



2017

## WHAT INDUCES FEMALE KICKING IN *CALLOSOBRUCHUS MACULATUS*? DISENTANGLING THE EFFECTS OF MALE TRAITS ON FEMALE MATING DECISIONS

William I. Licht

University of Kentucky, wili222@g.uky.edu

Digital Object Identifier: <https://doi.org/10.13023/ETD.2017.342>

[Right click to open a feedback form in a new tab to let us know how this document benefits you.](#)

---

### Recommended Citation

Licht, William I., "WHAT INDUCES FEMALE KICKING IN *CALLOSOBRUCHUS MACULATUS*? DISENTANGLING THE EFFECTS OF MALE TRAITS ON FEMALE MATING DECISIONS" (2017). *Theses and Dissertations--Entomology*. 38.

[https://uknowledge.uky.edu/entomology\\_etds/38](https://uknowledge.uky.edu/entomology_etds/38)

This Master's Thesis is brought to you for free and open access by the Entomology at UKnowledge. It has been accepted for inclusion in Theses and Dissertations--Entomology by an authorized administrator of UKnowledge. For more information, please contact [UKnowledge@lsv.uky.edu](mailto:UKnowledge@lsv.uky.edu).

## **STUDENT AGREEMENT:**

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained needed written permission statement(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine) which will be submitted to UKnowledge as Additional File.

I hereby grant to The University of Kentucky and its agents the irrevocable, non-exclusive, and royalty-free license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless an embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

## **REVIEW, APPROVAL AND ACCEPTANCE**

The document mentioned above has been reviewed and accepted by the student's advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student's thesis including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

William I. Licht, Student

Dr. Charles W. Fox, Major Professor

Dr. Charles W. Fox, Director of Graduate Studies

WHAT INDUCES FEMALE KICKING IN *CALLOSOBRUCHUS MACULATUS*?  
DISENTANGLING THE EFFECTS OF MALE TRAITS ON FEMALE MATING  
DECISIONS.

---

THESIS

---

A thesis submitted in partial fulfillment of the  
requirements for the degree of Master of Science in the  
College of Agriculture, Food and Environment  
at the University of Kentucky

By

William I. Licht

Lexington, Kentucky

Director: Dr. Charles W. Fox, Professor of Entomology

Lexington, Kentucky

2017

Copyright © William I. Licht 2017

## ABSTRACT OF THESIS

### WHAT INDUCES FEMALE KICKING IN *CALLOSOBRUCHUS MACULATUS*? DISENTANGLING THE EFFECTS OF MALE TRAITS ON FEMALE MATING DECISIONS.

Sexual conflict over mating duration drives the evolution of male and female adaptations that facilitate the manipulation of mating interactions in their favor. This conflict drives the evolution of traits that improve the fitness of the focal sex despite inflicting costs on mates. However, males can express multiple traits that increase and decrease female fitness simultaneously. When the effects of male traits on female fitness increase or decrease with duration of exposure, female traits that influence mating duration are selected upon. Females of *Callosobruchus maculatus*, a bruchid bean beetle, kick mates to forcefully end copulation. Although both negative effects of male genital spines and positive effects of ejaculatory materials on female fitness have been documented, it is not yet clear how these male traits interact to influence the timing of female kicking. In this study, we observed the effect of male genital spine size, ejaculate size and mating history, and manipulated mating duration to disentangle the effects of male traits on the timing of female kicking behavior. We found that male mating history and mate body size dimorphism predicted the timing and duration of female kicking, but that male ejaculate size and spine length did not predict female kicking timing.

Keywords: Sexual conflict, mating duration, *Callosobruchus maculatus*, genital spines, female resistance

---

William I. Licht

---

6/9/2017

WHAT INDUCES FEMALE KICKING IN *CALLOSOBRUCHUS MACULATUS*?  
DISENTANGLING THE EFFECTS OF MALE TRAITS ON FEMALE MATING  
DECISIONS.

By

William I. Licht

---

Dr. Charles W. Fox

*Director of Thesis*

---

Dr. Charles W. Fox

*Director of Graduate Studies*

---

July 21<sup>st</sup> 2017

## Table of Contents

List of Tables .....	iv
List of Figures .....	v
Chapter One: .....	1
Introduction.....	1
Materials & Methods .....	4
Beetle Populations: .....	4
Experiment I: .....	4
Experiment II: .....	6
Experiment III: .....	8
Analysis.....	9
Experiment I.....	9
Experiments II and III.....	10
Results.....	10
Experiment I: Female scarring and response to male traits .....	10
Effects of male traits on the timing of kicking .....	10
Kicking duration .....	12
Spine length and female damage .....	13
Experiment II: Pumping and kicking manipulation experiment.....	14
Influence of pumping on scarring and fecundity .....	14
Influence of kicking on scarring and fecundity .....	15
Experiment III: Pumping, kicking and transfer of ejaculates .....	16
Discussion .....	18
Female kicking timing and duration and the effects of male traits .....	18
Effects of pumping and kicking on scarring and fecundity .....	21
Why do females kick?.....	22
References.....	33
Vita.....	38

## List of Tables

1.1. Results of AICc best fit general linear mixed effects models to explain variation in kicking timing and duration, ejaculate size and female scarring from the matings examined in Experiment I. ....	24
1.2. Experiment II results of AICc best fit general linear mixed effects models for female scarring.....	25

## List of Figures

Figure 1. The duration (in seconds) of the pumping (A) and kicking (B) stages of mating in a male's first through fourth mates for six populations of the seed beetle, <i>Callosobruchus maculatus</i> . A) Pumping durations for all matings for each population. B) Kicking durations for all matings for each population. ....	26
Figure 2. The size of ejaculates for all matings for each population of the seed beetle, <i>Callosobruchus maculatus</i> .....	27
Figure 3. Spine length and damage in experiment I. Aedeagal spine length (A) and damage done to the female bursa (B) for six populations of <i>Callosobruchus maculatus</i> .....	28
Figure 4. Effects of spine length and male mass on damage in experiment I. A) Regressions of spine length vs damage caused in first matings for the six populations measured. B) regressions of male mass vs damage caused in first and fourth matings for the six populations measured. ....	29
Figure 5. Pumping manipulation treatment results. Symbols represent means of manipulative treatments. A) Proportion of females with scarring in pumping treatments. B) proportion of females ovipositing in pumping treatments. C) Amount of scar tissue found on female reproductive tracts in pumping treatments. D) Fecundity means for pumping treatments.....	30
Figure 6. A) Proportion of matings where ejaculates entered female bursae in dissection experiment treatments. B) Mean number of pumps before the onset of kicking pooled from dissection and kicking experiments for each population. ....	31
Figure 7. The degree of bursal scarring (A) and fecundity of fertilized females (B) from of the pumping and kicking manipulation study.....	32

## *Introduction*

Male or female traits decrease the fitness of their mates, they are pitted against one another in an evolutionary game (Parker 1979, Arnqvist and Rowe 2013). This sexual conflict leads to males and females both evolving behaviors and morphology that assist in manipulating mating interactions in their favor. For males, traits that increase success in copulation (Tatarnic et al. 2014), remove competitors' sperm (Cordero-Rivera 2016, Waage 1979, Haubruge et al. 1999), and inhibit female remating (Elias et al. 2014, Yamane et al. 2015, Crudgington et al. 2005) can be favored by selection. Female behaviors and morphology that counteract the male traits (Ronkainen et al. 2005, Green et al. 2013) are generally favored by selection given the cost is not too high (Arnqvist and Rowe 2013). Male traits that improve male fitness can evolve despite being directly harmful to their mates (Tatarnic et al. 2014, Le Boeuf and Mesnick 1991), intensifying sexual conflict (Parker 1979). Males also express traits, such as nuptial gifts (Lewis et al. 2011, Al-Wathiqui 2016, Lehmann and Lehmann 2016), that improve female fitness by providing females with chemical protection from predators (Gwynne 2008) or nutrients that can be used for egg production or somatic maintenance (DiRienzo and Marshall 2013, Fox and Moya-Laraño 2009). Experiments have observed and manipulated the effects of antagonistic (Hotzy et al. 2012, Polak and Rashed 2010) and mutualistic traits (DiRienzo and Marshall 2013, Savalli and Fox 1999), but few attempts have been made to simultaneously measure the effects of both mutualistic and antagonistic traits on female behavior.

Mating duration, a character over which males and females often hold opposing interests, is mediated by both male and female traits (Edward et al. 2014, Rowe 1992). Whether or not long matings are in a female's interest depends upon the nature of male traits; females likely have an optimal mating duration that balances the costs and benefits of mating (Arnqvist and Nilsson 2000). Females may balance the costs and benefits of mating through adjustment of remating latency by avoiding (Pineaux and Turgeon 2017, Wey et al. 2015, Krupa et al. 1990) or resisting males when the benefit of mating is outweighed by costs (Green et al. 2013, Rowe 1992, Watson and Arnqvist 1998, Arnqvist 1989), or by terminating matings early (Lieshout et al. 2014, Edvardsson and Tregenza

2005). The timing of female attempts to terminate mating should thus respond plastically to the traits of the male that mediate his influence on female fitness. However, when males have multiple sex-associated traits that vary as to whether they improve (nutritious seminal compounds, South and Lewis 2010; Fox 1993; nuptial gifts, Gwynne 2008) or impair female fitness (where male genitals are harmful, Tataric et al. 2014; ejaculates contain manipulative compounds, Yamane et al. 2008 and see Lieshout et al. 2014; and toxic compounds in male ejaculates, Wigby and Chapman 2005), it becomes more difficult to assess the trait to which females are responding. Understanding how females adjust mating behavior in response to multiple male traits is necessary to disentangle the driving forces behind female mating decisions.

Males of the seed beetle, *Callosobruchus maculatus* (Coleoptera: Chrysomelidae: Bruchinae), simultaneously express both antagonistic and mutualistic traits. The male intromittent organ (aedeagus) has spines that penetrate and scar their mate's reproductive tract (bursa) (Crudginton and Siva-Jothy 2000), reducing female lifespan (Wigby and Chapman 2004) and facilitating diffusion of ejaculates that contain manipulative compounds into the female hemolymph (Hotzy et al. 2012). These manipulative compounds induce mating refractory periods (Yamane et al. 2008, 2015) and oviposition (Yamane and Miyatake 2010) when microinjected into females. Ejaculates of *C. maculatus* also contain nutrients that improve female longevity and fecundity (Fox 1993, Savalli and Fox 1999, Edvardsson 2007). Females can improve their fecundity and lifespan by remating, but over-exposure to males can be detrimental as male harassment decreases female fitness (Fox 1993; Gay et al. 2009). During copulation males forcefully inflate and deflate (pump) their aedeagus, which presses their aedeagal spines against the bursal wall (personal observation); the consequences of this behavior have not been investigated directly, but Crudginton and Siva-Jothy (2000) suggest that this alternation between inflation and deflation increases the damage done to the female reproductive tract.

