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**Mechanisms underlying sexual selection and  
sexual conflict in *Drosophila melanogaster***

Thesis submitted for PhD  
by Jon Linklater  
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**“Love is the answer, but while you are waiting for the answer, sex raises some pretty good questions.”**

Woody Allen

*For Mama and Papa*

## Abstract

The revelation that females of many taxa mate with multiple males has broadened perspectives in sexual selection to encompass the study of conflicts of interest between males and females over mating and reproduction. Conflict often exists between the sexes over reproductive decisions such as how often to mate, because the optimum mating rate may often be higher for males than for females. Selection is expected to favour traits in males that manipulate female mating frequency and that confer an advantage over competing males, particularly in relation to post-copulatory competition. A significant fraction of male post-copulatory success in *Drosophila melanogaster* is determined by the action of accessory gland proteins (Acps) that are transferred by males to females during mating. Acps have significant effects on female behaviour and physiology and have been shown to play a role in mediating sexual conflict. Exploring the role of the male accessory glands and of Acps in moulding evolutionary interactions between the sexes and in determining overall fitness remains an important task and one that will illuminate the mechanisms underlying sexual selection and sexual conflict.

In this thesis I use *Drosophila melanogaster* as a model system to ask how reproductive traits, strategies and morphologies have been shaped by sexual selection and sexual conflict. I show that traits related to ejaculate investment and depletion have evolved in lines of *D. melanogaster* with an evolutionary history of exposure to high or low levels of sperm competition and mating opportunities. I then describe how I initiated, propagated and analysed lines selected for increased and decreased male accessory gland size. I describe the direct and correlated responses to selection and show that accessory gland size is associated with male post-copulatory success but not with the magnitude of female mating costs. I further probe the nature of female mating costs by analysing the contribution to them of a single Acp, Acp62F. Finally, I summarise my thesis and discuss future directions for investigating the mechanisms of sexual selection and sexual conflict in *Drosophila*.

## Table of Contents

|  |    |
|--|----|
| Abstract .....   | 4  |
| List of Figures .....  | 10 |
| List of Tables.....  | 13 |
| Declaration .....  | 16 |
| Acknowledgements.....  | 16 |
| Chapter 1. General Introduction.....   | 18 |
| 1.1 Sexual selection .....   | 18 |
| 1.2 The evolution of polyandry .....   | 20 |
| 1.3 Evolutionary consequences of polyandry: how males adapt to female<br>promiscuity .....                     | 21 |
| <u>Adaptations of sperm and of ejaculate size in response to sperm competition</u><br><u>(Chapter 3)</u> ..... | 22 |
| <u>Adaptations in male genitalia in response to sperm competition.</u> .....                                   | 26 |
| <u>Behavioural adaptations in response to sperm competition</u> .....  | 26 |
| <u>Remote mate guarding in response to sperm competition</u> .....   | 27 |
| <u>Conflict between the sexes</u> .....  | 27 |
| 1.4 Evidence of sexual conflict .....  | 29 |
| 1.5 The mating system of <i>Drosophila melanogaster</i> .....  | 30 |
| 1.5.1 <i>Sperm competition in Drosophila melanogaster</i> .....  | 31 |
| 1.5.2 <i>Accessory gland proteins (Acps) of Drosophila melanogaster</i> .....                                  | 31 |
| 1.5.3 <i>Role of Acps in Drosophila melanogaster</i> .....   | 32 |
| <u>Decline in female attractiveness, receptivity to mating and increase in egg<br/>laying</u> .....            | 32 |
| <u>Sperm storage and displacement</u> .....  | 33 |
| <u>Immunological properties of seminal fluid</u> .....   | 34 |
| 1.5.4 <i>Acps, the cost of mating and sexual conflict</i> .....  | 34 |
| <u>Acp62F and the cost of mating in <i>Drosophila melanogaster</i> (Chapter 6)</u> .....                       | 35 |
| 1.6 Outline of Thesis.....   | 37 |
| Chapter 2 General Materials and Methods .....  | 39 |
| 2.1 Fly Culturing.....   | 39 |
| 2.1.1 <i>Bottles and vials (stocks and experiments)</i> .....  | 39 |
| 2.1.2 <i>Standard larval density culture</i> .....   | 39 |
| 2.2 Media.....   | 40 |



|   |   |    |
|---|---|----|
| 2.2.1   | <i>Sugar-Yeast medium</i> .....   | 40 |
| 2.2.2   | <i>Maize-Yeast medium</i> .....   | 40 |
| 2.2.3   | <i>Grape juice medium</i> .....   | 40 |
| 2.3   | <i>Stocks</i> .....   | 41 |
| 2.3.1   | <i>Wild-type Drosophila melanogaster</i> .....  | 41 |
| 2.3.2   | <i>Manipulating sperm competition by experimental evolution of sex ratio (Chapter 3)</i> .....                                      | 41 |
| 2.3.3   | <i>sparkling poliert stock (Chapter 4)</i> .....  | 42 |
| 2.3.4   | <i>Flies lacking Acp62F – gene silencing of Acp62F by RNAi (Chapter 6)</i> ..   | 42 |
| 2.3.5   | <i>Flies lacking Acp62F – null mutants (Chapter 6)</i> .....  | 43 |
| 2.4   | <i>General Methods</i> .....  | 45 |
| 2.4.1   | <i>Mating and courtship observations</i> .....  | 45 |
| 2.4.2   | <i>Egg counts and egg to adult viability</i> .....  | 45 |
| 2.4.3   | <i>Morphological measurements of accessory glands, testis and body size</i> .....   | 45 |
| 2.4.4   | <i>Western Blot analysis</i> .....  | 46 |
| Chapter 3 Ejaculate depletion patterns evolve in response to experimental |   |    |
|   | <i>manipulation of sex ratio in Drosophila melanogaster</i> .....   | 49 |
| 3.1   | <i>Abstract</i> .....   | 49 |
| 3.2   | <i>Introduction</i> .....   | 50 |
| 3.3   | <i>Materials and Methods</i> .....  | 52 |
| 3.3.1   | <i>Stocks and Cultures</i> .....  | 52 |
| 3.3.2   | <i>Experimental procedures</i> .....  | 53 |
| (i)   | <i>Progeny sired by males from the MB and FB lines mated to 5 wild-type virgin females in series</i> .....                          | 53 |
| (ii)  | <i>The time to mating and mating duration of males from the MB and FB lines mated to 5 wild-type virgin females in series</i> ..... | 53 |
| (iii)   | <i>The accessory gland and testis size of males from the MB and FB lines mated to 5 wild-type virgin females in series</i> .....    | 54 |
| 3.3.3   | <i>Statistical analysis</i> .....   | 54 |
| 3.4   | <i>Results</i> .....  | 55 |
| 3.4.1   | <i>Progeny sired by males from the MB and FB lines mated to 5 wild-type virgin females in series</i> .....                          | 55 |

