing required identification of the best combination of gene promoter and 3'UTR for expression in spermatogonia. The *Drosophila β2 tubulin* promoter was mostly active after the spermatogonial stage in maturing spermatocytes and consequently little homing (1%) was observed with the I-SceI gene (11). High HEG activity and efficient homing (61%) was achieved with the testes-specific *Rcd-1r* gene promoter and βTub56D 3'UTR. High HEG activity and homing (42-48%) was also found with the *vasa* promoter and either the *vasa* 3' UTR or βTub56D 3'UTR. The *vasa* promoter is active in the germline (12). Interestingly, homing in *Drosophila* was much less efficient at 18 °C than at 25 °C (11).

**Gene Drives based on CRISPR/Cas9**

In 2013 and 2014 there were several reports describing efficient site-directed mutagenesis in *D. melanogaster* using CRISPR/Cas9 (5, 13-15). Cas9 is an RNA-guided nuclease that was first identified as part of a viral-defense system in bacteria (16-18). In *Streptococcus pyogenes*, the tracrRNA is required for Cas9 activity and the crRNA guides Cas9 cleavage through specific base-pairing with target DNA (16, 18). The functions of the tracrRNA and crRNA can be replaced with a single guide RNA (gRNA) in *D. melanogaster* and other species (19-21). The gRNA contains 20 nt of homology to the targeted gene. A protospacer adjacent motif (PAM), which is typically NGG, must follow the 20 bp spacer sequence in the target gene. Mutations, which are typically small deletions and insertions, are generated as a consequence of errors in the repair process by the cells NHEJ machinery (19, 21). The efficiency of Cas9-mediated gene editing in *D. melanogaster* was improved through the development of transgenic lines that expressed Cas9 in the germline using either the *nanos* or *vasa* gene promoters (15, 22). The system was further improved through use of *U6* gene promoters to drive expression of the gRNA (14). The *U6:3* gene promoter was reported to be particularly efficient (23).

CRISPR/Cas9-mediated gene drives have been demonstrated in *D. melanogaster* (24), *An stephensi* (25) and *An. gambiae* (26). For the *D. melanogaster* experiments, Cas9 expression was

driven by the *vasa* gene promoter and *vasa* 3' UTR (24). As noted above, the combination of *vasa* promoter and 3'UTR was effective for homing of the I-SceI HEG in *D. melanogaster* (11). The *U6:3* promoter was used for expression of a gRNA directed against the *yellow* gene. The specific gRNA had been previously shown to promote efficient site-directed mutagenesis at the *yellow* locus (13). Males and females were obtained that contained the *vasa-Cas9* gene inserted at the specific site within the *yellow* gene. Drive was remarkably efficient, with 95-100% of the offspring of these flies carrying the *yellow* gene with the *vasa-cas9* insertion. Thus homing was significantly more efficient than observed with I-SceI. This could reflect differences in experimental design or differences in the local chromatin environment that may influence Cas9-induced DSB and/or HDR. With regard to the latter, it has been reported that Cas9 site-directed mutagenesis of the *white* gene in *D. melanogaster* is less efficient than the *yellow* gene (13). It was suggested that this may be due to differences in the local chromatin environment. Thus it remains to be shown if Cas9-mediated gene drives for other genes in *Drosophila* will be as efficient as reported for *yellow*.

In the *An. stephensi* gene drive experiments, expression of Cas9 was controlled with the promoter and 3'UTR/flanking from the *An. stephensi vasa* gene (25). A promoter from the *An. stephensi U6A* gene was used to express the gRNA, which was targeted against the *kynurinine hydroxylase* gene. This provided an easily recognizable phenotype as loss-of-function mutations in *kynurinine hydroxylase* result in a white eye phenotype. In addition, the authors included the dominant *3xP3-DsRed* fluorescent protein marker, which is expressed in eyes and neural tissue (optic nerve, cerebral ganglia, ventral nerve ganglia and anal papillae) and can be readily detected in larvae. The fluorescent marker was used to distinguish HDR and NHEJ events as the latter would produce white eye mosquitoes but without red fluorescence. Gene drive was very efficient (98%) in the initial generations in both males and female germline. In $G_4$, NHEJ events appear to occur at a higher frequency than HDR events in crosses of a wild type male with transgenic female. HDR events were much more common with a transgenic male crossed to wild type female. The authors suggest this is due to maternal expression of Cas9 loading the egg with active Cas9 and gRNA. After fertilization, it was proposed that Cas9 induces a DSB in the paternal *kynurinine hydroxylase* before the paternal and maternal alleles are in close physical proximity. Consequently NHEJ repair events predominate. To recover HDR events, it was suggested that Cas9 expression be limited to the male germline, such as by using the promoter from the β2-tubulin promoter. As discussed, above male expression of I-SceI led to efficient homing in *An. gambiae* (9).

The *An. gambiae* gene drive experiments had a similar experimental design. Cas9 expression was driven by the *An. gambiae vasa2* promoter and a *An. gambiae U6* promoter was used to express a gRNA (26). The homing gene cassette included the dominant *3xP3-DsRed* marker gene. The gRNAs were targeted against three recessive genes thought to be essential for female fertility. As noted above, Burt had previously suggested that recessive genes essential for fertility would be good targets for a population suppression gene drive (1). Two of the genes chosen were orthologs of the *Drosophila* genes *yellow-g* and *nudel*, which are known to be required for fertility but expressed in the somatic follicle cells. Thus expression of Cas9 in germline cells would not disrupt these genes in the somatic cells in which they are expressed. HDR could occur in the germline and knockout both copies of the targeted gene but the egg would develop normally as the somatic cells would remain heterozygous and express the gene. Targeted disruption confirmed that the three recessive genes were essential for female fertility. High levels of homing were observed for all three loci in males and females (87-98%). A

decrease in homing performance was not detected over 4 generations. However, the fertility of heterozygous females was much less than wild type at each of the three loci (0-9%). The authors suggest this is due to somatic expression of Cas9 in heterozygous females. Modeling suggested that the low fertility would prevent the *vasa-Cas9* gene from driving through a population with the possible exception of the targeted gene with the mildest reduction in fertility (9% of wild type). Cage experiments showed a moderate level of gene drive at this gene, with the disrupted gene increasing from 50% to 75% of the population over 4 generations. The authors note that such a strong reproductive load will select for resistance mutations such a small deletions generated by NHEJ repair of the Cas9-induced DSB. It was suggested that fertility would be improved by using a different promoter that is more restricted to the germline, such as *nanos* in *D. melanogaster* (23). Restricting Cas9 expression to the male germline would also appear to be advantageous.

**Biosafety of CRISPR/Cas9 gene drives**

In their 2014 article, Esvelt and colleagues outlined a variety of uses for CRISPR/Cas9 gene drives in human health, agriculture and the environment (7). They described how natural variation or errors induced by the NHEJ repair pathway could inhibit a drive. They suggest the best approach to avoid development of resistance is to target multiple sites in a single gene. Specifically, targeting multiple sites within the 3' ends of essential genes as any repair event that deletes target sites would be too deleterious to compete with the drive. Importantly, the authors foresaw that the potential efficiency of CRISPR/Cas9 gene drive systems posed a significant challenge for containment in the laboratory. They suggested parallel development of a "reversal" gene drive that would restore the original locus and would be recoded such that it would be immune to the original drive as the gRNAs would not anneal with the recoded sequence.