Females of *C. maculatus* can influence the duration of their exposure to male genital spines by kicking and forcefully dislodging males (Crudginton and Siva-Jothy 2000, but see Wilson and Tomkins 2014). Females prevented from kicking their mates

during mating suffer more severe copulatory scarring, indicating that females may be kicking to reduce damage inflicted by spines (Crudgington and Siva-Jothy 2000). Females assisted in terminating matings by rupturing male genitals at the onset of kicking exhibit shorter remating latency, equal fecundity, but slightly shorter lifespans than females engaged in uninterrupted matings (Lieshout et al. 2014a). This suggests that females terminate matings to reduce the volume of ejaculatory materials received by kicking their mates (Lieshout et al. 2014b; Wilson and Tomkins 2014). Despite the efforts dedicated to understanding the motivations behind kicking behavior in *C. maculatus*, it is not yet known what triggers kicking. Elucidating what triggers female kicking may bring us closer to understanding why females kick their mates, and how females assess the costs and benefits of mating.

We sought to understand how females decide when to terminate mating and how male traits affect those decisions by investigating the influences a mutualistic male trait (ejaculate size) and an antagonistic male trait (spine size) have on the timing and duration of female kicking behavior. We quantified effects of spine length, ejaculate size and male mating status on female kicking decisions in six populations of *C. maculatus* that varied in mean spine length. We then manipulated aedeagal pumping and quantified its effects on the timing of female kicking, the transfer of ejaculates, fecundity and amount of bursal scarring. We also examined the effect of pumping on the frequency of ejaculate transfer, and the frequency at which ejaculates are successfully transferred at the time of female kicking. Finally, we tested the prediction that females change the timing of copulatory resistance in response to male traits. Determining whether female *C. maculatus* choose to end mating based on thresholds for exposure to antagonistic or mutualistic traits informs us as to how females balance the costs and benefits of mating.

## *Methods*

### Beetle populations

*Callosobruchus maculatus* (Coleoptera, Chrysomelidae, Bruchinae) are cosmopolitan pests that attack seeds of various species of beans (Fabaceae). Larvae develop inside beans, emerge from seeds as adults, then mate and lay eggs directly on seeds. We used beetles from six populations of *C. maculatus* for the comparative kicking study (Ofuya, Mali, Lossa, Benin, Leic and Zaire) and four populations for the manipulative studies (Ofuya, Mali, Leic and Zaire). Beetles were shared with us by the Miller Lab at Rice University in February 2015. Populations varied as to when they were collected from the field; Leic in 1975, Mali in 1984, Lossa, Zaire and Benin in 2000, and Ofuya in 2010. All populations had already been reared in laboratory conditions for well over 50 generations (Ofuya) and 100 generations (other populations) prior to our experiments. We maintained these populations in the lab at 25°C with a 15:9 day:night cycle for 4 generations on cowpea seeds prior to the breeding of families for the experiments.

### Experiment I: Female scarring and response to male traits.

The purpose of this study was to assess whether male traits covary within and among populations with the onset of female kicking behavior and the severity of harm done to females, and whether these relationships vary among populations and among males of differing mating status (virgin versus multiply mated males). To answer these questions, we examined the relationships of antagonistic (spine length) and mutualistic (ejaculate size) male traits with female behavior (kicking timing), female harm (bursal scarring) and female resistance to copulation (kicking duration). The effect of male status was measured by sequentially mating each male to four virgin females.

All beetles were reared for one generation at one beetle per seed (to eliminate effects of larval competition on body size and other traits) on seeds of cowpea (*V. unguiculata*). Virgin adult males and females were collected from isolated seeds within

12 hours of their emergence from the seed. Because male *C. maculatus* emerge from their host seed with only partially filled seminal vesicles (Fox et al. 1995a), all beetles were isolated in individual 35mm petri dishes without seeds and allowed to mature for 48 hours before use in experiments.

Virgin males and females were weighed immediately before mating and then paired in 5mm wide flat-bottomed glass test tubes with cotton plugs. Mating was videotaped under a dissection scope. After the first mating finished, males were reweighed to determine the amount of mass they lost (a measure of ejaculate size). The male was then mated sequentially to three additional virgin females (a total of four matings), with each mating videotaped and the male reweighed after each mating. After each mating, the female was placed individually in a 100mm petri dish filled with approximately 75 cowpea seeds for 6-10 days, after which females were dissected to quantify bursal scarring. Males were kept in isolated petri dishes in incubators and dissected for aedeagal spine measurements within 6 days of their final mating. I quantified pumping phase and kicking phase durations by marking the time point of intromission (insertion of the aedeagus), onset of kicking by females, and evulsion of the aedeagus in each mating video. I will henceforth refer to the time between intromission and the onset of kicking as the pumping phase duration, and the time from onset of kicking to the separation of mating pairs as the kicking phase duration. Do note that pumping behavior does stop with onset of kicking.

To quantify damage done to the female during mating, the female genital tract was dissected, and both lateral sides of each female bursa were photographed under 50X magnification. Scarring was measured using NIH image software (Abramoff et al. 2004). Lateral sides were chosen for measurement because the majority of scarring was restricted to lateral sides of the bursae. Scar tissue was measured by tracing the visible surface area of haemocyte clusters using the freehand selection tool in NIH ImageJ. Because scarring did not differ significantly between the first and fourth females mated to each male, we did not quantify scarring in second or third females.

The ventral side of each male's aedeagus was photographed using a confocal microscope at 100X magnification, and spine length was measured using NIH image

software (ImageJ). Spine length was calculated as the mean length ( $\mu\text{m}$ ) of the six largest spines for each male (modified from Rönn and Hotzy 2012). We chose to measure lateral spine length because the majority of scarring in bursae was located ventrolaterally.

Sample sizes for first, second, third and fourth matings in each population were  $N=24,24,23,21$  for Ofuya,  $N= 26,24,22,19$  for Mali,  $N= 31,30,26,22$  for Lossa,  $N= 37,31,27,23$  for Benin,  $N= 26,26,22,23$  for Leic, and  $N= 29,28,26,22$  for Zaire.

### Experiment II: Pumping and kicking manipulation experiment.

To investigate how male aedeagal pumping and female kicking affect female fecundity and harm to females, we manipulated either the number of pumps males completed or the duration of kicking in each mating. I tested four hypotheses: (i) that pumping is harmful to females, (ii) that harm to females increases with kicking duration, (iii) that females do not kick until ejaculates are transferred, and (iv) that pumping facilitates the transfer of ejaculatory materials- by manipulating either the number of aedeagal pumps or duration of kicking in each mating and quantifying the effects of pump number and kicking duration on scar tissue in females (i, ii) and fecundity (iii, iv).

Beetles were reared in the same manner as in the previous study. Virgin beetles were isolated in 35mm petri dishes within 12 hours of emergence where they remained for 48-60 hours prior to mating. Males and females were weighed immediately before mating, and then enclosed together in 35mm petri dishes under dissection scopes. Upon intromission, petri dish lids were carefully removed to allow ablation.

Male copulatory rocking was quantified in each mating. Males rock back and forth while in copula even before the onset of female kicking. The pivot point of rocking is located at the two terminal segments of the male abdomen which are compressed and relaxed, squeezing the large pump-like seminal vesicle in the basal abdominal segment, thereby inflating and deflating the aedeagus, apparently through hydrostatic pressure (personal observation). In pilot matings we determined that each rocking cycle coincided with an inflation/deflation cycle. Pumps were counted by watching mating pairs under a dissection microscope. I manipulated number of pumps rather than the duration of the

pumping phase because each pump corresponded to a period of increased pressure against the bursa, and because I was concerned males might vary in rate of pumping. In the pumping manipulation treatments, we manipulated the number of aedeagal pumps in each mating by severing the base of the male's abdomen, as in Edvardsson and Canal (2006), after a predetermined number of pumps (5, 20, 40, or 60). Kicking manipulation treatments were conducted similarly, but instead we waited until females began kicking and severed the base of each male's abdomen after females had kicked for 60, 120 or 180 seconds. The number of pumps prior to female kicking was counted as above, but we did not count pumps during kicking due to how erratically mating couples moved during the kicking phase. We simultaneously euthanized males by severing the cephalic and thoracic ganglion with forceps. After ablation, females were left undisturbed until they removed the male aedeagus from their genital tract, after which they were placed individually into 100mm petri dishes filled with approximately 75 cowpea seeds and allowed to oviposit. Females were transferred to new 100mm dishes of seeds after 48 hours. After 4 days, females were transferred to empty petri dishes and held for two days before dissection (to guarantee haemocyte clusters were visible; Hotzy and Arnqvist 2009) to quantify bursal scarring, as above.

To ensure we isolated the effect of pumping from kicking, the 11 females (3.2% of those in the pumping manipulation treatments) that kicked their mates prior to severing of male genitals were omitted from analyses. Sample sizes for beetles interrupted after 5, 20, 40 and 60 pumps were,  $N=21,20,20,18$  for Ofuya,  $N=17,19,20,21$  for Mali, and  $N=21,23,23,17$  for Leic, and  $N=17,17,17,15$  for Zaire.

Due to the high frequency at which females kicked males out prior to male ablation in kicking manipulation treatments (31%, 77% and 72% in 60, 120 and 180 second treatments for Mali, and 50%, 63% and 53% respectively for Leic), we omitted Mali and Leic from models comparing pumping and kicking treatments. We also omitted the Ofuya 180 second treatment (33% failed to manipulate) and Zaire 120 and 180 second treatments (40% and 42% failed to manipulate respectively) for this same reason. Thus, our kicking treatment analyses only included Zaire females from 60 second (1 of 15 omitted due to failure to manipulate) and Ofuya females from 60 second and 120 second treatments (2 of 19 and 1 of 17 omitted respectively). Sample sizes for 5, 20, 40, 60 pump and both 60 and 120 second kick treatments were  $N=21,20,20,18,17,16$  for Ofuya,  $N=17,17,17,15,14$  for Zaire (As mentioned previously, Zaire's 120 second kick treatment was omitted from analysis).

### Experiment III: Pumping, kicking and transfer of ejaculates.

To investigate the effect of pumping on ejaculate transfer and observe the progress of ejaculates at the onset of female kicking, we terminated matings at fixed pump numbers or the onset of kicking and quantified whether ejaculates had successfully entered the bursa. We tested the hypotheses- that male pumping behavior facilitates the transfer of ejaculates (1), that females kick upon receipt of ejaculates (2), and that females kick after fewer pumps when mating with males from large-spined populations than when mated with males from small-spined populations (3)- by manipulating the number of pumps in each mating.