|   |   |    |
|---|---|----|
| 3.4.2   | <i>The time to mating and mating duration of males from the MB and FB lines mated to 5 wild-type virgin females in series</i> | 55 |
| 3.4.3   | <i>The accessory gland and testes sizes of males from the MB and FB lines mated to 5 wild-type virgin females in series</i>   | 55 |
| 3.5   | Discussion  | 65 |
| Chapter 4 Accessory gland size responds to artificial selection and is associated with male reproductive success  |   | 68 |
| 4.1   | Abstract  | 68 |
| 4.2   | Introduction  | 69 |
| 4.3   | Materials and Methods   | 70 |
| 4.3.1   | <i>Artificial selection for large and small accessory glands</i>  | 71 |
| 4.3.2   | <i>Stocks</i>   | 72 |
| 4.3.3   | <i>Direct and correlated responses to selection on male accessory gland size</i>  | 73 |
| 4.3.4   | <i>The effects of selection for accessory gland size on male post-copulatory success</i>                                      | 73 |
|   | (i) The effect of selection for accessory gland size on pre and post-mating competitive male reproductive success             | 73 |
|   | (ii) The effects of selection on post-mating sperm displacement ability   | 74 |
| 4.4   | Results   | 74 |
| 4.4.1   | <i>Direct and correlated responses to selection on male accessory gland size</i>  | 75 |
|   | (ii) Testis size  | 75 |
|   | (iii) Body size   | 76 |
| 4.4.2   | <i>The effects of selection for accessory gland size on male post-copulatory success</i>                                      | 76 |
|   | (i) The effect of selection for accessory gland size on the pre and post-mating competitive male reproductive success:        | 76 |
|   | (ii) The effects of selection on post-mating sperm displacement ability:  | 76 |
| 4.5   | Discussion  | 88 |
| Chapter 5 The effect of accessory gland size on the survival and fitness of female <i>Drosophila melanogaster</i> |   | 92 |
| 5.1   | Abstract  | 92 |
| 5.1   | Introduction  | 93 |
| 5.2   | Materials and Methods   | 95 |

|  |   |     |
|--|---|-----|
| 5.2.1  | <i>Females</i> .....  | 95  |
| 5.2.2  | <i>Males</i> .....  | 95  |
| 5.2.3  | <i>The effect of accessory gland size on female survival</i> .....  | 95  |
| 5.2.4  | <i>The effect of accessory gland size on female fecundity and egg to adult viability</i> .....  | 95  |
| 5.2.5  | <i>Mating and courtship frequency</i> .....   | 96  |
| 5.3  | <b>Results</b> .....  | 97  |
| 5.3.1  | <i>The effect of accessory gland size on female survival</i> .....  | 97  |
| 5.3.2  | <i>The effect of accessory gland size on female fecundity and egg to adult viability</i> .....  | 97  |
| 5.3.3  | <i>Mating and courtship frequencies</i> .....   | 98  |
| 5.4  | <b>Discussion</b> .....   | 103 |
| <b>Chapter 6 The effect of Acp62F delivery on female lifespan and reproductive success</b> ..... |   | 106 |
| 6.1  | <b>Abstract</b> .....   | 106 |
| 6.2  | <b>Introduction</b> .....   | 107 |
| 6.3  | <b>Materials and Methods</b> .....  | 109 |
| 6.3.1  | <i>Generation of Acp62F RNAi knockdown and control males</i> .....  | 109 |
| 6.3.2  | <i>Generation of Acp62F null mutants</i> .....  | 110 |
| 6.3.3  | <i>Acp62F levels in RNAi knockdown, null and control males</i> .....  | 110 |
| 6.3.4  | <i>Acp62F RNAi experiment 1: Effect of Acp62F on female fitness</i> .....   | 110 |
| 6.3.5  | <i>Acp62F RNAi experiment 2: Effect of Acp62F on female fitness</i> .....   | 111 |
| 6.3.6  | <i>Acp62F null experiment: Effect of Acp62F on female fitness</i> .....   | 112 |
| 6.4  | <b>Results</b> .....  | 112 |
| 6.4.1  | <i>Acp62F levels in RNAi knockdown, null and control lines</i> .....  | 112 |
| 6.4.2  | <i>Acp62F RNAi experiment 1: Effect of Acp62F on female fitness</i> .....   | 113 |
| 6.4.3  | <i>Acp62F RNAi experiment 2: Effect of Acp62F on female fitness</i> .....   | 114 |
| 6.4.4  | <i>Acp62F null experiment: Effect of Acp62F on female fitness</i> .....   | 115 |
| 6.5  | <b>Discussion</b> .....   | 131 |
| <b>Chapter 7 General discussion of the thesis and future research directions</b> .....           |   | 134 |
| 7.1  | <b>Introduction – summary of the thesis work</b> .....  | 134 |
| 7.2  | <b>Ejaculate depletion patterns in male <i>Drosophila melanogaster</i> selected under increased and decreased levels of sperm competition (Chapter 3)</b> ..... | 135 |
| 7.3  | <b>Accessory gland size responds to selection and is associated with male reproductive success (Chapter 4)</b> .....  | 138 |

|     |   |     |
|-----|---|-----|
| 7.4 | The effect of accessory gland size on the survival and fitness of female<br><i>Drosophila melanogaster</i> (Chapter 5) .....                    | 140 |
| 7.5 | The effect of Acp62F delivery on female lifespan and reproductive success<br>(Chapter 6).....   | 142 |
|     | References .....  | 145 |
|     | Appendix I Ejaculate depletion patterns evolve in response to experimental<br>manipulation of sex ratio in <i>Drosophila melanogaster</i> ..... | 161 |
|     | Appendix II Mating and immunity in invertebrates .....  | 170 |