Although Esvelt *et al.* (7) had highlighted the need for safeguards, the ease and efficiency of the Cas9-mediated gene drive in *D. melanogaster* (24) was a surprise to many of our colleagues and has led to wide discussion of the risks of gene drives. A study is underway by the National Academies of Sciences, Engineering and Medicine on the science, oversight, governance and ethics of gene drive research (nas-sites.org/gene-drives/). The PD recently participated in a workshop held at NCSU on gene drives and developing a framework for research and governance (Raleigh, NC, February 24-26, 2016, https://research.ncsu.edu/ges/researchers/gene-drives-grant/). Recently, scientists working on Cas9 published an article in *Science* on "Safeguarding gene drive experiments in the laboratory" (27). They recommended:
1) Developing "split gene drive systems" in which only the gRNA or Cas9 is flanked by the homology arms of the targeted gene. For example, target *yellow* in *D. melanogaster* by flanking the *U6:3*-gRNA gene only with homology arms from *yellow* with *vasa-Cas9* integrated onto a separate chromosome. The *U6:3*-gRNA will only drive if the chromosome with the Cas9 gene is also inherited. With a limited release (e.g. 0.1%) the drive would be predicted to quickly stop and would be lost from the population if there are any fitness costs associated with the transgene. While a split drive system would significantly reduce the risks, a recombination event that places the Cas9 gene next to the gRNA would create a functional Cas9+gRNA drive system. An alterative molecular confinement strategy would be to target a gene that is absent from wild type insects (e.g. the *3xP3-DsRed* marker gene).

2) Perform gene drive experiments outside the ecological range of the organism (e.g. *Anopheles* mosquito in Boston). *D. suzukii* is well established in North Carolina and Panama is well within the ecological range of *C. hominivorax*. So this form of containment is not available to us.
3) Use a strain that cannot reproduce with wild organisms. Such strains have not been made for *C. hominivorax* or *D. suzukii*.
4) Physical barriers. In practice, this could be a higher level of containment than is currently recommended for transgenic strains of the species of interest. For example, *D. suzukii* transgenic strains can be maintained in arthropod containment level 1, the lowest level. Using additional containment recommended by Akbari *et al.* (27) such as air blast fans and higher precautions to prevent escape (e.g sealing possible escape routes) is part of arthropod containment level 2.
It was suggested that gene drive experiments in the laboratory employ at least two of the recommended types of containment (e.g. split drives and physical barriers).

At the recent gene drive workshop in Raleigh, we suggested an additional molecular containment strategy of controlling Cas9 expression using a conditional system (28). The tetracycline transactivator (tTA)/tetO conditional expression system (29) is currently used for suppression of female-lethal genes in engineered strains designed for genetic control programs (30, 31). An example of the design of a conditional Cas9 gene drive system is shown in Figure 2. Rather than tTA, this system uses reverse tTA (rtTA) as rtTA will only bind DNA in the presence of tetracycline or its derivative doxycycline (32). Thus gene drive will not occur outside containment because the diet in the field would lack tetracycline.

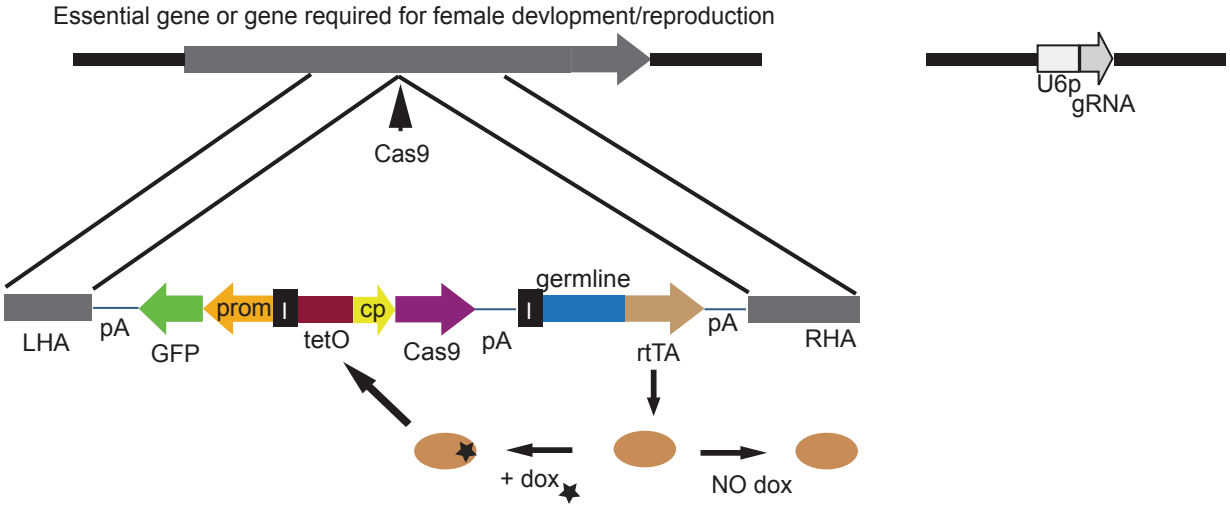



**Figure 2. Conditional "tet-ON" split Cas9-mediated gene drive.** Key: rtTA, reverse tetracycline transactivator; germline, germline promoter such as *vasa*; I, insulator; LHA and RHA are left and right homology arms complementary to the targeted gene.

In theory, releasing a few insects carrying a very efficient population suppression gene drive could be sufficient to suppress a pest population after many generations. In practice, growers would likely desire a faster response, which could be achieved by releasing more insects carrying the gene drive. For example, a ratio 1:10, modified:wild type insect would lead to pest suppression in 10-20 generations (28). If Cas9 expression was controlled with tTA, large numbers of insects could be reared on diet with tetracycline but the drive would be functional in their offspring as they would consume a diet lacking tetracycline. Therefore an additional advantage of using rtTA to develop a gene drive system is that it could easily be modified to a drive suitable for field application by simply replacing rtTA with tTA.

***D. suzukii*: an invasive pest of soft-skinned fruits**

*D. suzukii* larvae are capable of infesting a wide range of host fruit but appear to be most significant pests in stone fruits (peach, cherry, and plum) and berries (caneberries, blueberries, and strawberries) (33, 34). Unlike most other *Drosophila* species, female *D. suzukii* use their highly developed serrated ovipositor to pierce the skin of soft fruits and lay their eggs inside the fruit (35). *D. suzukii* has a short generation time and multiple generations per year (36). In contrast, the larger tephritid fruit flies (*Rhagoletis* sp.) native to North America and Europe have only one generation per year. *D. suzukii* is endemic in Asia but in 2008 the fly was found in California and Europe. Since then, *D. suzukii* has spread rapidly and is now found in temperate regions in North America and Europe (37). In the USA, any fruit that contain developing *D. suzukii* larvae can cause an entire shipment to be rejected. Growers are currently using broad spectrum insecticides to protect fruit from damage caused by *D. suzukii.* For example, growers in North Carolina are using more frequent insecticide applications to manage this invasive fruit fly but the effectiveness of these treatments is weather dependent. It is also anticipated that *D. suzukii* will develop resistance to some of the more commonly used insecticides (34). Therefore, non-chemical means for controlling *D. suzukii* are needed.

***C. hominivorax*, a devastating obligate parasite of livestock and the target of the first successful use of the sterile insect technique**

*C. hominivorax* is a serious pest of warm-blooded animals (38, 39). Females lay their eggs in open wounds or a natural orifice. The hatched larvae then feed on the animal's living tissue. Animals with severe screwworm infestations may die if untreated. However, most cases are less severe but they are economically important because the animal suffers weight loss and carcasses and hides are damaged. Screwworm populations are limited in their range by cold seasonal temperatures: the insect cannot survive freezing temperatures and cannot overwinter successfully under temperate conditions. The pre-eradication range was between 35 and -35 latitudes in the Western Hemisphere.