Beetles were reared and prepared as in the pumping and kicking manipulation studies. Mating pairs were randomly assigned to six treatments; matings were interrupted after 5, 20, 40, 60 pumps, or allowed to mate until the female kicked. Mating events were then terminated at the appropriate pump number by severing the male's basal abdominal segment as above. In contrast to the methods above, both males and females were simultaneously euthanized. We assessed the proportion of males that transferred ejaculate by dissecting male and female reproductive organs in copula and photographing the progress of male ejaculates through the transparent bursal wall using a dissection scope

camera. We characterized ejaculates as transferred as soon as ejaculatory material passed the tip of the aedeagus into the bursa.

Sample sizes for mating pairs interrupted after 5, 20, 40, 60 pumps, or at the onset of kicking, were  $N=21,20,19,18,23$  for Ofuya,  $N=18,15,20,18,18$  for Mali,  $N=18,20,19,19,21$  for Leic, and  $N=18,16,17,16,19$  for Zaire.

## Analysis

### Experiment I

All statistical analyses were run in R version version 3.0.3 (R Development Core Team, 2014). Data from experiment I was analyzed using the ‘lmer’ function in the ‘lme4’ package (Bates et al. 2014) for general linear mixed effect models, and the lmerTest package (Kuznetsova et al. 2014) was used to test hypotheses associated with general linear mixed effect models.

I began analysis with a simple model (trait ~ population + male mating history + male ID) to test whether pumping phase duration, kicking phase duration, ejaculate size, and female scarring varied among populations. I then used a general linear mixed effects models to assess relationships between my dependent variables, pumping phase duration, kicking phase duration, ejaculate size, and female scarring, and independent variables, population, male mating history, male body mass, female body mass, male spine length, male ID (the only random factor), and appropriate interactions (table 1.1). I used the top-down procedure to eliminate variables that were not significantly contributing to the model as in Zurr et al. (2010), but using corrected Akaike information criterion values for best fit model selection. The ‘beyond optimal’ model I started with was of the form  $y \sim$  population + male mating history + male body mass + female body mass + mate dimorphism + male spine length + random effect male ID, except in cases where independent variables overlapped with dependent variables (ejaculate size was not included in the ‘beyond optimal’ model for ejaculate size), or where there was no reason to expect an effect (ejaculate size was not included in the model predicting female

scarring). No transformations were used on data from this experiment, as variables appeared to be reasonably normally distributed within populations.

## Experiments II and III

Data from experiments II and III were analyzed using R's default parameters for general linear models when dependent variables were continuous, as was the case with surface area of female scarring and fecundity. Hypotheses regarding linear models with binomial dependent variables, as in the models assessing the proportion of females scarred, laying eggs, or receiving some ejaculatory material in each treatment, were tested using likelihood ratio statistics. As above, I used the top-down procedure to eliminate variables that were not contributing significantly to each model. The 'beyond optimal' models for this experiment started with the form of  $y \sim \text{population} + \text{treatment} + \text{male body mass} + \text{female body mass} + \text{dimorphism} + \text{population by treatment interaction}$ . The surface area of female scarring was log transformed for this experiment's data set.

## *Results*

### *Experiment I: Variation in female scarring and female response to mate traits*

#### Effects of male traits on the timing of kicking

Immediately after intromission, males begin pumping (the pumping phase of mating) and continue to do so throughout copulation. During this time males are likely transferring ejaculates, and simultaneously pressing their spines against the female's bursa. We define pumping duration as this time period, prior to the onset of female kicking. The duration of the pumping phase of mating increased with each subsequent mating (figure 1A, table 1.1; mate order effect in linear model;  $F_{3,441}=39.6$ ,  $P<0.0001$ ). Averaged across populations, the pumping duration of fourth matings was 133% longer than first matings (when the male was virgin), with the duration of pumping in second and third matings being intermediate between that of first and fourth matings. Pumping duration also varied among populations (figure 1A; pumping duration  $\sim$  population + mate order; population effect;  $F_{5,169}=9.40$ ,  $P<0.0001$ ). The variation among populations

remained significant in our best fit model (table 1.1; population effect in linear model;  $F_{5,167}=9.1$ ,  $P<0.0001$ ); indicating that females from different populations may vary in kicking timing after controlling for male traits. Zaire had the longest pumping durations; on average, 81% longer than Mali, the population with the shortest pumping durations.

As predicted, ejaculate size decreased with each sequential mating (figure 2; table 1.1; main effect in linear model,  $F_{3,574}=248.5$ ,  $P<0.0001$ ); the size of male ejaculates in their first mating were, on average, 346% larger than the size of male ejaculates in their fourth mating. Given the increase in pumping duration with this decrease in ejaculate size, it first appeared that females kick in response to ejaculatory materials. However, after taking into account variation due to male mating history, variation in ejaculate mass did not predict variation in pumping duration (table 1.1; ejaculate mass effect in linear model;  $F_{1,488}=0.05$ ,  $P=0.83$ ). Thus, it is unclear whether ejaculate size cues female kicking. Ejaculate size also varied among populations (ejaculate size  $\sim$  population + mate order; population effect;  $F_{5,620}=11.5$ ,  $P<0.0001$ ). The variation among populations remained significant in our best fit model (table 1.1; population effect in linear model;  $F_{5,574}=7.4$ ,  $P<0.0001$ ); indicating that populations vary in male ejaculate size even after controlling for male size. Virgin males from Ofuya, the population that had the largest ejaculates, transferred ejaculates that were, on average, 36% larger than those transferred by Zaire males, the population with smallest ejaculates (figure 2). The degree of sexual dimorphism predicted the onset of kicking (table 1.1; dimorphism effect in linear model,  $F_{1,516}=9.8$ ,  $P=0.002$ ). The larger a female is in comparison to her mates, the longer she waits before kicking ( $\beta=152.4$  seconds/times larger a female is than her mate,  $t_{516}=2.75$ ,  $P=0.006$ ).

Variation in spine length did not predict variation in pumping duration; the influence of spine length on pumping duration was non-significant when included in the linear model (table 1.1; spine length effect in linear model;  $F_{1,160}=0.8$ ,  $P=0.36$ ), and the among-population variation in pumping duration remained highly significant. Nor did spine length predict ejaculate mass (table 1.1; spine length effect in linear model,  $F_{1,574}=0.4$ ,  $P=0.55$ ). Therefore, we have no evidence to suggest that spine size influences the onset of kicking or that there is a tradeoff between ejaculate size and spine size. As

expected, larger males contributed larger ejaculates (table 1.1; male mass effect in linear model,  $F_{1,574}=40.9$ ,  $P<0.0001$ ). Female size, however, did not influence ejaculate size, and was not included in the best fit model (Likelihood Ratio Test;  $X^2_{14}=15.5$ ,  $P=0.34$ ), and, when added it was non-significant ( $F_{1,573}=2.7$ ,  $P=0.10$ ).

### Kicking duration

The duration of female kicking – the period of time in which females use their hind legs to push males off – also changed with each subsequent mating, generally decreasing in subsequent matings (figure 1B; table 1.1; mate order effect in linear model;  $F_{3,435}=6.0$ ,  $P<0.0001$ ) in contrast to pumping phase duration, which increased in subsequent matings. Kicking duration in fourth matings was 60% shorter on average than in first matings (figure 1B). Kicking duration also varied among populations (kicking duration ~ population + mate order; population effect;  $F_{5,151}=5.2$ ,  $P=0.0002$ ). The variation among populations remained significant in our best fit model after adding relevant covariates (figure 1B; table 1.1; population effect in linear model;  $F_{5,142}=4.6$ ,  $P<0.001$ ); indicating that the duration of persistence/resistance struggles between males and females vary among populations. Mali females took the longest to dislodge their mates; on average kicking for 125% longer than Lossa females, which had the shortest kicking durations. Unlike pumping duration, there was no significant interaction between population and mate number in the best fit model (Likelihood Ratio Test;  $X^2_{15}=21.5$ ,  $P=0.12$ ) and, when added, it was non-significant ( $F_{15,416}=1.5$ ,  $P=0.11$ ). The variation in kicking duration did not support the hypothesis that males with longer spines would resist female kicking for longer periods of time (table 1.1; non-significant spine length effect in the linear model;  $F_{1,135}=1.5$ ,  $P=0.23$ ). This is inconsistent with our prediction that longer spines would help males stay in copula under pressure of female kicking. However, sexual dimorphism in body size contributed to predicting kicking duration (table 1.1; dimorphism effect,  $F_{1,394}=9.0$ ,  $P<0.001$ ). The larger females were in comparison to their mates, the faster they evulsed their mate ( $\beta=-76.6$  seconds/times larger a female is than her mate,  $t_{307}=-3.00$ ,  $P=0.003$ ), indicating that body size may mediate the outcome of struggles over mating duration.

### Spine length and female damage

The size of spines on the male aedeagus varied among populations of *C. maculatus* (figure 3A; one-way analysis of variance,  $F_{5,165} = 13.7$ ,  $P < 0.0001$ ). Zaire and Leic males had the largest spines; their spines were, on average, 48% longer than those of the two smallest-spined populations, Ofuya and Mali (figure 3A). Populations also varied in the amount of scar tissue that females had in their bursa after mating to virgin males (solid circles in figure 3B; table 1.1; population effect in linear model,  $F_{5,141} = 3.7$ ,  $P = 0.004$ ). However, the variation within populations did not support the hypothesis that males with longer spines inflict more harm on their mates; the effect of spine size on bursal damage was non-significant (table 1.1; non-significant effect of spine length on damage;  $F_{1,136} = 0.36$ ,  $P = 0.55$ ) and the relationship between spine size and damage across populations was at best weak. Interestingly, despite there being no significant effect of male spine length on bursal damage in the best fit model, there was an interaction between population and spine length, suggesting that the relationship between spine length and bursal damage *within* populations varies among populations (table 1.1; significant spine length by population interaction in linear model;  $F_{5,138} = 3.0$ ,  $P = 0.01$ ). This can be seen in figure 4A, which shows that the relationship between male spine size and bursal damage in first matings is negative for most populations (contrary to expectations), but slightly positive for Benin. There was also no evidence that mating history of males (virgin males versus thrice previously mated males) influenced the damage done to females (table 1.1;  $F_{1,196} = 2.5$ ,  $P = 0.12$ ). Pumping duration did not influence the amount of harm on females either (table 1.1; pumping duration effect;  $F_{1,257} = 1.1$ ,  $P = 0.29$ ). There was also no evidence that duration of the kicking phase predicted harm; kicking duration was not included in the best fit model to explain bursal damage (table 1.1, Likelihood Ratio Test;  $\chi^2_1 = 1.7$ ,  $P = 0.20$ ), and was non-significant ( $F_{1,254} = 1.7$ ,  $P = 0.20$ ) when added to the best fit model. There was a suggestive trend where male body mass contributed to explaining the variation in bursal damage (table 1.1; male mass effect in linear model;  $F_{1,135} = 135$ ,  $P = 0.02$ ); larger males inflicted more damage than small males (figure 4B).