## List of Figures

|   |     |
|---|-----|
| <b>Figure 2.1</b> The crosses used to produce Acp62F knockdown and control males.....   | 47  |
| <b>Figure 2.2</b> Construction of control and experimental males for the Acp62F null experiment.....  | 48  |
| <b>Figure 3.1</b> Mean ( $\pm$ SE) total number of progeny produced by males from the MB and FB lines mated in series to 5 virgin wild type females.....  | 57  |
| <b>Figure 3.2</b> Median ( $\pm$ interquartile range) time to mating of each mating for females 1-5 mated to FB and MB males. ....  | 58  |
| <b>Figure 3.3</b> Median ( $\pm$ interquartile range) mating duration of each mating for females 1-5 mating with MB and FB males.....   | 59  |
| <b>Figure 3.4</b> (a) Mean ( $\pm$ SE) accessory gland size ( $\text{mm}^2$ ) and (b) testes size for males from FB and MB lines that were either not mated or mated to 5 females in series.....  | 60  |
| <b>Figure 4.1</b> Direct response to bi-directional selection on male accessory gland size plotted as mean accessory gland size ( $\text{mm}^2$ ) against generation of selection..   | 78  |
| <b>Figure 4.2</b> Relative accessory gland size (mean $\pm$ SE) of large, small and unselected accessory gland selection lines at generation 16 and 38, plotted as relative accessory gland size (accessory gland size/body size). .... | 79  |
| <b>Figure 4.3</b> Relative testis size (mean $\pm$ SE) of large, small and unselected accessory gland selection lines at generation 16 and 38, plotted as relative testis size (testis size/body size). ....                            | 80  |
| <b>Figure 4.4</b> Body size (mean $\pm$ SE) of large, small and unselected accessory gland selection lines at generation 16 and 38. ....  | 81  |
| <b>Figure 4.5</b> Total progeny (mean $\pm$ SE) sired by large, unselected or small accessory gland selection line males in competition with <i>sparkling</i> males..   | 82  |
| <b>Figure 5.1</b> The effect of exposure to large, small and unselected accessory gland line males on female lifespan. ....   | 99  |
| <b>Figure 5.2</b> Age-specific egg production and viability of eggs of wild-type females continuously exposed to large, small or unselected accessory gland males. ...  | 100 |

- Figure 6.1** Western Blot analysis of Acp62F levels in (a) RNAi knockdown (*Acp26AaP-Gal4; UAS-Acp62F-IR1b*, *Acp26AaP-Gal4; UAS-Acp62F-IR3A*) and control males (*Acp26AaP-Gal4; UAS-Acp62F-IR3C*, *Acp26AaP-Gal4; +*) and (b) *Acp62F* null and control males. .... 118
- Figure 6.2** Effect of Acp62F on female lifespan (RNAi experiment 1). Cumulative survival probability of females mated to Acp62F knockdown (*Acp26AaP-GAL4; UAS-Acp62F-IR1b* and *Acp26AaP-GAL4; UAS-Acp62F-IR3A*) or control (*Acp26AaP-GAL4; UAS-Acp62F-IR3C*) males, against time (days). .... 119
- Figure 6.3** Age-specific egg production (a) and viability (b) of eggs laid by wild-type females continuously exposed to Acp62F knockdown or control males (RNAi experiment 1. (a) median ( $\pm$  inter-quartile range) number of eggs laid per 24 hours by females continuously exposed to knockdown (*Acp26AaP-GAL4; UAS-Acp62F-IR1b* and *Acp26AaP-GAL4; UAS-Acp62F-IR3A*) and control (*Acp26AaP-GAL4; UAS-Acp62F-IR3C*) males (b) median ( $\pm$  inter-quartile range) egg-adult viability for the eggs laid by the females shown in (a). .... 120
- Figure 6.4** Effect of Acp62F on female lifespan (RNAi experiment 2). Cumulative survival probability of females mated to Acp62F knockdown (*Acp26AaP-GAL4; UAS-Acp62F-IR1b* and *Acp26AaP-GAL4; UAS-Acp62F-IR3A*) and control (*Acp26AaP-GAL4; UAS-Acp62F-IR3C* and *Acp26AaP-GAL4; +*) males, against time (days). .... 121
- Figure 6.5** Age-specific production of eggs laid by females continuously exposed to Acp62F knockdown (*Acp26AaP-GAL4; UAS-Acp62F-IR1b* and *Acp26AaP-GAL4; UAS-Acp62F-IR3A*) or control (*Acp26AaP-GAL4; UAS-Acp62F-IR3C* and *Acp26AaP-GAL4; +*) males (RNAi experiment 2) (median  $\pm$  inter-quartile range number of eggs laid per 24 hours). .... 122
- Figure 6.6** Effect of Acp62F on female lifespan (Acp62F Null experiment). Cumulative survival probability of females mated to Acp62F knockout (*Acp62F<sup>lbb</sup>*, *Acp62F<sup>lbc</sup>*, *Acp62F<sup>lc</sup>*) and control (*Acp62F<sup>Tandem</sup>*, *Acp62F<sup>lcCtrl</sup>*) males, against time (days). .... 123
- Figure 6.7** Age-specific egg production and viability of eggs of wild-type females continuously exposed to Acp62F knockout or control males (Acp62F null experiment). (a) median ( $\pm$  inter-quartile range) number of eggs laid per 24 hours by females continuously exposed to Acp62F null (*Acp62F<sup>lbb</sup>*, *Acp62F<sup>lbc</sup>*, *Acp62F<sup>lc</sup>*) or control (*Acp62F<sup>Tandem</sup>*, *Acp62F<sup>lcCtrl</sup>*) males, (b) median ( $\pm$  inter-

quartile range, error bars) egg-adult viability for the eggs laid by the females  
shown in (a). ..... 124