Edward Knipling realized that if large numbers of sterile males could repeatedly be released into wild populations, it would eventually eliminate population reproduction and lead to eradication (40, 41). This genetic control method is now generally referred to as the sterile insect technique or SIT. The program initiated by Knipling and his colleague Raymond Bushland began with the release of sterilized insects in Florida in the late 1950s. Subsequently, the SIT approach was used to eradicate screwworm from all of the USA. However, Texas farmers faced an ongoing threat of invasion of screwworm from Mexico. To alleviate this threat, SIT was used to eradicate the fly from Mexico in a joint program with the government of Mexico. Lastly, the program was extended to eradicate screwworm from all of Central America (42). To prevent re-infestation from South America, sterilized flies are currently being constantly released in a "buffer zone" in Southern Panama and along the border with Colombia. The screwworm mass rearing facility is in Pacora, Panama and is run by the U.S.-Panamanian Commission for the Eradication and Prevention of Screwworms (Comisión Panamá–Estados Unidos para la Erradicación y Prevención del Gusano Barrenador del Ganado or COPEG). Currently about 25 million flies are reared each week. SIT is more efficient if only sterile males are released as they only mate with fertile females in the field rather than the co-released sterile females (43). Consequently, we have developed male-only transgenic strains that carry a tetracycline-repressible female lethal gene (see below).

**SPECIFIC OBJECTIVES**

1) Create conditional female to male transformation and female sterile CRISPR/Cas9 split gene drives in *D. suzukii*
2) Create conditional female to male transformation CRISPR/Cas9 split gene drives in *C. hominivorax*
3) Evaluate the potential for population suppression in cage experiments under permissive and non-permissive conditions
4) Evaluate the potential for stopping a drive by releasing flies that are immune to Cas9

**RATIONALE AND SIGNIFICANCE**

CRISPR/Cas9-mediated gene drives offer a potentially very efficient genetic mechanism for controlling populations of insect pests. However, they pose a challenge for regulators as, by their very nature, they are designed to not be contained. We propose to evaluate conditional expression of Cas9 as a means of containment in the lab. In addition drive systems will be split for initial testing. Having established drives in the lab we can then test the potential for stopping a drive by releasing strains that have been recoded to be immune to Cas9 digestion.

This research will provide information for regulators on the use of conditional control of Cas9 expression and split systems for containment of population suppressive gene drives in the laboratory. In the long term, replacing rtTA with tTA would provide a conditional CRISPR/Cas9 gene drive that could be suppressed in containment but would drive in the field. This would allow mass rearing facilities to raise sufficient flies to achieve a relatively rapid population suppression in the targeted area. The systems could be more cost effective than alternative genetic control strategies such as SIT and release of insects with conditional dominant female lethal genes (fsRIDL) because many fewer flies would need to reared and released to achieve population suppression.

Screwworm remains a problem throughout most of tropical South America and on some Caribbean islands, notably Cuba and Jamaica. A more efficient SIT program with male-only release would likely be sufficient for eradication and/or suppression of island populations and west of the Andes. East of the Andes presents a considerable area with no appreciable geographic barriers and ideal habitat for screwworm. A one-time release of flies carrying an efficient gene drive system throughout the region could potentially be an effective means for population suppression.

We are not aware of accurate measurements of *D. suzukii* population sizes, but trapping data suggests populations can be high, particularly in the warmer months (34). It is possible that a male-only SIT or fsRIDL approach could be successful if attempted early in the growing season when populations appear to be relatively low and in conjunction with intensive application of conventional methods for controlling *D. suzukii*. However, an efficient gene drive system could be more effective and significantly lower cost as fewer flies would need to be released.

**Program Areas:** Management Practices to Minimize Environmental Risk of GE Organisms

**Priority Areas:** Evaluation of safeguards (e.g. reversal drives, immunization) for controlling the spread of gene drives during research to understand the effect of the desired genetic change on organisms and populations

**Magnitude of the issues and their relevance to stakeholders and to federal regulatory agencies**

It appears to be relatively straightforward to adapt CRISPR/Cas9 technology to a pest species of interest if techniques have been developed for delivering nucleic acid to the germline. For example, as transgenic technologies were previously developed for *D. suzukii* and *L. cuprina*, it was

relatively easy for my laboratory to use CRISPR/Cas9 for site-directed mutagenesis in these species (see below). For those pests that are amenable to CRISPR/Cas9 genome editing, the expectation is that gene drive systems will be developed over the next few years. These could provide cost-effective, species-specific genetic methods for suppression of pest species. Consequently, we envision there would be considerable stakeholder interest once the effectiveness of the Cas9 gene drives are demonstrated in some pest species. An application for a field trial of a genetically engineered pest species with a Cas9 gene drive would be a considerable challenge for regulatory agencies. Currently, transgenic insects strains that are approved for field trial carry self-limiting genetic systems, and consequently are considered low risk. For example, Oxitec's diamondback moth strain with a tetracycline-repressible female lethal transgene (44) is approved for field trials in upstate New York (permit 13-297-102r). A Cas9-based gene drive will not be self-limiting and would be difficult to contain. Hence, we believe the research described in this application on containment and reversal drives are highly relevant to regulatory agencies. The availability of a strain carrying a reversal drive at the time of a field trial of a Cas9 gene drive would reduce risk associated with a non-reversible gene drive system.

## RECENTLY PUBLISHED AND UNPUBLISHED RESEARCH FROM THE PDs LABORATORY THAT SUPPORT THIS PROPOSAL

### CRISPR/Cas9-mediated targeted mutagenesis in *D. suzukii*

The CRISPR/Cas9 system was used to introduce site-specific mutations in the *D. suzukii white* (*w*) and *Sex lethal* (*Sxl*) genes. Since our work has been recently published (45), we will only summarize the highlights:

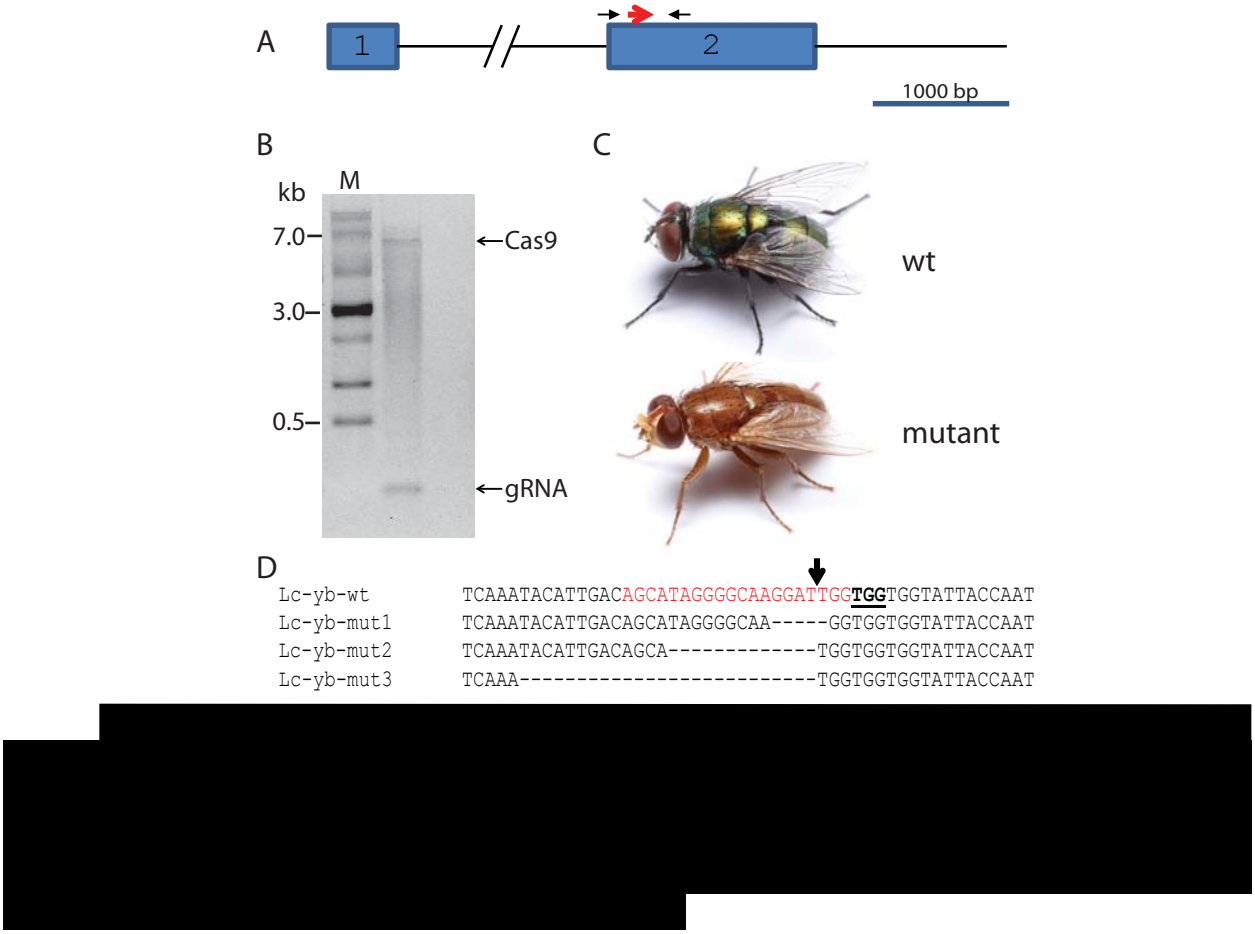
- gRNA expression was driven by the *D. melanogaster U6:3* promoter (22).
- *D. suzukii* embryos were co-injected with the *U6:3-gRNA* plasmid DNA and the *vasa-Cas9* plasmid DNA previously used in *D. melanogaster* (15).
- From embryos injected with gRNA plasmid targeting the *white* gene, $G_0$ adults were obtained that produced males hemizygous for *white* mutations with white eyes. The mutations were heritable. DNA sequencing confirmed that mutations occurred within the nucleotide sequence complementary to one of the gRNAs, likely due to repair errors
- Some of the $G_0$ females that developed from Cas9/Sxl gRNA-injected embryos showed abnormal genitalia. DNA sequencing confirmed that these females carried small deletion mutations within the *Sxl* gene at the targeted site.