### *Experiment II: Pumping and Kicking Manipulation Experiment*

The results of the above-reported study found no evidence to suggest spine length, kicking duration, or pumping duration affect damage to females. However, due to the observational nature of this experiment, we were not able to isolate the effects of pumping and kicking duration on damage to females. Thus, we conducted a manipulative experiment consisting of 6 different treatments: where matings were interrupted after 5, 20, 40 or 60 pumps, or allowed to proceed until kicking and then interrupted after kicking for 60 or 120 seconds. All matings in the kicking treatments were allowed to progress longer than any mating in a pumping treatment. With this experiment, we disentangle the effects of pumping and kicking on female fecundity and damage to females.

The previous results also indicated that the amount of time females allow males to pump before kicking is influenced by the size of ejaculates transferred, but it was not clear how pumping behavior related to ejaculate transfer, so we conducted a manipulative experiment (experiment III) to assess the direct effects of male pumping on ejaculate transfer as well.

#### Influence of pumping on scarring and fecundity

The frequency of scarring in female reproductive tracts increased with the number of times males pumped prior to experimental ablation (figure 5A; treatment effect; frequency of scarring  $\sim$  line + treatment + female mass;  $X^2_3=37$ ;  $P<0.0001$ ); females from 60 pump treatments were scarred 158% more often than females from 5 pump treatments (figure 5A). However, the effect of pumping on the amount of female scarring varied among populations (table 1.2; population by treatment interaction in linear model;  $F_{9,289}=3.0$ ,  $P=0.002$ ); the slope at which damage increased across treatments varied among populations, as can be seen in figure 5C. On average, females from 60 pump treatments suffered 448% more scarring than females from 5 and 20 pump treatments while females in the 40 pump treatment had an intermediate amount of scarring. This supports the hypothesis that male pumping behavior is harmful to females; greater amounts of pumping resulted in higher frequency and severity of harm to females. There

was also variation in frequency and severity of damage between populations (figures 5A and 5C; table 1.2; population effect in linear model;  $X^2_3=9.8$ ,  $P=0.02$ , and  $F_{3,289}=9.9$ ,  $P<0.0001$  respectively). Zaire males, one of the two large-spined populations, inflicted harm 67% more often and 82% more severely than the other three populations (figure 5C). As in the previous experiment, Leic males did not inflict more harm than males from small-spined populations despite their large spine size. This suggests that spine length may predict the severity to which a male will harm his mate in some populations, but that factors other than spine length contribute to the degree of damage inflicted on females as well. In contrast to the results of our observational experiment, sexual dimorphism was a significant predictor of harm to females (table 1.2; dimorphism effect,  $F_{1,289}=5.8$ ,  $P=0.016$ ); females had less bursal scarring when they were larger than their mate ( $\beta=-0.23 \mu\text{m}^2$  scar tissue/times larger a female is than her mate,  $t=-2.41$ ,  $P=0.016$ ).

In all four populations measured, the proportion of females that laid at least one egg, and thus were fertilized, varied among treatments (fertilization  $\sim$  population + treatment + male mass,  $X^2_3=183.6$ ,  $P<0.0001$ ). The proportion of females that laid eggs increased with pumping treatment; only 5% of females from 5 and 20 pump treatments laid eggs ( $n=161$ ), while 45% of females in 40 pump treatments laid eggs and 89% of females from 60 pump treatments laid eggs (figure 5B). This suggests that the speed of ejaculate transfer varies among mating pairs, and that sperm is almost always transferred after 40 pumps but rarely before then. Total fecundity of fertilized females also varied and increased with pumping treatment (fecundity  $\sim$  population + treatment + male mass,  $F_{3,104}=4.67$ ,  $P=0.004$ ). Females from 60 pump treatments, on average, laid 42% more eggs than females from 40 pump treatments (figure 5D).

#### Influence of kicking on scarring and fecundity

Contrary to our predictions, allowing matings to progress past the onset of kicking did not consistently increase scarring in female bursae; there was no significant difference in scarring between 60 second and 120 second kicking treatments, nor was

there a significant difference in scar tissue between 60 pump and kicking treatments (figure 7A; Tukey HSD,  $P>0.05$ ). However, females from 60 pump, and both kicking treatments had significantly more scarring than 5, 20 or 40 pump treatments (figure 7A; Tukey HSD,  $P<0.05$ ).

Zaire and Ofuya, the two populations compared here, differed in the amount of scar tissue found in female bursae (figure 7A; bursal scarring ~ population + treatment; population effect;  $F_{1,184}=18.93$ ,  $P<0.0001$ ). Zaire females, on average (5 pump to 60 second kicking treatments), had 141% more scar tissue than Ofuya females. This mirrors the results of our observational study, where Zaire females had more scarring after mating than Ofuya females.

Treatments also varied in female fecundity (figure 7B; fecundity ~ population + treatment + male mass; treatment effect;  $F_{5,91}=9.68$ ,  $P=0.002$ ). As discussed above, females from 5, 20 and 40 pump treatments laid very few if any eggs on average. In contrast, females from the 60 pump and both kicking treatments laid the most eggs. Fecundity increased with treatment, though the increase from 60 pump to kicking treatments was not statistically significant (figure 7B; Tukey HSD,  $P>0.05$ ). All females in kicking treatments were fertilized, supporting our hypothesis that females do not kick until receiving some ejaculatory material. However, as the overwhelming majority of females from 60 pump treatments were fertilized as well, it also appears that females do not immediately begin kicking upon receipt of ejaculatory materials.

### *Experiment III: Pumping, kicking and transfer of ejaculates*

The proportion of matings where male ejaculates progressed past the aedeagus' opening varied across treatments (ejaculate transfer ~ treatment + female mass; treatment effect;  $\chi^2_4=203.0$ ,  $P<0.0001$ ). As predicted, the probability of ejaculate transfer increased with the number of pumps. Ejaculate transfer was rare in 5 and 20 pump treatments, intermediate in 40 pump treatments and frequent in 60 pump and kicking treatments (88%; figure 6A). Ejaculates appear to be transferred at around 40 or more pumps. These results are in line with the oviposition data above, and further support the hypothesis that

pumping facilitates the transfer of ejaculates; however it is possible that another unmeasured behavior may be responsible for this trend as we were unable to disentangle pumping from time. Despite the ejaculate breaching the aedeagus in 88% of 60 pump treatments, females from all populations often waited over 60 pumps before kicking (figure 6B). This disconnect in the timing of female kicking and the frequency of ejaculate transfer across populations further supports the hypothesis that the receipt of ejaculates does not immediately trigger female kicking behavior. However, 6% of females kicked before ejaculatory materials had breached the aedeagus and entered the bursa. Indicating that in some matings females kick prior to the receipt of ejaculates. The number of pumps prior to the onset of kicking varied among populations (pumps to kick  $\sim$  population + male mass; population effect;  $F_{3,272}=7.5$ ,  $P<0.0001$ ); with Mali females differing from the other three populations by receiving more pumps before kicking (figure 6B). Females from short-spined populations did not consistently wait for more pumps than long-spined populations, as Ofuya females did not differ from Zaire and Leic in the number of pumps prior to kicking. Thus, as in our first study, it does not appear that spine length predicts pumping duration. Variance in male mass predicted the number of pumps prior to female kicking (pumps to kick  $\sim$  population + male mass; male mass effect,  $F_{1,272}=11.7$ ,  $P<0.001$ ), and supported the hypothesis that larger males induce kicking faster (with fewer pumps) than smaller males ( $\beta=-13.5$  pumps per mg male mass,  $t_{272}=-3.42$ ,  $P=0.0007$ ).

Populations did not vary in the frequency of ejaculate transfer (ejaculate transfer  $\sim$  population + treatment; population effect;  $X^2_3=1.18$ ,  $P=0.759$ ); furthermore, population was not included in the best fit model (Likelihood Ratio Test;  $X^2_3=1.38$ ,  $P=0.71$ ), and was non-significant when added ( $X^2_3=1.38$ ,  $P=0.71$ ). This parallels the frequency of fertilization results in the pumping and kicking data above and indicates that the speed of ejaculate transfer is similar among populations. The probability of ejaculate transfer varied with female size (ejaculate transfer  $\sim$  treatment + female mass; female mass effect;  $X^2_1=4.7$ ,  $P=0.03$ ). Larger females successfully received ejaculates more often than smaller females ( $\beta=40\%$  more likely per mg female mass,  $z=2.14$ ,  $P=0.033$ ).

## *Discussion*

Sexual conflict over mating drives the evolution of sex traits that improve either sex's ability to shift mating interests back in their favor. This conflict is continuously complicated by the evolution of additional sex traits in either sex. Here, we disentangled the effects of sex traits that improve (ejaculates) or detract (aedeagal spines) from female fitness on the timing and duration of female copulatory resistance. Our results show that the timing of female kicking is influenced by body size and male mating history, but not by ejaculate size or aedeagal spine size. Females mated to virgin males kicked sooner and took longer to evulse their mates than did females mated to previously-mated males. Females also delayed kicking longer, and evulsed their mates faster, when they were larger than their mates. However, variation in aedeagal spine length (despite being harmful to females) and nuptial gift size (despite positively affecting female fecundity) did not influence the timing of female kicking. We found that female kicking does not appear to exacerbate scarring, and confirmed that male pumping behavior is harmful to females, but we were unable to determine whether harm inflicted by pumping triggers female kicking. Thus, it appears that the timing of kicking and duration of struggles over mating duration in *C. maculatus* are explained largely by male pumping behavior, the relative body size of mates, and male mating history.

### Female kicking timing and duration and the effects of male traits

As predicted, male status was a strong predictor of female latency to kicking; females mating with virgin males kicked sooner and took longer to evulse their mate than did females mated to previously-mated males. This shift in female latency to kick may be explained by two hypotheses: First, females may delay kicking when mating to previously-mated males because they are waiting for more ejaculate (satiation hypothesis). Previously-mated males transferred smaller ejaculates than virgin males. Given the fitness benefits of receiving larger ejaculates in *C. maculatus* (Fox 1993, Savalli and Fox 1999, Edvardsson 2007), it would make sense that females wait longer to receive larger ejaculates. Though there was not a statistically significant increase in female fecundity when matings continued past the onset of kicking in our study, other studies have shown longer matings can improve female fecundity (Lieshout et al. 2014).

However, if females were adjusting kicking timing in response to ejaculate size, variation in ejaculate size among males of like-mating status should have predicted kicking timing, but it did not. Furthermore, we noted that when mating pairs were dissected at the onset of kicking, ejaculates had just begun passing through the aedeagus; if females were kicking upon detecting a volume of ejaculate, they would likely wait until ejaculates were in the bursa copulatrix before kicking.