## List of Tables

|  |    |
|--|----|
| <b>Table 3.1.</b> Effect sizes of linear mixed effects ANOVA model (by REML) on the total number of progeny sired by FB and MB males mated to 5 wild-type virgin females in series.....  | 61 |
| <b>Table 3.2.</b> Effect sizes of linear mixed effects ANOVA model (by REML) on the mating duration (log transformed) of FB and MB males mated to 5 wild-type virgin females in series.....  | 62 |
| <b>Table 3.3.</b> Mixed effects ANOVA of (a) body (wing), (b) relative testis and (c) relative accessory gland size of unmated males from replicate MB and FB lines, with selection regime (fixed effect) and replicate line nested within selection regime (random effect). ....  | 63 |
| <b>Table 3.4.</b> Effect sizes of mixed effects ANOVA of (a) absolute accessory gland size and (b) absolute testis size (log transformed) of males from replicate MB and FB lines that were not mated or that were mated to 5 females in series, with status (not mated versus mated 5 times in succession without recovery), selection regime and status x regime (fixed effects) and replicate line nested within selection regime (random effect). .... | 64 |
| <br><b>Table 4.1</b> Nested ANOVA of relative accessory gland size from the large, small and unselected lines at (a) generation 16 and (b) generation 38, with selection regime (fixed effect) and replicate line nested within selection regime (random effect). ....   | 83 |
| <b>Table 4.2</b> Nested ANOVA of body size of males from the large, small and unselected lines artificially selected for male accessory gland size at (a) generation 16 and (b) generation 38, with selection regime (fixed effect) and replicate line nested within selection regime (random effect).....   | 84 |
| <b>Table 4.3</b> Mating frequencies of <i>sparkling</i> females mating with selection line and <i>sparkling</i> males. ....  | 85 |
| <b>Table 4.4a</b> General Linear Model of progeny sired by males from large, unselected and small males in competition with <i>sparkling</i> males with regime set as a factor.....  | 86 |
| <b>Table 4.4b</b> Nested ANOVA of total progeny sired by males from the large, small and unselected lines, with selection regime (fixed effect) and replicate line nested within selection regime (random effect).....   | 86 |



|   |     |
|---|-----|
| <b>Table 5.1</b> General Linear Model analysis on (a) the number of eggs laid by females mated to males from large, unselected or small accessory gland lines and (b) the egg to adult viability of those eggs with replicate, Day and the interaction between replicate and Day as factors.....  | 101 |
| <b>Table 5.2</b> Mating and courtship frequencies of between females continuously housed with males selected for large, small or unselected accessory gland size. ....  | 101 |
| <b>Table 6.1</b> General Linear Model analysis of (a) the number of eggs laid by males mated to males with knocked down or control Acp62F levels and (b) the egg to adult viability of those eggs (RNAi experiment 1).....  | 125 |
| <b>Table 6.2</b> General Linear Model analysis of the number of eggs laid by males mated to males with knocked down or control Acp62F levels in RNAi experiment 2.....  | 126 |
| <b>Table 6.3</b> General Linear Model analysis on (a) the number of eggs laid by females mated to each of the groups in the Acp62F null experiment and (b) the egg to adult viability of those eggs. Group and the interaction between group and day are set as factors .....   | 127 |
| <b>Table 6.4</b> Courtship and mating frequencies of females continuously housed with Acp62F RNAi knockdown and control males (RNAi experiment 1). Chi-square tests were used to test for differences in (a) total mating and (b) total courtship frequencies for females mated to Acp62F knockdown ( <i>Acp26AaP-GAL4;UAS-Acp62F-IR1b</i> , <i>Acp26AaP-GAL4;UAS-Acp62F-IR3A</i> ) and control ( <i>Acp26AaP-GAL4;UAS-Acp62F-IR3C</i> ) males.....   | 125 |
| <b>Table 6.5</b> Courtship and mating frequencies of females continuously housed with Acp62F RNAi knockdown or control males (RNAi experiment 2). Chi-square tests were used to test for differences in (a) total mating and (b) courtship frequencies for females housed with <i>Acp26AaP-GAL4;UAS-Acp62F-IR1b</i> or <i>Acp26AaP-GAL4; +</i> males; or for differences in (c) total mating and (d) courtship frequencies for females housed with <i>Acp26AaP-GAL4;UAS-Acp62F-IR3A</i> or <i>Acp26AaP-GAL4;UAS-Acp62F-IR3C</i> males. .... | 129 |
| <b>Table 6.6</b> Courtship and mating frequencies of females continuously housed with Acp62F null and control males. Chi-square tests were used to test for differences in (a) total mating and (b) total courtship frequencies for females   |     |

mated to Acp62F null ( $Acp62F^{lbB}$ ,  $Acp62F^{lbC}$ ,  $Acp62F^{lc}$ ) or control  
 ( $Acp62F^{Tandem}$ ,  $Acp62F^{lcCtrl}$ ) males..... 130

## **Declaration**

I declare that the work presented in this thesis is my own except where duly noted.

.....

Jon Linklater

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## **Chapter 1. General Introduction**

### **1.1 Sexual selection**

Darwin's (1871) recognition that male–male competition and female choice could favour the evolution of exaggerated male traits detrimental to male survival remains among the greatest insights in evolutionary biology. The paradigm of sexual selection attempts to explain the existence of sex-specific characters and how the variation within those secondary sexual characters determines the mating and reproductive success of (usually) males. Darwin's (1871) thesis, and the subsequent (traditional) models of sexual selection that followed much later (e.g. Fisher 1930; Lande 1981; Kirkpatrick 1982; Kokko et al. 2002), sought to explain the evolution of sexual traits (mainly in males), the preference for such traits (mainly by females) and how variation within such traits determines mating and reproductive success. Males tend to be the competing sex and females the choosing sex because receptive females tend to be scarce and represent a limited resource thereby creating a male-biased operational sex ratio (the ratio of sexually receptive males to receptive females) (Emlen and Oring 1977). Females are constrained by the time and resources required to make large eggs and/or by substantial investment in parental care (Trivers 1972). Males on the other hand produce smaller gametes, sperm, which may be energetically cheaper per unit to produce (i.e. each individual sperm is cheaper to produce than each egg). Even considering that ejaculates as a whole are likely to be more costly to produce than has traditionally been thought (Dewsbury 1982), the differences in per gamete investment and parental care lead to a disparity in reproductive potential, with males having a higher potential reproductive rate i.e. they can fertilise significantly more eggs than are contained within a single female. In turn, the difference in the number of receptive mates (i.e. there are more receptive males than females) leads to competition among males for access to relatively few sexually receptive females, and allows females to choose among competing males (Trivers 1972).