Given that *Sxl* is essential for female viability and development but not for males, we suggested that this could be the basis for a gene drive system for suppression of *D. suzukii* populations. However, for reasons discussed below we now believe that other genes would be better targets for a CRISPR/Cas9 gene drive.

### CRISPR/Cas9-mediated targeted mutagenesis in *L. cuprina* and *C. hominivorax*

Since we cannot work with *C. hominivorax* in Raleigh, we generally use *L. cuprina* as a model calliphorid for developing and evaluating genetic technologies. For example, *piggyBac* vectors for germline transformation (46) and male-only genetic systems were developed in *L. cuprina* (47, 48) and have subsequently been shown to function effectively in *C. hominivorax* (see below). Thus, we have mostly been testing CRISPR/Cas9 technology in *L. cuprina* but with the aim of transferring knowledge gained to *C. hominivorax*. Initially, we targeted the red fluorescent protein marker gene (*DsRed-express 2* or simply *RFPex*) in the *L. cuprina slam5* line (49). This line was selected since the transgene is X-linked and thus targeted mutagenesis in

males would result in loss of fluorescence. Embryos were injected with a mixture of Cas9 capped and polyadenylated RNA and a mix of two guide RNAs for *RFPex*. The RNAs were made in vitro using commercial kits, as used in *D. melanogaster* (50) and injected into the posterior end of embryos. We observed that >50% of the larvae that developed from injected embryos showed loss of fluorescence at the posterior end. PCR and DNA sequencing confirmed that loss of fluorescence was due to targeted mutagenesis of the *RFPex* gene. Encouraged by these results, *C. hominivorax* embryos were injected with a mixture of Cas9 and *RFPex* guide RNAs. This work was done in Panama in the biosecure facility. The transgenic line used was autosomal, as to date no X-linked lines have been obtained. On average 20% of the larvae that developed from injected embryos showed loss of fluorescence, indicating both *RFPex* alleles had been cut and mis-repaired.




Lastly, we have targeted the *L. cuprina* ortholog of the *Drosophila pof* gene, which we think is required for X chromosome dosage compensation in calliphorids (51). In this case we

wanted to select for the insertion of a marker gene in *Lcpof* as a result of HDR of a Cas9-mediated DSB. A construct was designed with a red fluorescent protein marker gene flanked on each side by about 1kb of DNA from the *Lcpof* gene. *L. cuprina* embryos were injected with a mixture of the construct and a gRNA that targeted a sequence in an exon that encodes the conserved RNA binding domain. The 30 $G_0$ that developed from injected embryos were crossed with wild type and the offspring were screened for the red fluorescent protein marker. One cross produced fluorescent offspring, which were subsequently back-crossed to the parental strain. DNA was isolated from some of the offspring of this backcross and analyzed by PCR and Sanger DNA sequencing. The analysis confirmed integration of the red fluorescent protein gene at the site in *Lcpof* targeted by Cas9.

**Tetracycline transactivator-regulated gene expression in *L. cuprina* and *C. hominivorax***

Transgenic *L. cuprina* male-only strains have been developed with either a single component or two component genetic system (Fig. 4) (47, 48). In the single component system, tTA expression is autoregulated with the gene promoter containing multiple copies of the tTA binding site (tetO) (47). Only female express very high levels of tTA protein due to sex-specific splicing of the transcript. The tTA gene contains the sex-specific intron from the *C. hominivorax* transformer (*Chtra*) gene. In the two-component system, the *Chtra* intron is within a cell death gene such as the *Lucilia* ortholog of the *D. melanogaster hid* gene (48). The lethal gene promoter contains tTA binding sites. A promoter from a cellularization gene drives tTA expression in the early embryo. *L. cuprina* sexing strains carrying the single or two component system show 100% female lethality on normal diet but females are fully viable on diet supplemented with tetracycline (47, 48). Lethality is dominant and occurs at the pupal stage with the single component system but at the embryo stage with the two component system. The latter is advantageous as early lethality would result in considerable savings in diet cost in a mass rearing facility.

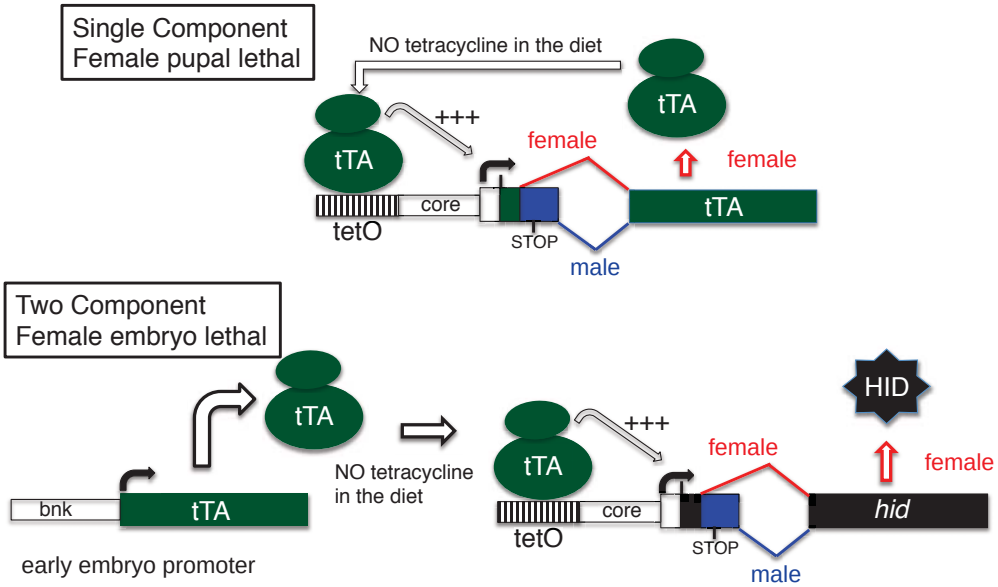



**Figure 4. Single and Two-component tetracycline-repressible female lethal genetic systems (from (31)).**

Over 20 *C. hominivorax* strains were made with a single component system (52). Eight

lines how 100% female lethality on normal diet but females are fully viable on diet with tetracycline. The strains have been evaluated with a series of tests measuring biological parameters important for mass rearing such as pupal weight, egg hatch etc. The most promising strains have been further evaluated for male sexual behavior and competitiveness. Two strains are being tested under mass rearing conditions [redacted]