Alternatively, the delay may be due to reduced male vigor in pumping; increasing male exhaustion from mating might influence the effect of pumping on injection of manipulative ejaculatory compounds and/or rate of harm to females. Our manipulative experiment showed that both the frequency and severity of female scarring increased with male pumping. However, there was no significant difference in scarring between females mated to virgin versus previously-mated males despite the prolonged exposure to male pumping; suggesting that the effect of pumping on scarring decreases with male mating status. This reduction in the effect of pumping on harm would theoretically also result in a decreasing hypodermic effectiveness of spines. It is established that aedeagal spines in *C. maculatus* facilitate the transmission of male accessory proteins into female hemolymph (Hotzy et al. 2012) and that some of these accessory proteins induce female refractory periods (Yamane et al. 2015, Yamane and Kimura 2008). If females adjust kicking timing in response to manipulative compounds, reduced hypodermic effectiveness of pumping may be responsible for the increased latency to kicking in previously-mated males. We discuss this hypothesis below. Further supporting the hypothesis that male vigor is responsible for increased kicking latency in previously-mated males, the duration of successful male resistance to female kicking decreased with mate number, and we observed in ablation treatments that females waited far longer to evulse males and that kicking was sluggish (personal observation), more closely resembling the female grooming behavior typically demonstrated post copula. Future research may fully disentangle the effects of male inflicted harm from manipulative seminal proteins by microinjecting accessory protein extracts as in (Yamane et al. 2015), but into females copulating with males of varying mating status, rather than into non-mating females. This design would allow researchers to control for the effect of ejaculate receipt and test its effect on the onset of kicking. Regardless of whether the reason male

status influences female latency to kicking is due to differences in harm or transmission of ejaculates, it is apparent that male mating history affects the timing of female kicking behavior and that the reason for this effect is not necessarily tied to changes in ejaculate size.

Populations varied in both female latency to kicking and duration of kicking. We were not surprised to find this variation as males and females are constantly coevolving traits that assist in manipulating mating interactions in their favor (Arnqvist and Rowe 2013). We were surprised, however, that we found no meaningful interactions between population and any of the traits we measured. It may be that females evolve to adjust latency to kicking, and/or that the populations differed in degree of manipulative effectiveness of and female resistance to ejaculates. Reciprocal crosses of populations differing in latency to kicking and kicking duration might shed light as to whether these differences are due to male and/or female adaptations. For example, if females from high kick latency populations reduce latency to kicking when mated to males from low kick latency populations, it indicates that females are adjusting latency to kicking in response to some male traits.

Interestingly, and perhaps intuitively, sexual dimorphism in body size predicted both latency to female kicking and the duration of kicking. When females were larger than their mates, they waited longer before starting to kick but also dislodged their mates more rapidly than when their mates were similar or larger in size compared to themselves. There are multiple hypotheses that could explain this result: When females are larger than their mates, they may have higher thresholds for ejaculate satiety, may receive less harm from pumping, or it may take longer for manipulative compounds to induce kicking when females are larger than their mates. Though dimorphism did not predict the severity of scarring in experiment I, in which matings were uninterrupted, it did predict the severity of harm in experiment II where we manipulated the number of pumps in matings. The lack of effect of dimorphism on scarring in experiment I may be due to females kicking in response to manipulative compounds; larger females would kick later than smaller females and thus the scarring may equalize between highly and less dimorphic mating pairs. That both male and female size is important in determining

the outcome of persistence and resistance struggles over mating duration has long been established (Rowe 1992), but this is the first evidence of the importance of sexual dimorphism in body size in struggles over mating duration in *C. maculatus*.

Contrary to our predictions, there was no consistent effect of spine length on the timing of kicking; though the effect of spine length on harm varied among populations. Others have shown females evolve resistance or tolerance to increasingly aggressive male traits (Arnqvist and Rowe 2002, Wigby and Chapman 2004, Rönn et al. 2007), and we did not use a reference population for females in these experiments to control for those effects. As in Rönn and Hotzy (2012), we also found there was no evidence that longer genital spines improved male resistance to kicking. This counter-intuitive result suggests that male spine size does not evolve to improve male resistance to kicking. Also contrary to our predictions, male spine length and ejaculate size did not covary. Hotzy (2011) also found this when comparing large and small spined selection lines. It remains puzzling that spine length and ejaculate size do not covary, as larger spines improve injection of seminal compounds into female hemolymph (Hotzy et al. 2012).

#### Effects of pumping and kicking on scarring and fecundity

The reason for female kicking and its effects on female fitness have been the subject of debate (Van Lieshout 2014; Wilson and Tomkins 2014; Crudgington and Siva-Jothy 2000), however it was still unclear whether kicking itself exacerbates harm to females. We compared female scarring and fecundity in matings experimentally terminated before and after female kicking. Damage to females increased as males pumped, and the act of kicking did not significantly modify the degree of scarring in females, rather than increasing the damage to female bursae as suggested by Wilson and Tomkins (2014). Though there was a trend for damage to increase the longer the pair was mated, this appears more likely due to the increased exposure to male pumping rather than kicking. Females in kicking treatments received 50% more pumps than females our highest pump treatment. Thus, unless the effect of pumping changed dramatically prior to kicking, the difference in scarring between the last pump manipulation treatment and kicking manipulation treatments is not likely due to kicking. We also observed that

fecundity increased with kicking duration, as in Van Lieshout et al. (2014), indicating that longer struggles between males and females likely result in larger ejaculates being transferred.

### Why do females kick?

The hypothesis proposed by Van Lieshout et al. (2014) is that females kick to reduce the amount of male manipulative proteins transferred, and thus the male effect on their refractory period, so that they may remate for increased indirect genetic benefits. This assumes the indirect genetic benefits of remating outweigh the direct benefits of receiving larger ejaculates. Theory indicates that the indirect benefits from good genes and sexy sons are minimal when compared to direct fitness costs associated with mating (Arnqvist and Rowe 2013). However, it is possible that females are better able to metabolize manipulative seminal compounds in smaller volumes and optimize their ‘foraging’ for ejaculatory resources by kicking males off and thus minimizing refractory periods. While we also observed that fecundity increased with the duration of kicking, and agree it could be due to the receipt of larger ejaculates, our data do not support this hypothesis. Female kicking did not seem to be cued by the amount of ejaculate received in our experiment (as discussed above). Even if female kicking can be cued by reaching either ejaculatory satiety or some threshold for waiting, then we should have seen variation in ejaculate size among males of like mating status predict the onset of kicking. Furthermore, females waited exceedingly long periods of time before dislodging males that were ablated prior to kicking (as discussed above), indicating pumping itself serves an active role in inducing female kicking.

Instead of females kicking in response to ejaculate size, it seems more plausible that female kicking is a response to manipulative seminal compounds. If females are kicking in response to manipulative compounds in the ejaculate, it would explain why females kick and reduce the size of ejaculates despite the fitness advantages of receiving larger ejaculates (Lieshout et al. 2014); manipulative compounds in the ejaculate can induce behaviors detrimental to females but beneficial to male fitness (Arnqvist and Rowe 2013). An over-functionality of manipulative compounds, where a male trait inflicts direct costs for both males and females by serving its purpose too effectively, may

be responsible for female kicking behavior. Similar mechanisms have been documented in other species; for example, the Acp26Aa seminal protein in *Drosophila melanogaster* induces release of eggs from ovaries faster than optimal, resulting in inefficient fertilization where some eggs are oviposited faster than they can be inseminated (Chapman et al. 2001, Prout and Clark 2000). In this case, manipulative compounds inducing mating refractory periods may have become too effective and began inducing female kicking during copulation. Female mate kicking behavior closely resembles the kicking behavior females use to deter mating attempts during mating refractory periods (personal observation), which can be induced without exposure to aedeagal spines by injecting seminal compounds (Yamane and Kimura 2008); indicating that female kicking is not a response to harmful spines, but to seminal compounds. It is already clear that seminal compounds induce refractory periods in *C. maculatus* (Yamane et al. 2015, Yamane and Kimura 2008), and that genital harm itself does not seem to induce female refractory periods in *C. maculatus* (Morrow et al. 2003). Furthermore, if female kicking is induced by male accessory proteins, then the increased latency to kick in previously-mated males may be explained by a reduction in hypodermic effectiveness via reduced pumping vigor. Our data cannot directly test this hypothesis, and theory has indicated this is a complicated question to answer (Arnqvist and Rowe 2013); nonetheless, our results do imply that female kicking in *C. maculatus* may be a non-adaptive consequence of over-effectiveness in male adaptations for sperm competition (Arnqvist and Rowe 2013), or an adaptation to minimize harm inflicted by a male sperm competition adaptation.

Table 1.1. Results of AICc best fit general linear mixed effects models to explain variation in kicking timing and duration, ejaculate size and female scarring from the matings examined in Experiment I. Models were run using the lmer package in R(v3.0.3) and hypothesis tests conducted with the lmerTest package.

Term	Onset of kicking			Kicking duration			Ejaculate size			Female bursal scarring		
	df(num,den)	F	P	df(num,den)	F	P	df(num,den)	F	P	df(num,den)	F	P
Population	5,167	9.09	<b>&lt;0.0001</b>	5,142	4.56	<b>&lt;0.001</b>	5,574	7.39	<b>&lt;0.0001</b>	5,141	3.67	<b>0.004</b>
Spine length	1,160	0.84	0.360	1,136	1.74	0.180	1,574	0.35	0.554	1,136	0.36	0.548
Pumping duration	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1,257	1.12	0.292
Mate order	3,441	39.58	<b>&lt;0.0001</b>	3,435	5.98	<b>&lt;0.001</b>	3,574	248.50	<b>&lt;0.0001</b>	1,196	2.50	0.115
Male mass	-	-	-	-	-	-	1,574	40.89	<b>&lt;0.0001</b>	1,144	6.60	<b>0.011</b>
Female mass	-	-	-	-	-	-	-	-	-	-	-	-
Dimorphism	1,516	7.55	<b>0.006</b>	1,394	9.02	<b>0.003</b>	-	-	-	-	-	-
Ejaculate mass	1,488	0.06	0.809	1,507	2.63	0.105	N/A	N/A	N/A	N/A	N/A	N/A
Line by spine length interaction	-	-	-	-	-	-	-	-	-	5,139	3.01	<b>0.013</b>
Line by mate order interaction	15,432	2.48	<b>0.002</b>	-	-	-	-	-	-	-	-	-
Pumpingduration by mate order	N/A	N/A	N/A	N/A	N/A	N/A	-	-	-	1,233	4.74	<b>0.030</b>

Table 1.2. Experiment II results of AICc best fit general linear mixed effects models for female scarring. Models were run using the glm function in R(version3.0.3).