Male-male competition and female choice can be important drivers of evolutionary change and their results can be manifested in extraordinary adaptations. For example, males of many bird species often possess elaborate or large plumage displays that serve to attract females but which may have effects that are counter to

survival. Darwin and his successors studied the processes of pre-copulatory selection and why such traits should exist, framing the processes of sexual selection in terms of competition and conflict between members of the same sex (usually males) or on the preference (usually by females) for 'quality/attractive' mates, generating a paradigm of study that persists today. With the behavioural ecology revolution of the 1970s and the appreciation of selection acting at an individual level (Dawkins 1976; Parker 1979; Trivers 1972) came an understanding of the conflicts of interests that might exist between males and females. For example, the importance of post-copulatory sexual selection was considered in the conflicts between males that extend beyond the point of copulation and in the importance of paternity assurance and the morphological, behavioural and ejaculate characteristics that might be shaped by such selection pressures (Parker 1970). The traditional perspectives on sexual selection, those of a cooperative and harmonious relationship between males and females, were also challenged by the idea that some sexually selected characters could be shaped by conflicts of interest between males and females (e.g. Dawkins 1976; Parker 1979; Trivers 1972). Following advances in molecular techniques (Burke and Bruford 1987) which revealed the frequency of female promiscuity, theoretical and empirical attention in the 1990s explored the possibility that antagonistic intersexual conflict and coevolution could drive the evolution of male and female reproductive traits.

The revelation that females of many taxa mate with multiple males has shifted perspectives in sexual selection to consider how males are expected to adapt to female promiscuity. An appreciation of the conflicts of interest over mating and reproduction has inspired a reinterpretation of the evolutionary dynamic between males and females. Males and females have divergent interests in reproduction. Selection is expected to favour traits in males that manipulate, for example, female mating frequency and that confer advantage over competing males, particularly in relation to post-copulatory competition because such traits will result in greater fitness for males possessing the trait. An increasing number of organisms have been used to explore manifestations of sexual conflict over a range of reproductive decisions and its influence on mating systems (Chapman et al. 2003b). Of particular interest are the conflicts identified in *D. melanogaster* since the genetics and mating system of this species are relatively well understood and many genetic and molecular tools exist and are being developed to investigate research questions in

this area. Males transfer cocktail of seminal fluid proteins that have dramatic consequences for female behaviour, physiology and reproduction and which can also reduce female survival (Chapman et al. 1995). Exploring this conflict, the role of the seminal fluid, and particularly *accessory gland proteins* (Acps) (a diverse and often rapidly evolving set of proteins that are transferred to females during mating and that have dramatic effects on male and female fitness) as traits intimately involved with fitness and the evolutionary interactions between the sexes, remains an important challenge in elucidating the mechanisms underlying sexual selection and sexual conflict.

## **1.2 The evolution of polyandry**

Females of many animal species mate with more than one male during their lifetime (Jennions and Petrie 2000; Zeh and Zeh 2001). This is puzzling given females can often obtain all the sperm they require to fertilise all of their eggs in one mating. While males can generally increase their fitness by mating with many mates (Bateman 1948), females may not need to mate multiply to maximize their lifetime reproductive success. However, females of most species do mate multiply either with the same male or with different males (polyandry) suggesting that females may benefit and increase their fitness by multiple mating. Although females may receive sufficient sperm from a single mating to fertilize their eggs, other substances transferred with sperm, for example Acps, may provide cues about male quality or about the size of other direct benefits. The presence of sperm and accessory gland products, for example, can increase female egg production and fertility and this may be sufficient to explain the evolution of polyandry despite the negative effects of remating (Arnqvist and Nilsson 2000). In species with nuptial feeding direct benefits may be more obvious. Theoretical studies suggest that females may benefit indirectly by providing genetic benefits that increase offspring fitness through good genes or increasing genetic diversity (for a review see Simmons 2005; Yasui 1998). For example, females may employ a 'bet-hedging' strategy against genetic incompatibility of maternal and paternal haplotypes (Simmons 2005; Yasui 1998) or as a way of avoiding inbreeding (Tregenza and Wedell 2000). Genetic compatibility may also be important in species in which selfish genetic elements are abundant (Zeh and Zeh 1996, 1997), for example, those species in which the maternally inherited *Wolbachia* can generate cytoplasmic incompatibility resulting in embryo

cessation (Charlat et al. 2007). Such sex ratio distortion in *Drosophila* (Atlan et al. 2004) and stalk-eyed flies (Wilkinson and Fry 2001) decreases sperm competition ability in Wolbachia-infected males, so females benefit from multiple mating which increases their fertility (Wilkinson et al. 2003).

Whatever the explanation invoked to explain the evolution of polyandry, the consequences for males include conflicts with females, for example over (re)mating rates, and with rival males in sperm competition. Inspired in large part by the pioneering work of Geoff Parker in the 1970s, both sperm competition and sexual conflict have received much theoretical and empirical attention in recent times, particularly with the advance of genetic and molecular techniques which have allowed researchers to start to reveal the mechanisms underpinning both phenomena. The following sections describe the evolutionary consequences of female promiscuity for males and examine some of the ways in which males have adapted in response to conflicts between and within the sexes.