[redacted]

**EXPERIMENTAL PLAN**

[redacted]

**Initial evaluation of guide RNAs**. Prior to making Cas9 gene constructs it is first necessary to identify gRNAs that efficiently target the gene of interest. For the *DsSxl* gene, this was done by injecting *D. suzukii* embryos with Cas9 RNA and a mix of in vitro synthesized

gRNAs (45). The presence of insertions/deletions at the targeted sites were identified using the heteroduplex mobility assay of PCR products from the *DsSxl* gene from $G_0$ adult genomic DNA. The same approach could be used to identify gRNAs that efficiently target the *D. suzukii tra*, [redacted] These lines are currently being bred to homozygosity. A transgenic line expressing Cas9 RNA could provide more efficient targeting as observed in *D. melanogaster* (15). Another potential modification is to use high resolution melt analysis (HRMA) for PCR products from $G_0$ individuals rather than the heteroduplex mobility assay. HRMA is reported to be the quickest and most sensitive method for identifying mutations in mosaic insects (50). HRMA analysis software has been recently purchased for a Biorad quantitative thermal cycler that is available to the PD.

**Germline transformation and analysis of lines.** Gene constructs (Fig. 2) will be made using standard procedures. Homology arms for the targeted gene will be designed such that they are within 10bp of the Cas9 cut site. About 1kb will be used for each arm. Transgenic lines that carry *U6:3-gRNA* transgenes will be made using a *piggyBac* marker with a *polyubiquitin-GFP* marker gene. Lines will be bred to homozygosity by selecting for brightly fluorescent individuals. Transgenic lines carrying the targeted Cas9 transgene will be made by injecting embryos with plasmid DNA and synthesized gRNA and Cas9 RNA. Transgenic individuals will be identified using the *polyubiquitin-DsRed* marker gene. The lines will be maintained by selecting for homozygous males and heterozygous females as homozygous females will be sterile or transformed into males. [redacted]

**Possible pitfalls.** At 12.7 kb, the size of the Cas9-rtTA-GFP gene cassette (Fig. 2) may be too large for successful Cas9-mediated integration into the targeted gene. In *D. melanogaster*,

studies with *P*-element-induced DSBs found that up to 11 kb of DNA can be copied by HDR (66). The largest gene construct we have inserted is a 5.5 kb DsRed marker gene into the *Lcpof* gene in *L. cuprina* (see above). If we do not obtain any lines with the Cas9 gene cassette inserted into one or more of the genes listed in Table 1, the alternative approach is make a construct with the much smaller *U6:3-gRNA-polyubiquitin-gfp* gene cassette (4 kb) with homology arms. The Cas9-rtTA-DsRed gene cassette would be cloned into a *piggyBac* vector and transgenic lines obtained by standard means.

As discussed above, Cas9 activity in the soma in females would reduce fertility. If the fertility is too low, gene drive will not occur. [redacted] However, given drive appears to be restricted to the spermatogonial stage, the drive frequency may be too low to be effective.

**2) Conditional CRISPR-Cas9 split gene drives in *C. hominivorax* that disrupt female development**

**Promoters for Cas9 and gRNA expression**. The development of gene drive systems in *D. suzukii* described above clearly benefits from the availability of germline and *U6* gene promoters that have been well characterized in *D. melanogaster*. In our experience *Drosophila* promoters generally function poorly in blow flies. Thus developing a gene drive system in *C. hominivorax* will be require the isolation and characterization of suitable blow fly gene promoters. We generally design and test gene constructs first in *L. cuprina* as it is easy to manipulate and a close relative of *C. hominivorax*. If the system being tested is active we then send it to Panama for evaluation in *C. hominivorax.* To date, all gene constructs developed and tested in *L. cuprina* have been functionally active in *C. hominivorax.* We have identified orthologs of the *vasa* and *nanos* genes in the *L. cuprina* genome (67). The 5' and 3' ends of the *L. cuprina vasa* and *nanos* genes were identified by performing 5' and 3' RACE analysis using RNA from 0-2h old embryos. A gene construct was made using the *Lcvasa* promoter driving Cas9 with the 3' UTR and flanking from the *Lcnanos* gene. About 2kb of DNA upstream of the 5' end of the *Lcvasa* gene was included in the gene construct. Three transgenic lines were very recently obtained by *piggyBac*-mediated transformation and they are being bred to homozygosity. RT-PCR analysis of RNA isolated from 0-2h old embryos indicates that the Cas9 gene is expressed in each line. Cas9 activity will be assessed by injecting embryos with gRNA targeting the *yellow* gene. The $G_0$ adults will be crossed with a homozygous *yellow* strain and the offspring screened for yellow flies. Targeted mutagenesis of the *yellow* gene would indicate that the transgenic lines are expressing Cas9 in the germline.

Four *U6* genes have also been identified in *L. cuprina* genome. The genes are closely linked on one scaffold. Constructs will be made with putative *U6* promoters driving expression of a gRNA for *yellow*. Activity of the constructs will be evaluated by co-injecting embryos with *U6-gRNA* plasmid DNA and Cas9 RNA. As before, the $G_0$ adults will be crossed with a homozygous *yellow* strain to determine if there was targeted mutagenesis of the *yellow* gene, indicating expression of the *yellow* gRNA from the *U6* gene promoter. Alternatively, heteroduplex mobility or HRMA assays will be performed on PCR products obtained from DNA of $G_0$ individual flies.

**gRNA evaluation**. [redacted]

[redacted] Targeted mutagenesis will be assessed by heteroduplex mobility analysis PCR products from the DNA of $G_0$ flies that develop from injected embryos. Mutagenesis will be confirmed by DNA sequencing of PCR products.

**Germline transformation and analysis of lines.** The experimental strategy will be essentially the same as for *D. suzukii* (see above). Rather than the GFP and DsRed marker genes being driven by the *Drosophila polyubiquitin* promoter, we will use the *L. cuprina hsp83* promoter (46, 47). We have used the *Lchsp83-ZsGreen* and *Lchsp83-DsRed-express 2* marker genes to identify many *L. cuprina* (well over 200) and *C. hominivorax* (over 50) transgenic lines. Thus the Cas9 gene cassette will contain the DsRed marker gene, tetO-Cas9 gene and rtTA gene driven by the *Lcvasa* promoter with *Lcnos* 3'. [redacted] With correct targeting all homozygous individuals will develop as males. The *LcU6:Chtra* gene construct will be in a *piggyBac* vector with a *Lchsp83-ZsGreen* marker gene. Gene drive will be assessed as proposed for *D. suzukii*, by crossing homozygous Cas9 males and homozygous gRNA transgenic females on doxycycline. The $F_1$ will be crossed to wild type and larval offspring analyzed for the percentage that inherit the red marker.