Term	Female bursal scarring		
	df(num,den)	<i>F</i>	<i>P</i>
Population	3,289	9.93	<b>&lt;0.0001</b>
Treatment	3,289	2.65	<b>0.049</b>
Dimorphism	1,289	5.83	<b>0.016</b>
Population:Treatment	9,289	3.04	<b>0.002</b>

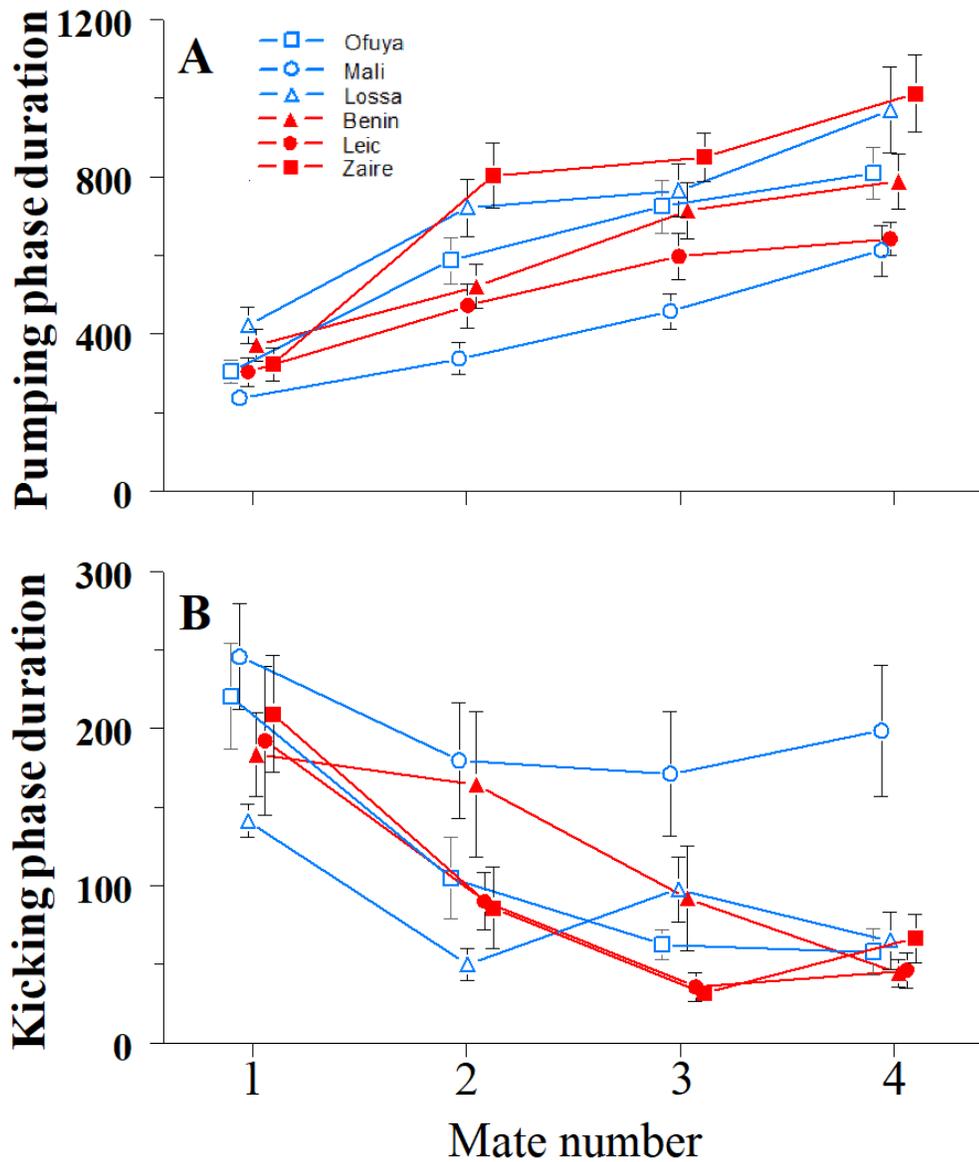


Figure 1. The duration (in seconds) of the pumping (A) and kicking (B) stages of mating in a male's first through fourth mates for six populations of the seed beetle, *Callosobruchus maculatus*. Closed symbols represent longer-spined populations while open symbols represent shorter-spined populations. Error bars represent standard error. A) Pumping durations for all matings for each population. Mean pumping duration increased with mating order for each population. B) Kicking durations for all matings for each population. Mean kicking duration decreased with mating order in all populations.

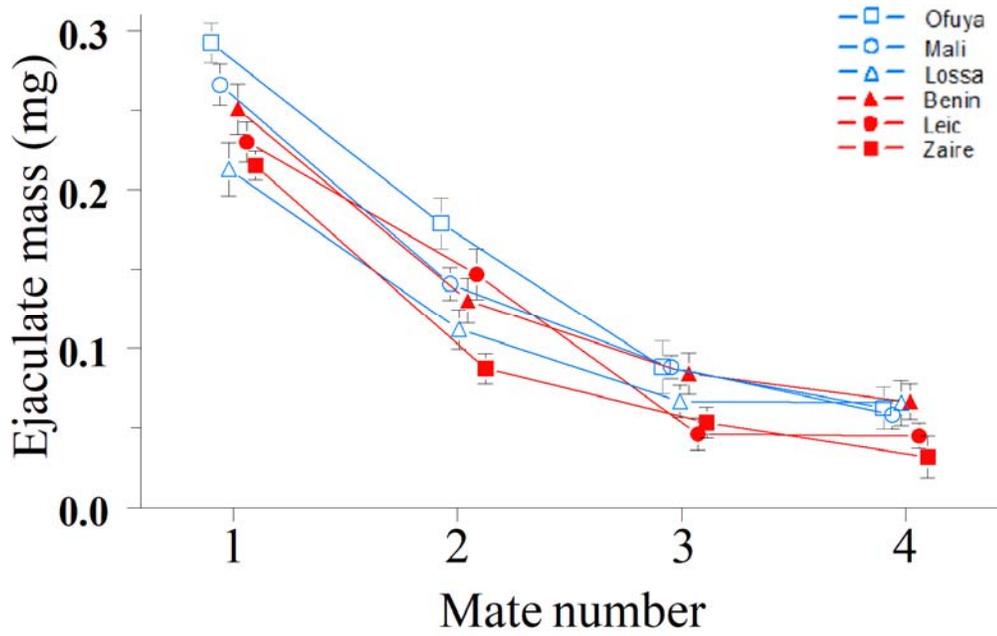


Figure 2. The size of ejaculates for all matings for each population of the seed beetle, *Callosobruchus maculatus*. Populations identified in the legend are ordered from smallest-spined (Ofuya) to largest-spined (Zaire). Error bars represent standard error. Ejaculate size decreased with mating number.

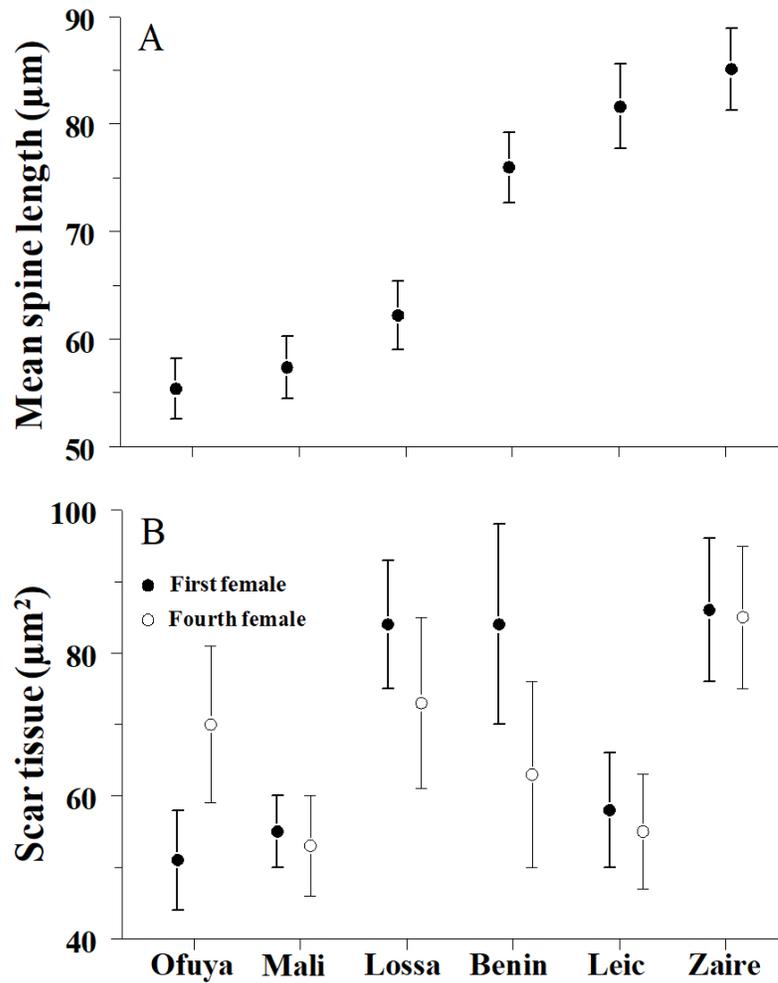


Figure 3. Spine length and damage in experiment I. Aedeagal spine length (A) and damage done to the female bursa (B) for six populations of *Callosobruchus maculatus*. Bars are standard errors. In A, populations are ranked from shortest to longest spines. In B, closed circles (●) are means for damage caused in first matings while open circles (○) are damage caused in fourth matings.

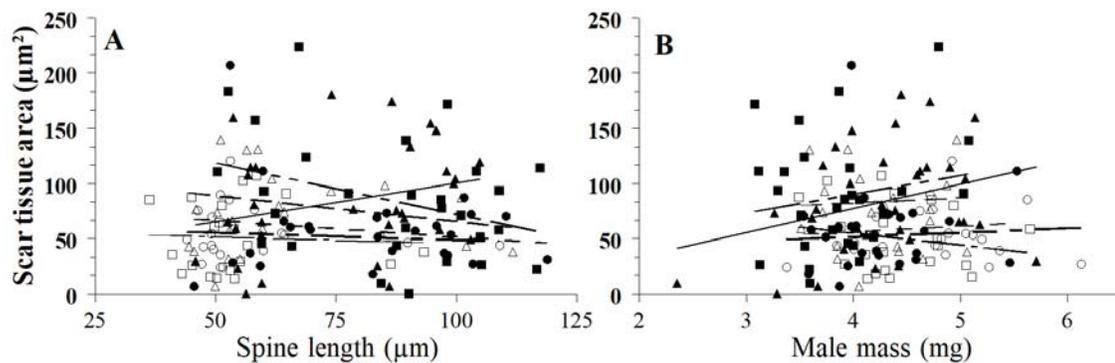


Figure 4. Effects of spine length and male mass on damage in experiment I. A) Regressions of spine length vs damage caused in first matings for the six populations measured. B) regressions of male mass vs damage caused in first and fourth matings for the six populations measured. Open squares represent measurements for Ofuya mating pairs, open circles represent measurements for Mali mating pairs, open triangles represent measurements for Lossa mating pairs, closed triangles represent measurements for Benin mating pairs, closed circles represent measurements for Leic mating pairs, and closed squares represent measurements for Zaire mating pairs. The effect of spine length on female scarring differed significantly among populations. Females mated to larger males had more scarring than females mated to smaller males.