### **1.3 Evolutionary consequences of polyandry: how males adapt to female promiscuity**

The acquisition of mating partners or mating opportunities is not sufficient to provide males with high reproductive success. In the widespread, perhaps ubiquitous, situations where females mate with more than one partner, males must also compete for access to fertilisation opportunities after copulation, i.e. male sperm must compete to be stored and to be used in fertilisations, in preference to any sperm of competing males. Parker (1970) first realised the existence, and evolutionary significance, of sexual selection through sperm competition in his studies on the yellow dung fly, *Scathophaga stercoraria*. He recognised that competition between males for fertilisations will continue after mating if the sperm of two or more males overlap near the site of fertilisation in females. The process of sperm competition is defined as 'the competition between the sperm of two or more males for the fertilisation of a given set of ova' (Parker 1970; Parker 1998). Sperm competition is taxonomically widespread and has been reported in birds, mammals, fish, amphibians, reptiles and invertebrates, and is particularly common in the insects (see Birkhead and Møller 1998). Much attention in sperm competition research has focussed on insects because of the prevalence in this group of multiple



mating and because females often store viable sperm for long periods. These features allow the sperm from different males to be stored concurrently. However, despite nearly 40 years of experimental research, the precise mechanisms by which sperm of one male out-compete the sperm of rival male are unclear even when there are techniques available to directly visualise sperm in competition (Civetta 1999; Price et al. 2000). Although the exact mechanisms of sperm competition remain elusive, sperm competition is widely recognised as a major and pervasive force in evolution. Sperm competition generates adaptations that enable males to pre-empt potential threats to paternity or that facilitate success when sperm are in competition. Males may increase their success in sperm competition by making a greater investment in sperm size or number (Parker 1990a), investing in different types of sperm (Cook and Gage 1995), by delaying remating by females (e.g. Gillott 2003) or by donating nuptial gifts (Engqvist and Sauer 2001). In the following section, I consider how males have adapted in the face of sperm competition and sexual conflict arising from female promiscuity.

### *1.3.1 Adaptations of males to sperm competition*

#### *Adaptations of sperm and of ejaculate size in response to sperm competition* *(Chapter 3)*

When the sperm of two or more males are present within the female reproductive tract, sexual selection will favour adaptations that increase the probability that sperm are used in fertilisations. Perhaps the most obvious selection pressure is that which acts to increase or decrease sperm number (Parker 1982). In this regard, the response to selection arising from sperm competition has been assessed by testing for an increase in male ejaculate investment (the number of sperm transferred per mating) with elevated sperm competition risk (the probability the females in the population have mated or will mate again) and/or elevated sperm competition intensity (the average number of males competing for a given set of eggs; Parker 1982). Inter-specific comparative studies have tested for an association between the level of sperm competition experienced and the relative level of investment in spermatogenesis. The theoretical prediction is that male investment in ejaculates (and therefore reproductive tissue) should increase with increasing risk of sperm competition. The data are consistent with this expectation; males in polyandrous

mating systems have relatively larger testes (primates, Harcourt et al. 1981; birds, Møller and Ninni 1998; amphibians, Jennions and Passmore 1993; butterflies, Gage 1994; Karlsson 1995; bats, Hosken 1997; fish, Stockley et al. 1997) and are hence capable of producing more sperm (Gage 1994; Møller 1988). For example, Stockley et al. (1997) contrasted closely related fish species that differed in the intensity of sperm competition (assessed according to the degree of polygamy or communal spawning) and found positive associations between sperm competition intensity and relative gonad weight and sperm numbers. (Hosken 1997) also found a positive association between testis mass and social group size (an indicator of sperm competition risk) in bats. In insects, a simple association between female mating frequency and testis size might not be expected given the diversity of sperm competition mechanisms (Simmons and Siva-Jothy 1998). For example, sperm number may be less important where sperm displacement may occur by mechanical means (Waage 1979). Selection could also act on accessory gland size rather than testis size if seminal secretions rather than sperm are involved in sperm displacement. Nevertheless there are species in which associations between mating frequency and testis size do exist. Gage (1994) found that in 74 species of butterfly, representing five families, relative testis size was significantly positively correlated with the degree of polyandry (measured as spermatophore counts per female). Similarly, Karlsson (1995) found that the degree of polyandry was significantly positively correlated with residual ejaculate weight across 21 species of butterfly.

Intra-specific studies in a number of taxa provide direct evidence that males show plastic responses and allocate their ejaculates according to the degree of sperm competition risk (e.g. in snails, Oppliger et al. 1998; insects, Cook and Wedell 1996; Gage and Baker 1991; Martin and Hosken 2002; Simmons et al. 1993; Wedell and Cook 1999; birds, Pizzari et al. 2003) or according to sperm competition intensity (e.g. in bushcrickets, Simmons and Kvarnemo 1997; fish, Pilastro et al. 2002). For example, male butterflies, *Pieris rapae*, exhibit a plastic response to sperm competition risk. Males increase both the number of fertilising and non-fertilising sperm, and increase the number of fertilising sperm as a proportion of total sperm when they encounter a virgin female on their second mating (Cook and Wedell 1996). Mated males also provide more sperm to previously mated females as compared to virgin females (Wedell and Cook 1999). Similarly, male bushcrickets, *Requena verticalis*, increase sperm number when paternity assurance is low

(Simmons et al. 1993). In field-collected bushcrickets, males mating with older females transferred greater sperm numbers, as predicted by theory, since there is a higher probability that older females will have already mated (Simmons et al. 1993).

Support for the prediction that sperm competition selects for increased investment in sperm also comes from intra-specific studies of male polymorphisms. For example, 'minor morph' males that sneak matings must always compete with the sperm of 'major morph' males: as expected, the residual gonad mass and ejaculate volume of sneak males was found to be significantly higher than for major morphs in beetles and fish (Gage et al. 1995; Simmons et al. 1999). For example, in *Onthophagus* dung beetles, males of a major morph monopolize and guard females in tunnels beneath dung. The major morphs experience lower levels of sperm competition as compared to the minor morph males that sneak matings (Simmons et al. 1999).

These major morph males are subject to sperm competition only when the frequency of minor morph males is high and therefore generally experience much lower sperm competition than sneak males that must always compete with the sperm of major males. In *O. binodis*, 31% of males are of the minor morph, and, as predicted, the residual testis mass and ejaculate volume of minor males is significantly higher than for major morphs (Simmons et al. 1999). These trends were not seen in a second study species, *O. taurus*, in which the percentage of minor morphs in the population (57%), and therefore the risk of sperm competition to major morphs, was higher.