**Possible pitfalls.** The *Lcvasa* promoter may not lead to expression of Cas9 in the *L. cuprina* or *C. hominivorax* germline. If so, the *Lcnanos* and/or *Lcbicoid* promoters could be isolated and evaluated in *L. cuprina*. In *Drosophila*, the regulatory elements required for expression of *bcd* in the germline are located within 100 bp of the start of transcription (71). If the *Lcvasa* promoter is active in *L. cuprina* but not in *C. hominivorax*, we will identify the orthologous *vasa*, *nanos* and *bcd* genes in the *C. hominivorax* genome. We expect to assemble and annotate the genome of a highly inbred strain by the end of 2016. If Cas9 is expressed in the female germline but the transcript not targeted to the posterior end of the developing oocyte, we will then replace the *Lcnanos* 3' UTR and 3' flanking with the *Lcvasa* or *Chvasa* 3' sequences. These will be obtained by DNA synthesis as PCR of this region of the *Lcvasa* gene has been problematic due to high AT content and difficulty with designing PCR primers that do not have predicted secondary structures. The *L. cuprina* genome does not appear to have an ortholog of the *Rcd-1r* gene. Expression of the Cas9 gene in the male spermatogonial cells will require identification of a suitable promoter, which is likely beyond the timeframe of this proposal. Other possible pitfalls regarding size of gene construct and HDR are similar as discussed above for *D. suzukii*.

**3) Evaluation of the potential for population suppression in cage experiments under permissive and non-permissive conditions**

The experiments described above will identify which combination of Cas9 target [redacted] and gRNA transgenic line gives the highest frequency of gene drive. This optimal combination will be used for population suppression experiments with *D. suzukii* and *C. hominivorax*. The design of these experiments is similar to those that have been performed to show that release of males carrying a dominant female lethal gene into a cage containing a stable population of insects will lead to population suppression (72, 73). We are currently undertaking such experiments with transgenic screwworm lines in Panama with large cages (4 $m^3$) that are used for mass rearing. A major difference is that a much lower ratio of introduced transgenic males to wild type insects would need to be used for population suppression with a Cas9-

mediated gene drive. For example a ratio of 1:10 compared to 10:1 used for a male-only strain (i.e. 100 times fewer males). Further only a single release of males with a gene drive should be necessary. The protocol for these experiments has been modified from the protocol we have been following for the screwworm experiments:

1. 4 cages will be set with 250 pupae from a transgenic strain homozygous for *U6-gRNA* transgene. This is the "wild type" population. Two cages are "test" and two cages are "control". In one set of test and control cages, the flies are raised on diet with doxycycline. For the other set, the flies are raised on diet without doxycycline.
2. Pupae are added each week to each cage to maintain a population of about 400 flies. This is typically around 100 but is determined each week.
3. Egg production is monitored weekly by weighing.
4. Once conditions have been established to maintain a stable population, 40 males homozygous for a Cas9 gene drive will be added to the test cage. The exact number of Cas9 males added will be based on the efficiency of gene drive obtained from the initial evaluation of gRNA and Cas9 lines.
5. At the time of addition of Cas9 males, the number of pupae added to the test cage will be reduced based on egg production from the test and control cages. The percentage eggs that develop into fluorescent larvae will be monitored. This information will be used to determine the proportion of transgenic to non-transgenic pupae added to the cage. The number of pupae added to the control cages will be as previously.
6. The number of females in the cage will be monitored by collecting, counting and sexing dead flies at regular intervals.
7. The experiment will be repeated at least once

A similar experimental design would be used for *D. suzukii* experiments with some modifications. Egg production would be monitored by counting the eggs laid each day on diet. Rather than add pupae, immature male and female adults will be added as needed to maintain a stable population in the cages. Similarly, after addition of Cas9 males, "wild type" adults will be added to the test cage based on egg production from the test and control cages. We expect that by 8-10 generations the population in the test cage reared on doxycycline will become extinct (28).

**Possible Pitfalls.** It is possible that there may be some gene drive in absence of doxycycline due to basal (or "leaky") expression from the tetO/core enhancer/promoter. The gene drive may fail if flies are present in the wild type strain with sufficient nucleotide polymorphisms in the target region to reduce the efficiency of gRNA hybridization. Indeed, this is a general concern with CRISPR/Cas9 population suppression gene drives (7). To reduce the chance of resistance developing, it has been suggested that the drive system contain multiple gRNAs (e.g. four) homologous to the target gene (7). This would be a logical extension of the gene drives developed in this proposal.

**4) Evaluation of the potential for inhibition of gene drive by release of flies immune to Cas9 digestion.**

For these experiments, the gene being targeted in the above population suppression experiments must be recoded such that it is not cut by Cas9. This will be done by injecting embryos from the *U6-gRNA* strain (wild type in above cage experiments) with Cas9 RNA and a single stranded oligonucleotide (ssDNA). The ssDNA will include 40-60 nt homology arms

either side of the sequence homologous to the gRNA in the wild type gene. This has been shown to be sufficient for HDR in *D. melanogaster* (74). We will use a modified ssDNA as this could increase the frequency of HDR (75). The sequence homologous to the gRNA sequence will be altered at multiple positions to reduce annealing to the gRNA while not changing the encoded amino acid sequence. The altered sequence will introduce a unique restriction endonuclease site. Successful HDR can be determined by PCR of DNA obtained from a dissected wing or leg from $G_0$ adult followed by restriction endonuclease digestion. Alternatively, HRMA will be used to identify which $G_0$ flies carry the modified gene. These flies will be mated with wild type and $G_1$ offspring that inherit the modified gene will be identified by PCR of genomic DNA. A strain carrying the modified gene will then be bred to homozygosity.

The cage population suppression experiments will be performed as described above but with one modification. Forty males with the recoded target gene will be added to the cage 3 generations after addition of males carrying the Cas9 gene. This will simulate a recall of a gene drive release. The expectation is that, as the recoded targeted gene is not cut by Cas9, the recoded allele will increase in frequency in the cage. This is because females heterozygous or homozygous for the recoded gene will develop normally and be fertile and thus have a selective advantage. We will monitor the frequency of the recoded allele by PCR of DNA from dissected legs or wings and restriction endonuclease or HRMA analysis. The frequency of the gene drive Cas9 allele will be followed by screening for expression of the fluorescent protein marker gene.

**Possible Pitfalls.** The multiple substitutions in the gRNA complementary sequence in the recoded allele may not be sufficient to completely prevent Cas9 digestion and thus some gene drive may occur. An alternative approach is design a drive that targets the Cas9 gene (76). As the Cas9 gene is disrupted, drive will cease.

**Timeline**

| Objective | Year 1 | Year 2 | Year3 |
|---|---|---|---|
| Development and evaluation of conditional split drives in *D. suzukii* | → | → | |
| Development and evaluation of conditional split drives in *C. hominivorax* | → | → | |
| Population suppression cage experiments for *D. suzukii* and *C. hominivorax* | | → | → |
| Develop recoded resistant strain and evaluate in cage experiments | | → | → |