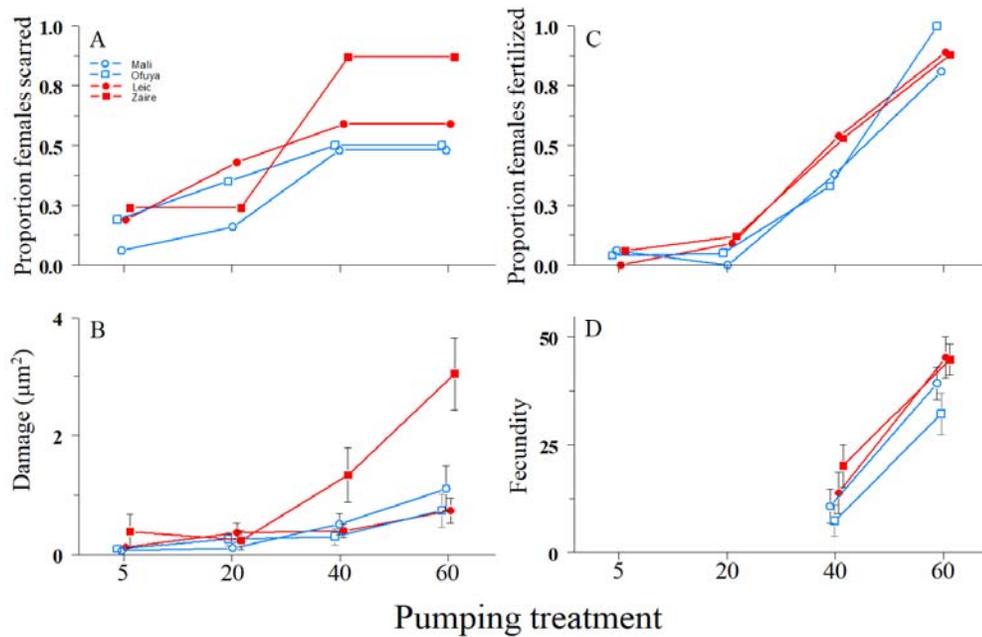


Figure 5. Pumping manipulation treatment results. Symbols represent means of manipulative treatments. Error bars represent standard error. A) Proportion of females with scarring in pumping treatments. B) proportion of females ovipositing in pumping treatments. C) Amount of scar tissue found on female reproductive tracts in pumping treatments. D) Fecundity means for pumping treatments. Fewer than 5 females in 5 and 20 pump treatments laid eggs.

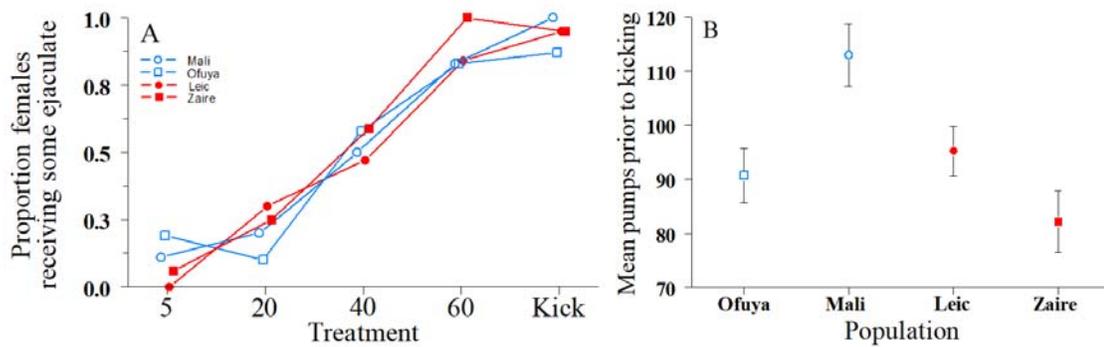


Figure 6. A) Proportion of matings where ejaculates entered female bursae in dissection experiment treatments. B) Mean number of pumps before the onset of kicking pooled from dissection and kicking experiments for each population. Error bars represent standard error. The proportion of matings where ejaculates entered female bursae increased with pump number.

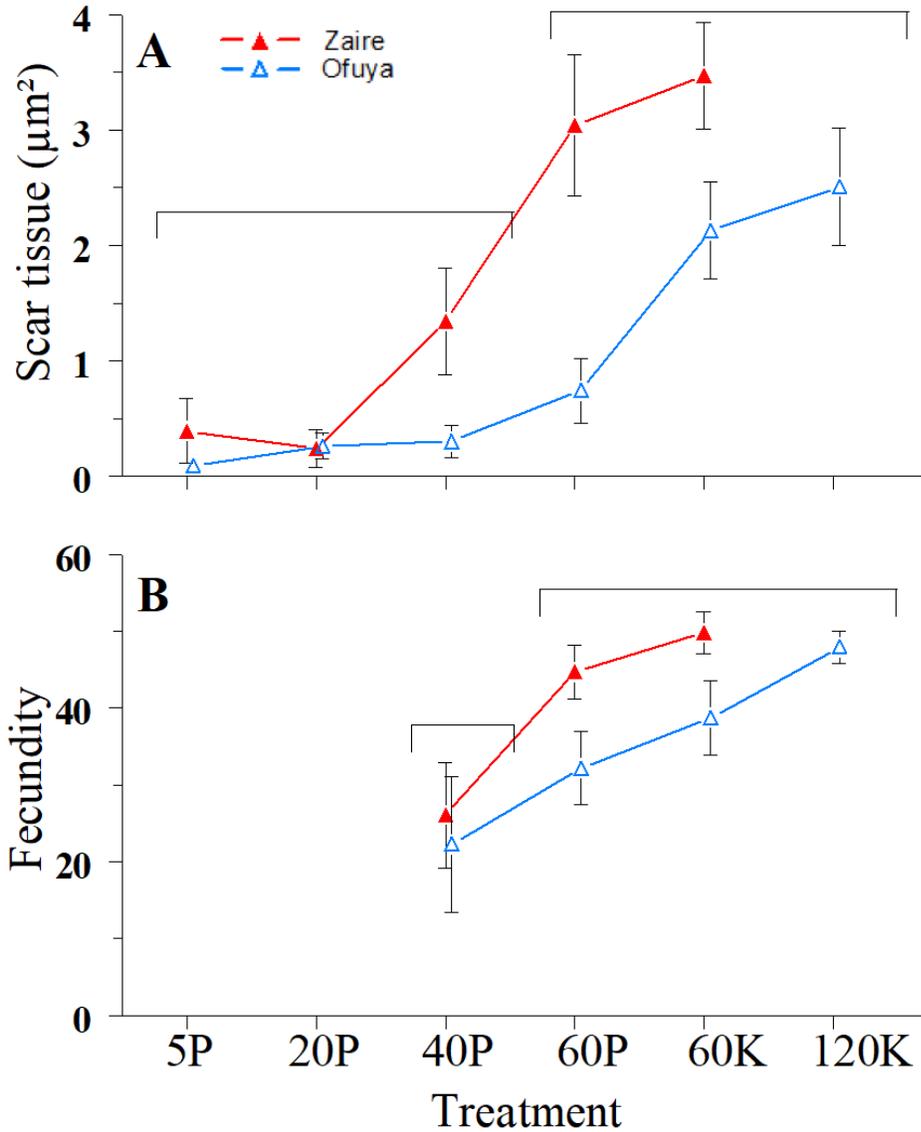


Figure 7. The degree of bursal scarring (A) and fecundity of fertilized females (B) from of the pumping and kicking manipulation study. Treatments are rank ordered with increasing mating duration; P represents pumping manipulation treatment (5, 20, 40 or 60 pumps) while K represents kicking manipulation treatment (60 second or 120 second). Closed red triangles represent treatment means from Zaire females, while open blue triangles represent treatment means for Ofuya females. Error bars represent standard error. Treatments under bars together were not significantly different from one another. Mean scar tissue and fecundity both increased with treatment in both populations. Zaire females had more scarring and laid more eggs than Ofuya females. Fewer than 5 females in 5 and 20 pump treatments laid eggs.

## References

- Al-Wathiqui, Nooria, et al. "Molecular characterization of firefly nuptial gifts: a multi-omics approach sheds light on postcopulatory sexual selection." *Scientific Reports*, 6(2016): 38556.
- Arnqvist, G. (1989). Multiple mating in a water strider: mutual benefits or intersexual conflict? *Animal Behaviour*, 38(5), 749-756.
- Arnqvist, G., & Rowe, L. (2002). Antagonistic coevolution between the sexes in a group of insects. *Nature*, 415(6873), 787-789.
- Arnqvist, G., & Rowe, L. (2013). *Sexual Conflict*. Princeton University Press.
- Arnqvist, G., & Nilsson, T. (2000). The evolution of polyandry: multiple mating and female fitness in insects. *Animal Behaviour*, 60(2), 145-164.
- Boulton, R. A., & Shuker, D. M. (2015). The costs and benefits of multiple mating in a mostly monandrous wasp. *Evolution*, 69(4), 939-949.
- Bates, D., Maechler, M., Bolker, B., & Walker, S. (2014). lme4: Linear mixed-effects models using Eigen and S4. *R package version*, 1(7), 1-23.
- Cayetano, L., Maklakov, A. A., Brooks, R. C., & Bonduriansky, R. (2011). Evolution of male and female genitalia following release from sexual selection. *Evolution*, 65(8), 2171-2183.
- Chapman, T., Herndon, L. A., Heifetz, Y., Partridge, L., & Wolfner, M. F. (2001). The Acp26Aa seminal fluid protein is a modulator of early egg hatchability in *Drosophila melanogaster*. *Proceedings of the Royal Society of London B: Biological Sciences*, 268(1477), 1647-1654.
- Crudgington, H. S., Beckerman, A. P., Brüstle, L., Green, K., & Snook, R. R. (2005). Experimental removal and elevation of sexual selection: does sexual selection generate manipulative males and resistant females? *The American Naturalist*, 165(S5), S72-S87.
- Cordero-Rivera, A. (2016). Sperm removal during copulation confirmed in the oldest extant damselfly, *Hemiphysalis mirabilis*. *PeerJ*, 4, e2077.
- DiRienzo, N., & Marshall, J. L. (2013). Function of the hemolymph nuptial gift in the ground cricket, *Allonemobius socius*. *Ethology*, 119(2), 104-109.
- Eady, P. E., Hamilton, L., & Lyons, R. E. (2007). Copulation, genital damage and early death in *Callosobruchus maculatus*. *Proceedings of the Royal Society of London B: Biological Sciences*, 274(1607), 247-252.