This is consistent with the general prediction of an association between testis size and the level of sperm competition (Simmons et al. 1999). Similarly, Atlantic salmon males become either large males that dominate spawning beds and guard females, or mature faster to become much smaller sneaker males (Gage et al. 1995). Gonad mass as a percentage of somatic mass, and sperm number per unit body mass, were higher in males of the sneaker morph (Gage et al. 1995). In certain contexts however, males are predicted to decrease their sperm expenditure when the number of males competing exceeds two, since the probability of paternity is low with increasing numbers of competing ejaculates (Ball and Parker 1997; Parker et al. 1996). In two gobiid fishes, for example, as the number of sneaker males attending a spawning increases, the number of sperm released by individual males decreases (Pilastro et al. 2002). Intra-specific ejaculate allocation has also been studied in response to artificial selection. Hosken and Ward (2001) placed replicate populations of the yellow dung fly, *S. stercoraria*, under monogamous or

polyandrous conditions. They subsequently documented a rapid (i.e. after 10 generations) evolution of testis size in response to the level of sperm competition.

Sperm size might also be expected to be a target of selection if changes in size increase the probability of fertilisation (Parker 1970). For example, sperm length may be under selection to increase if longer sperm are more competitive through being more motile (Gomendio and Roldan 1991) or by filling the female sperm storage organs more effectively and thereby reducing the likelihood of being displaced or usurped (see Simmons and Siva-Jothy 1998). Comparative studies have examined the adaptive significance of sperm length in a variety of taxa, including *Drosophila* (Pitnick and Markow 1994), concluding that relatively long sperm provide an advantage in sperm competition (Briskie and Montgomerie 1992; Briskie et al. 1997; Gage 1994; Gomendio and Roldan 1991; LaMunyon and Ward 1999; Ward 1998). However, this relationship has not always been found (Hosken 1997; Stockley et al. 1997) and the relationship between sperm size and number also remains unclear. Stockley et al. (1997) found evidence for a trade-off between sperm size and number, finding decreasing sperm length and increasing sperm number with increasing sperm competition across fishes. However, Arnaud et al. (2001) found no significant relationship in the red flour beetle suggesting that sperm size and number can evolve independently.

The production of more than one morphological type of sperm has been documented for a number of invertebrates, particularly in the Diptera and Lepidoptera; often, one type of sperm is fertilising and the other is non-fertilising (Silberglied et al. 1984; Swallow and Wilkinson 2002). A number of hypotheses have been proposed that link the occurrence of parasperm (one than one sperm type) with success in sperm competition. For example, in the butterfly, *P. napi*, males that transfer greater quantities of non-fertilising sperm induce a greater female refractory period in their partners (Cook and Wedell 1999). The non-fertilising sperm has been proposed to function as a filler of the female sperm storage organ, remaining in store and maintaining the refractory period and reducing the risk of sperm competition (Wedell 2001).

### Adaptations in male genitalia in response to sperm competition.

Adaptations in genitalia can also influence the success of males in sperm competition. There has been a recent expansion in studies investigating the role of sexual selection, and in particular that of sperm competition, in genital evolution (for a review see Hosken and Stockley (2004)). Arnqvist and Danielsson (1999) found that male genital sclerite shape affected fertilisation success in the water strider *Gerris lateralis*. Associations between male genital characters and sperm competition success have also been found in the dung beetle, *O. taurus* (House and Simmons 2003) and the fly *Dryomyza anilis* (Otronen 1998). In addition, Arnqvist (1998) found that genital shape divergence was greater in polyandrous compared to monoandrous species across beetles, flies, butterflies, moths and mayflies, implying a strong role for sexual selection in generating this diversity.

### Behavioural adaptations in response to sperm competition

Males may be able to combat the threat of sperm competition by guarding their mates from the attentions of rival males, either before or after copulation. Mate guarding can prevent females from remating and/or by deterring the courtship attempts of other males. Male waterstriders, for example, continue to grasp females following mating, rebutting the advances of rival males, increasing the opportunity for females to utilise their sperm and reducing the opportunity for sperm competition (Clark 1988; Rowe et al. 1994; Weigensberg and Fairbairn 1994).

In other species, males extend copulation as a way of excluding rival males until their mate is unreceptive or ready to lay eggs. Male fitness may be increased if such behaviour results in a greater number of sperm being transferred or stored (Alcock 1994; Dickinson 1986; Sakaluk 1984; Siva-Jothy and Tsubaki 1989) or if this results in a delay in female remating (Wedell 1993). Prolonged copulation may also facilitate the transfer of seminal fluid. In *D. melanogaster* extended copulation beyond the point of sperm transfer allows the full transfer of Acps that increase the inhibition of female receptivity and act to promote sperm displacement (Gilchrist and Partridge 2000). In gift-giving species, prolonged copulation may be related to nuptial gift size, with larger nuptial gifts resulting in greater male fitness (Sakaluk 1986; Svensson et al. 1990; Wedell 1993).

### Remote mate guarding in response to sperm competition

Males may also guard their mates remotely, through the actions of seminal fluid proteins that influence female physiology and behaviour (reviewed in Gillott 2003; Leopold 1976; Simmons and Siva-Jothy 1998). In *D. melanogaster*, for example, an Acp (section 1.5.3) in the seminal fluid suppresses female remating for several days, ensuring high levels of paternity for at least that length of time (Chen et al. 1988). Long refractory periods between matings are thought to be achieved in bush crickets and Lepidoptera by the transfer of large ejaculates, perhaps because large quantities of sperm and other ejaculate fluids may have evolved as “fillers” to increase the refractory period of females (Wedell 2001). In some species, mating plugs are formed by coagulation of the ejaculate in the female reproductive tract and may act to prevent the sperm of rival males entering storage. Mating plugs may increase the efficiency of sperm storage (e.g. Polak et al. 1998; Polak et al. 2001) or increase female refractoriness to mating (Contreras-Garduno et al. 2006; Lachmann 1998; Orr and Rutowski 1991; Polak et al. 1998; Polak et al. 2001) but do not necessarily prevent insemination by rival males (Moreira and Birkhead 2003).