**References Cited**


1. Burt A (2003) Site-specific selfish genes as tools for the control and genetic engineering of natural populations. *Proc Biol Sci* 270(1518):921-928.
2. Belfort M & Bonocora RP (2014) Homing endonucleases: from genetic anomalies to programmable genomic clippers. *Methods Mol Biol* 1123:1-26.
3. Deredec A, Burt A, & Godfray HC (2008) The population genetics of using homing endonuclease genes in vector and pest management. *Genetics* 179(4):2013-2026.
4. Stoddard BL (2011) Homing endonucleases: from microbial genetic invaders to reagents for targeted DNA modification. *Structure* 19(1):7-15.
5. Bassett AR & Liu JL (2014) CRISPR/Cas9 and genome editing in *Drosophila*. *J Genet Genomics* 41(1):7-19.
6. Beumer KJ & Carroll D (2014) Targeted genome engineering techniques in *Drosophila*. *Methods* 68(1):29-37.
7. Esvelt KM, Smidler AL, Catteruccia F, & Church GM (2014) Concerning RNA-guided gene drives for the alteration of wild populations. *Elife*:e03401.
8. Carroll D (2014) Genome engineering with targetable nucleases. *Annu Rev Biochem* 83:409-439.
9. Windbichler N, *et al.* (2011) A synthetic homing endonuclease-based gene drive system in the human malaria mosquito. *Nature* 473(7346):212-215.
10. Chan YS, Naujoks DA, Huen DS, & Russell S (2011) Insect population control by homing endonuclease-based gene drive: an evaluation in *Drosophila melanogaster*. *Genetics* 188(1):33-44.
11. Chan YS, Huen DS, Glauert R, Whiteway E, & Russell S (2013) Optimising homing endonuclease gene drive performance in a semi-refractory species: the *Drosophila melanogaster* experience. *PLoS One* 8(1):e54130.
12. Sano H, Nakamura A, & Kobayashi S (2002) Identification of a transcriptional regulatory region for germline-specific expression of *vasa* gene in *Drosophila melanogaster*. *Mech Dev* 112(1-2):129-139.
13. Bassett AR, Tibbit C, Ponting CP, & Liu JL (2013) Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. *Cell Rep* 4(1):220-228.
14. Gratz SJ, *et al.* (2013) Genome engineering of *Drosophila* with the CRISPR RNA-guided Cas9 nuclease. *Genetics* 194(4):1029-1035.
15. Kondo S & Ueda R (2013) Highly improved gene targeting by germline-specific Cas9 expression in *Drosophila*. *Genetics* 195(3):715-721.
16. Bhaya D, Davison M, & Barrangou R (2011) CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Annu Rev Genet* 45:273-297.
17. Jinek M, *et al.* (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337(6096):816-821.
18. Wiedenheft B, Sternberg SH, & Doudna JA (2012) RNA-guided genetic silencing systems in bacteria and archaea. *Nature* 482(7385):331-338.
19. Golic KG (2013) RNA-guided nucleases: a new era for engineering the genomes of model and nonmodel organisms. *Genetics* 195(2):303-308.
20. Hsu PD, Lander ES, & Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. *Cell* 157(6):1262-1278.

21. Mali P, Esvelt KM, & Church GM (2013) Cas9 as a versatile tool for engineering biology. *Nat Methods* 10(10):957-963.
22. Gratz SJ, *et al.* (2014) Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in *Drosophila*. *Genetics* 196(4):961-971.
23. Port F, Chen HM, Lee T, & Bullock SL (2014) Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in Drosophila. *Proc Natl Acad Sci U S A* 111(29):E2967-2976.
24. Gantz VM & Bier E (2015) Genome editing. The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations. *Science* 348(6233):442-444.
25. Gantz VM, *et al.* (2015) Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. *Proc Natl Acad Sci U S A* 112(49):E6736-6743.
26. Hammond A, *et al.* (2016) A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nat Biotechnol* 34(1):78-83.
27. Akbari OS, *et al.* (2015) BIOSAFETY. Safeguarding gene drive experiments in the laboratory. *Science* 349(6251):927-929.
28. Scott MJ, *et al.* (2016) Agricultural Production: Assessment of the Potential use of Cas9-mediated Gene Drive Systems for Agricultural Pest Control. *Journal of Responsible Innovation* submitted.
29. Gossen M & Bujard H (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* 89(12):5547-5551.
30. Koukidou M & Alphey L (2014) Practical applications of insects' sexual development for pest control. *Sex Dev* 8(1-3):127-136.
31. Scott MJ (2014) Development and evaluation of male-only strains of the Australian sheep blowfly, *Lucilia cuprina*. *BMC Genet* 15 Suppl 2:S3.
32. Urlinger S, *et al.* (2000) Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proc Natl Acad Sci U S A* 97(14):7963-7968.
33. Lee JC, *et al.* (2011) In Focus: Spotted wing drosophila, *Drosophila suzukii*, across perspectives. *Pest Manag Sci* 67(11):1349-1351.
34. Asplen M, *et al.* (2015) Invasion biology of spotted wing Drosophila (*Drosophila suzukii*): a global perspective and future priorities. *Journal of Pest Science* 88(3):469-494.
35. Lee JC, *et al.* (2011) The susceptibility of small fruits and cherries to the spotted-wing drosophila, *Drosophila suzukii*. *Pest Manag Sci* 67(11):1358-1367.
36. Tochen S, *et al.* (2014) Temperature-related development and population parameters for Drosophila suzukii (Diptera: Drosophilidae) on cherry and blueberry. *Environ Entomol* 43(2):501-510.
37. Cini A, *et al.* (2014) Tracking the invasion of the alien fruit pest *Drosophila suzukii* in Europe. *Journal of Pest Science* 87(4):559-566.
38. Krafsur ES (1998) Sterile insect technique for suppressing and eradicating insect population: 55 years and counting. *Journal of Agricultural Entomology* 15(4):303-317.
39. Alexander JL (2006) Screwworms. *J Am Vet Med Assoc* 228(3):357-367.
40. Knipling EF (1960) The eradication of the screw-worm fly. *Scientific American* 203(4):54-61.

41. Knipling EF (1955) Possibilities of insect control or eradication through the use of sexually sterile males. *J Econ Entomol* 48(4):459-462.
42. Vargas-Teran M, Hofmann HC, & Tweddle NE (2005) Impact of screwworm eradication programmes using the sterile insect technique. *Sterile Insect Technique. Principles and Practice in Area-Wide Integrated Pest Management*, eds Dyck VA, Hendrichs J, & Robinson AS (Springer, Dordrecht), pp 629-650.
43. Rendon P, McInnis D, Lance D, & Stewart J (2004) Medfly (Diptera: Tephritidae) genetic sexing: large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala. *Journal of Economic Entomology* 97(5):1547-1553.
44. Jin L, *et al.* (2013) Engineered female-specific lethality for control of pest lepidoptera. *ACS Synthetic Biology* 2(3):160-166.
45. Li F & Scott MJ (2016) CRISPR/Cas9-mediated mutagenesis of the *white* and *Sex lethal* loci in the invasive pest, *Drosophila suzukii*. *Biochem Biophys Res Commun* 469(4):911-916.
46. Concha C, *et al.* (2011) Efficient germ-line transformation of the economically important pest species *Lucilia cuprina* and *Lucilia sericata* (Diptera, Calliphoridae). *Insect Biochem. Mol. Biol.* 41(1):70-75.
47. Li F, Wantuch HA, Linger RJ, Belikoff EJ, & Scott MJ (2014) Transgenic sexing system for genetic control of the Australian sheep blow fly *Lucilia cuprina*. *Insect Biochem. Mol. Biol.* 51:80-88.
48. Yan Y & Scott MJ (2015) A transgenic embryonic sexing system for the Australian sheep blow fly *Lucilia cuprina*. *Sci Rep* 5:16090.
49. Edman RM, *et al.* (2015) Functional characterization of calliphorid cell death genes and cellularization gene promoters for controlling gene expression and cell viability in early embryos. *Insect Mol. Biol.* 24(1):58-70.
50. Bassett A & Liu JL (2014) CRISPR/Cas9 mediated genome engineering in *Drosophila*. *Methods* 69(2):128-136.
51. Linger RJ, Belikoff EJ, & Scott MJ (2015) Dosage Compensation of X-Linked Muller Element F Genes but Not X-Linked Transgenes in the Australian Sheep Blowfly. *PLoS One* 10(10):e0141544.
52. Concha C, *et al.* (2016) A transgenic male-only strain of the New World screwworm, *Cochliomyia hominivorax*, suitable for a genetic control program through SIT. *Proc. Natl. Acad. Sci. U S A* submitted.
53. Alphey L, Nimmo D, O'Connell S, & Alphey N (2008) Insect population suppression using engineered insects. *Adv. Exp. Med. Biol.* 627:93-103.
54. Heinrich JC & Scott MJ (2000) A repressible female-specific lethal genetic system for making transgenic insect strains suitable for a sterile-release program. *Proc. Natl. Acad. Sci. U S A* 97(15):8229-8232.
55. Rorth P (1998) Gal4 in the *Drosophila* female germline. *Mech Dev* 78(1-2):113-118.
56. Gdula DA, Gerasimova TI, & Corces VG (1996) Genetic and molecular analysis of the *gypsy* chromatin insulator of *Drosophila*. *Proc Natl Acad Sci U S A* 93(18):9378-9383.
57. Carballar-Lejarazu R, Jasinskiene N, & James AA (2013) Exogenous *gypsy* insulator sequences modulate transgene expression in the malaria vector mosquito, *Anopheles stephensi*. *Proc Natl Acad Sci U S A* 110(18):7176-7181.