- Edvardsson, M., & Tregenza, T. (2005). Why do male *Callosobruchus maculatus* harm their mates? *Behavioral Ecology*, 16(4), 788-793.
- Edvardsson M, Canal D (2006) The effects of copulation duration in the bruchid beetle *Callosobruchus maculatus*. *Behavioral Ecology* 17: 430–434
- Edvardsson, M. (2007). Female *Callosobruchus maculatus* mate when they are thirsty: resource-rich ejaculates as mating effort in a beetle. *Animal Behaviour*, 74(2), 183-188.
- Edward, D. A., Poissant, J., Wilson, A. J., & Chapman, T. (2014). Sexual conflict and interacting phenotypes: a quantitative genetic analysis of fecundity and copula duration in *Drosophila melanogaster*. *Evolution*, 68(6), 1651-1660.
- Elias, D. O., Sivalingham, S., Mason, A. C., Andrade, M. C., & Kasumovic, M. M. (2014). Mate-guarding courtship behaviour: tactics in a changing world. *Animal Behaviour*, 97, 25-33.
- Fox, C. W. (1993). Multiple mating, lifetime fecundity and female mortality of the bruchid beetle, *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Functional Ecology*, 203-208.
- Fox, C. W., & Jordi, Moya-Laraño (2009). Diet affects female mating behaviour in a seed-feeding beetle. *Physiological Entomology*, 34(4), 370-378.
- Gay, L., Eady, P. E., Vasudev, R. A. M., Hosken, D. J., & Tregenza, T. (2009). Costly sexual harassment in a beetle. *Physiological Entomology*, 34(1), 86-92.
- Gay, L., Hosken, D. J., Eady, P., Vasudev, R., & Tregenza, T. (2011). The evolution of harm—effect of sexual conflicts and population size. *Evolution*, 65(3), 725-737.
- Green, K. K., Kovalev, A., Svensson, E. I., & Gorb, S. N. (2013). Male clasping ability, female polymorphism and sexual conflict: fine-scale elytral morphology as a sexually antagonistic adaptation in female diving beetles. *Journal of the Royal Society Interface*, 10(86), 20130409.
- Gwynne, D. T. (2008). Sexual conflict over nuptial gifts in insects. *Annual Review of Entomology*, 53, 83-101.
- Haubruge, E., Arnaud, L., Mignon, J., & Gage, M. G. (1999). Fertilization by proxy: rival sperm removal and translocation in a beetle. *Proceedings of the Royal Society of London B: Biological Sciences*, 266(1424), 1183-1187.
- Hotzy, C. (2011). *The functional significance of genital spines in males of the seed beetle Callosobruchus maculatus* (Doctoral dissertation, uniwiien).

- Hotzy, C., Polak, M., Rönn, J. L., & Arnqvist, G. (2012). Phenotypic engineering unveils the function of genital morphology. *Current Biology*, 22(23), 2258-2261.
- Krupa, J. J., Leopold, W. R., & Sih, A. (1990). Avoidance of male giant water striders by females. *Behaviour*, 115(3), 247-253.
- Kuznetsova, A., Brockhoff, P. B., & Christensen, R. H. B. (2015). Package 'lmerTest'. *R package version*, 2(0).
- Le Boeuf, B. J., & Mesnick, S. (1991). Sexual behavior of male northern elephant seals: I. Lethal injuries to adult females. *Behaviour*, 116(1), 143-162.
- Lehmann, G. U. (2012). Weighing costs and benefits of mating in bushcrickets (Insecta: Orthoptera: Tettigoniidae), with an emphasis on nuptial gifts, protandry and mate density. *Frontiers in Zoology*, 9(1), 19.
- Lehmann, G. U., & Lehmann, A. W. (2016). Material benefit of mating: the bushcricket spermatophylax as a fast uptake nuptial gift. *Animal Behaviour*, 112, 267-271.
- Lewis, S., South, A., Burns, R., & Al-Wathiqui, N. (2011). Nuptial gifts. *Current Biology*, 21(17), R644-R645.
- Lieshout, E., McNamara, K. B., & Simmons, L. W. (2014). Why do female *Callosobruchus maculatus* kick their mates? *PloS one*, 9(4), e95747.
- Lieshout, E., McNamara, K. B., & Simmons, L. W. (2014). Rapid loss of behavioral plasticity and immunocompetence under intense sexual selection. *Evolution*, 68(9), 2550-2558.
- Mazerolle, M. J. (2011). Model selection and multimodel inference based on QAICc. Version 1.21.
- Morrow, E. H., Arnqvist, G., & Pitnick, S. (2003). Adaptation versus pleiotropy: why do males harm their mates? *Behavioral Ecology*, 14(6), 802-806.
- Pineaux, M., & Turgeon, J. (2017). Behavioural consistency in female resistance to male harassment in a water wtrider species. *Ethology*, 123(1), 83-93.
- Polak, M., & Rashed, A. (2010). Microscale laser surgery reveals adaptive function of male intromittent genitalia. *Proceedings of the Royal Society of London B: Biological Sciences*, rspb20091720.
- Prout, T., & Clark, A. G. (2000). Seminal fluid causes temporarily reduced egg hatch in previously mated females. *Proceedings of the Royal Society of London B: Biological Sciences*, 267(1439), 201-203.

- Reinhold, K., Engqvist, L., Consul, A., & Ramm, S. A. (2015). Male birch catkin bugs vary copula duration to invest more in matings with novel females. *Animal Behaviour*, *109*, 161-166.
- Ronkainen, K., Kaitala, A., & Huttunen, R. (2005). The effect of abdominal spines on female mating frequency and fecundity in a water strider. *Journal of Insect Behavior*, *18*(5), 619-631.
- Rönn, J. L., & Hotzy, C. (2012). Do longer genital spines in male seed beetles function as better anchors during mating? *Animal Behaviour*, *83*(1), 75-79.
- Rönn, J., Katvala, M., & Arnqvist, G. (2007). Coevolution between harmful male genitalia and female resistance in seed beetles. *Proceedings of the National Academy of Sciences*, *104*(26), 10921-10925.
- Rowe, L. (1992). Convenience polyandry in a water strider: foraging conflicts and female control of copulation frequency and guarding duration. *Animal Behaviour*, *44*, 189-202.
- Savalli, U. M., & Fox, C. W. (1998). Genetic variation in paternal investment in a seed beetle. *Animal Behaviour*, *56*(4), 953-961.
- Savalli, U. M., & Fox, C. W. (1999). The effect of male mating history on paternal investment, fecundity and female remating in the seed beetle *Callosobruchus maculatus*. *Functional Ecology*, *13*(2), 169-177.
- Schmid-Hempel, R., & Schmid-Hempel, P. (2000). Female mating frequencies in *Bombus spp.* from Central Europe. *Insectes Sociaux*, *47*(1), 36-41.
- South, A., & Lewis, S. M. (2011). The influence of male ejaculate quantity on female fitness: a meta-analysis. *Biological Reviews*, *86*(2), 299-309.
- Tatarnic, N. J., Cassis, G., & Siva-Jothy, M. T. (2014). Traumatic insemination in terrestrial arthropods. *Annual Review of Entomology*, *59*, 245-261.
- Waage, J. K. (1979). Dual function of the damselfly penis: sperm removal and transfer. *Science*, *203*(4383), 916-918.
- Watson, P. J., Stallmann, R. R., & Arnqvist, G. (1998). Sexual conflict and the energetic costs of mating and mate choice in water striders. *The American Naturalist*, *151*(1), 46-58.
- Wey, T. W., Chang, A. T., Montiglio, P. O., Fogarty, S., & Sih, A. (2015). Linking short-term behavior and personalities to feeding and mating rates in female water striders. *Behavioral Ecology*, *26*(4), 1196-1202.

- Wilson, C. J., & Tomkins, J. L. (2014). Countering counteradaptations: males hijack control of female kicking behavior. *Behavioral Ecology*, *25*(3), 470-476.
- Wigby, S., & Chapman, T. (2004). Female resistance to male harm evolves in response to manipulation of sexual conflict. *Evolution*, *58*(5), 1028-1037.
- Wigby, S., & Chapman, T. (2005). Sex peptide causes mating costs in female *Drosophila melanogaster*. *Current Biology*, *15*(4), 316-321.
- Worthington, A. M., & Kelly, C. D. (2016). Direct costs and benefits of multiple mating: Are high female mating rates due to ejaculate replenishment? *Behavioural Processes*, *124*, 115-122.
- Yamane, T., Miyatake, T., & Kimura, Y. (2008). Female mating receptivity after injection of male-derived extracts in *Callosobruchus maculatus*. *Journal of Insect Physiology*, *54*(12), 1522-1527.
- Yamane, T., & Miyatake, T. (2010). Induction of oviposition by injection of male-derived extracts in two *Callosobruchus* species. *Journal of Insect Physiology*, *56*(12), 1783-1788.
- Yamane, T., Goenaga, J., Rönn, J. L., & Arnqvist, G. (2015). Male seminal fluid substances affect sperm competition success and female reproductive behavior in a seed beetle. *PloS one*, *10*(4), e0123770.
- Zuur, A. F., Ieno, E. N., Walker, N. J., Saveliev, A. A., & Smith, G. M. (2009). Mixed effects models and extensions in ecology with R. Gail M, Krickeberg K, Samet JM, Tsiatis A, Wong W, editors. *New York, NY: Spring Science and Business Media*.

**Wil Licht**  
**Vita**

**Place of Birth:** Wahpeton, North Dakota

**Educational Background:**

B.S., Biology  
University of Minnesota Duluth  
Completed May 2013

**Professional Positions Held:**

August 2013-July 2016  
Graduate Research Assistant, Insect Genetics Lab, University of Kentucky  
[Dr. Charles Fox]

April 2010-August 2013  
Undergraduate Research Assistant, Insect Ecology Lab, University of Minnesota Duluth  
[Dr. Tim Craig]

**Awards and Professional Honors:**

Fall 2013  
University of Minnesota Duluth Swenson College of Science and Engineering Excellence in  
Academics and Research Award

Fall 2012  
University of Minnesota Duluth Swenson College of Science and Engineering Travel Grant  
University of Minnesota Duluth Undergraduate Presentation Funding Award

Undergraduate Research Opportunity Program (UROP) grant

Fall 2011  
Undergraduate Research Opportunity Program (UROP) grant