58. Handler AM & Harrell RA, 2nd (1999) Germline transformation of *Drosophila melanogaster* with the *piggyBac* transposon vector. *Insect Molecular Biology* 8(4):449-457.
59. Schupbach T (1985) Normal female germ cell differentiation requires the female X chromosome to autosome ratio and expression of *sex-lethal* in *Drosophila melanogaster*. *Genetics* 109(3):529-548.
60. Chau J, Kulnane LS, & Salz HK (2009) *Sex-lethal* facilitates the transition from germline stem cell to committed daughter cell in the *Drosophila* ovary. *Genetics* 182(1):121-132.
61. Hashiyama K, Hayashi Y, & Kobayashi S (2011) *Drosophila Sex lethal* gene initiates female development in germline progenitors. *Science* 333(6044):885-888.
62. Marsh JL & Wieschaus E (1978) Is sex determination in germ line and soma controlled by separate genetic mechanisms? *Nature* 272(5650):249-251.
63. Schupbach T (1982) Autosomal mutations that interfere with sex determination in somatic cells of *Drosophila* have no direct effect on the germline. *Dev Biol* 89(1):117-127.
64. 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*. *Developmental Cell* 6(1):145-155.
65. Hong CC & Hashimoto C (1996) The maternal nudel protein of *Drosophila* has two distinct roles important for embryogenesis. *Genetics* 143(4):1653-1661.
66. Johnson-Schlitz DM & Engels WR (2006) The effect of gap length on double-strand break repair in *Drosophila*. *Genetics* 173(4):2033-2038.
67. Anstead CA, *et al.* (2015) *Lucilia cuprina* genome unlocks parasitic fly biology to underpin future interventions. *Nat. Commun.* 6:7344.
68. Scott MJ, Pimsler ML, & Tarone AM (2014) Sex determination mechanisms in the Calliphoridae (blow flies). *Sex Dev* 8(1-3):29-37.
69. Concha C & Scott MJ (2009) Sexual Development in *Lucilia cuprina* (Diptera, Calliphoridae) Is Controlled by the *Transformer* Gene. *Genetics* 182(3):785-798.
70. Li F, Vensko SP, 2nd, Belikoff EJ, & Scott MJ (2013) Conservation and Sex-Specific Splicing of the *transformer* Gene in the Calliphorids *Cochliomyia hominivorax*, *Cochliomyia macellaria* and *Lucilia sericata*. *PLoS One* 8(2):e56303.
71. Ruez C, Payre F, & Vincent A (1998) Transcriptional control of *Drosophila bicoid* by Serendipity delta: cooperative binding sites, promoter context, and co-evolution. *Mech Dev* 78(1-2):125-134.
72. Wise de Valdez MR, *et al.* (2011) Genetic elimination of dengue vector mosquitoes. *Proc Natl Acad Sci U S A* 108(12):4772-4775.
73. Ant T, *et al.* (2012) Control of the olive fruit fly using genetics-enhanced sterile insect technique. *BMC Biol* 10:51.
74. Gratz SJ, Harrison MM, Wildonger J, & O'Connor-Giles KM (2015) Precise Genome Editing of *Drosophila* with CRISPR RNA-Guided Cas9. *Methods Mol Biol* 1311:335-348.
75. Renaud JB, *et al.* (2016) Improved Genome Editing Efficiency and Flexibility Using Modified Oligonucleotides with TALEN and CRISPR-Cas9 Nucleases. *Cell Rep* 14(9):2263-2272.
76. Wu B, Luo L, & Gao XJ (2016) Cas9-triggered chain ablation of cas9 as a gene drive brake. *Nat Biotechnol* 34(2):137-138.

# Facilities & Other Resources

**North Carolina State University**

The PD has a 916 $ft^2$ laboratory that includes a chemical hood and is well equipped for molecular biology. The PD has an office of 118 $ft^2$ and has a computer and printer. Additional office space of 117 $ft^2$ and 227 $ft^2$ is used for lab personnel and visitors to the PDs lab. The PD shares a 138 $ft^2$ laboratory that is set-up with state-of-the-art equipment for making transgenic insects with funding from the North Carolina Biotechnology Center. The PDs laboratory and the core insect transgenesis facility are located on the first floor of Thomas Hall in close proximity.

Four temperature and light controlled rooms of 35 $ft^2$ (each room) that meet either PC1 or PC2 level arthropod containment, are available to the PD. Additional insect rearing rooms that meet PC1 level containment are also available. The work proposed in this application has been approved by the NCSU biosafety committee to be carried out in a PC1 level facility.

**USDA-APHIS Pacora, Panama**

Within the Screwworm Production Facility Bio-secure (Level 2) Mass Rearing Facility at Pacora, Panama, (72,554 $ft^2$) the screwworm research unit occupies a 2720 $ft^2$ laboratory consisting of 4 large (30 cu. ft.) incubators for rearing larval screwworm, equipment and materials necessary for adult screwworms, and agreement from the USDA-APHIS Eradication Program for more space as necessary. In addition the facility has a large (30 ft. diameter by 9 feet high) outside cage and four smaller cages for initial studies for fitness and longevity of sterilized transgenic screwworms; 5 vehicles for field use; computer and programs for habitat analysis.

# Equipment

**North Carolina State University**

*The PDs laboratory (916 ft$^2$):* is well equipped for molecular biology including refrigerated centrifuges, -80 and -20 °C freezers, thermal cyclers, agarose and acrylamide gel electrophoresis, four computers, platform and flask shakers, air and water incubators, UV/VIS spectrophotometer, qubit fluorometer. There are several autoclaves, large low speed centrifuges, ultracentrifuges, a Biorad CFX384 thermal cycler for qPCR with software for HRMA and a water purification system available and in close physical proximity to the Scott lab at NCSU.

*The core insect transgenesis facility*: is equipped with a Leica M165FC fluorescence microscope with a Leica DFC500 digital camera, Olympus MVX10 fluorescence microscope with XM10 camera, high zoom-range Leica M125 stereo-microscope with video camera for microinjection, Leica stereo microscopes for aligning embryos for injection, Sutter P-2000 glass needle puller and FG-BV10-D beveller, Xenoworks micromanipulator, Xenoworks digital injector and Newport LW3036B-OPT vibration free table.

**USDA-ARS Screwworm Research Unit, Panama**

Laboratory equipment at Screwworm research unit includes: molecular genetic analysis (thermo-cycler, gel electrophoresis, gel digitizer); two Leica M165FC fluorescence microscopes, Sutter P-2000 glass needle puller, Xenoworks micromanipulator, Xenoworks digital injector, additional microscopes (dissecting, and compound); BioRad Gene Pulser II electroporation system; liquid nitrogen tanks and equipment for cryopreservation, storage, and recovery of cryopreserved screwworms, various field equipment (light meters, temperature/humidity meters, nets and traps, etc).