### Conflict between the sexes

As well as generating post-copulatory male-male competition, female promiscuity also has the consequence of generating conflicts between the sexes (e.g. Chapman et al. 2003b). Two manifestations of sexual conflict have been considered: intralocus and interlocus sexual conflict. Intralocus sexual conflict concerns traits that are expressed in both sexes for which the fitness optima are different for males and females. Selection acting to the advantage of one sex will act antagonistically and to the detriment of the other (Parker and Partridge 1998). Therefore adaptations that are beneficially to one sex may result in reduced fitness because of the suboptimal level of expression of that trait in the other sex (Rice 1984). One of the most striking demonstrations of intralocus conflict has been in *D. melanogaster*. Chippendale et al. (2001) sampled haploid genomes from a wild-type population, clonally amplified them, and then used these to produce sons and daughters expressing the same haploid genomes. Intersexual genetic correlations were then measured for juvenile and for adult fitness. There was a strong positive correlation between the fitness of males and females at the larval stage when the optima for most fitness traits are expected to be similar. However, after adult maturation and the divergence of

reproductive roles there was a significant negative correlation in reproductive success between haploid genomes expressed in both sexes, indicating extensive genome-wide polymorphism for sexually antagonistic alleles (Chippindale et al. 2001).

Interlocus sexual conflict involves conflicts over the outcome of male-female interactions. Such conflicts are likely to be greatest in sex-limited genes, though sex-limitation is not a necessity (Rice and Holland 1997). Conflicts can occur over interactions such as mating frequency, fertilisation, relative parental effort, female remating behaviour, female reproductive rate and clutch size (Chapman et al. 2003b). High levels of female promiscuity are unlikely to be beneficial to males, since additional mating introduces sperm competition that may result in a loss of paternity, thus reducing male fitness (Parker 1970). The divergence of evolutionary interests over female mating frequency is predicted to generate conflict which will favour selection for male traits that discourage re-mating (that threatens paternity) or that increase the probability of sperm use for fertilisations (e.g. Rice 2000). Males can benefit from such adaptive traits that increase their own reproductive success (for example, through increased sperm competition success or decreased female receptivity to extra-pair mating), even if such traits are costly to their partners (Parker 1979). When such adaptations occur, counter-adaptations are expected to evolve in females that will reduce the costs of male-induced harm. These are in turn countered by new mutations in males that tend to increase male, but not necessarily female, fitness in a cycle of 'sexually antagonistic co-evolution' (Morrow and Arnqvist 2003; Rice 2000; Rice and Holland 1997). Antagonistic coevolution is expected to generate rapid evolution in physiological and morphological traits and can the potential to lead to reproductive isolation (Parker and Partridge 1998).

The imposition of mating costs by males on females, such as via morphological adaptations that benefit one sex to the detriment of the other, has been reported in a number of species (e.g. *Drosophila*, Chapman et al. 1995; *Callosobruchus maculatus*, Crudgington and Siva-Jothy 2000; *Sepsis cynipsea*, Blanckenhorn et al. 2002). The nature of the selection maintaining harmful traits has generally been considered in terms of those beneficial male traits having negative side effects in females (Parker 1979; Rice 2000). Selection may act on female harm traits when the reproductive benefits to males outweigh reduction of reproductive success in

females (Rice 2000). Johnstone and Keller (2000) proposed that harmful traits themselves can evolve as a result of conflict over mating rate and that this may be a general means by which males manipulate female mating. If females reduce mating rates as a consequence, male induced harm can become an established phenotype since males enjoy reduced sperm competition and higher reproductive success (Johnstone and Keller 2000). However, since the primary function of male traits studied is not to induce harm in females, as appears to be true of seminal proteins in *Drosophila*, this adaptive hypothesis is drawn into question (see Morrow et al. 2003). Male induced harm may instead be a side effect of other male traits (Parker 1979).

#### **1.4 Evidence of sexual conflict**

Several lines of evidence support the occurrence of sexual conflict. Comparative studies of coevolved morphological traits or behaviours, for example, has provided evidence of rapid evolutionary change between populations, indicating that antagonistic coevolution can play an important role in the evolution of mating systems and reproductive trait morphology (e.g. Arnqvist and Nilsson 2000; Rowe and Arnqvist 2002). Population crosses that exploit the expectation that sexually antagonistic coevolution will proceed along separate coevolutionary trajectories have also been cited in support of sexual conflict and genetic tools have allowed investigation of the evolution of Acps and their role in sexual conflict in *D. melanogaster* (reviewed in Chapman et al. 2003b).

The occurrence of sexual conflict is also supported by a number of experimental evolution studies. By preventing co-evolution of female *Drosophila melanogaster* to males that were evolving against a standard female genotype, Rice (1996) saw that after 30 generations, whilst males rapidly adapted to the standard female genotype and increased their fitness relative to control males, females suffered a decline in longevity when mating with males from the adapting lines relative to females mated to the other adapting or control line males. In a further study, populations where sexual selection was removed by enforcing monogamy - thus removing mate and sperm competition, cryptic female choice and sexual conflict - males evolved to be less harmful and females less resistant to male-induced harm (Holland and Rice 1999). The evolution of reduced male harm in monogamous populations is predicted



by sexual conflict theory to result in higher fitness in females mated to monogamous compared to polygamous males. Indeed, this has been demonstrated in *D. melanogaster* (Pitnick et al. 2001b) and yellow dung fly, *S. stercoraria* (Martin et al. 2004). Moreover, females mated to monogamous males remated faster than females mated to promiscuous males and monogamous females evolved to remate less frequently (Pitnick et al. 2001a). This suggests that the promiscuous males had evolved to be more effective in inducing female non-receptivity than monogamous males and that monogamous females were less able to resist ejaculate manipulation (Pitnick et al. 2001a).

*D. melanogaster* has proven to be a particularly useful tool for investigating both sexual conflict and sexual selection. The following sections give background on its mating system and the questions under investigation in this thesis.

### **1.5 The mating system of *Drosophila melanogaster***

The fruit fly, *Drosophila melanogaster*, has long been established as a powerful model for testing many questions in evolutionary biology and recently, has been used for investigating sexual conflict and sexually antagonistic coevolution (e.g. Rice 1996; Holland and Rice 1999; Chippendale et al. 2001). Because it has been a long established model organism, much is known about *Drosophila* genetics, physiology and behaviour. Of particular use has been the ability to identify, phenotype and manipulate components of the accessory gland, which has proved valuable in trying to understand the genetic bases of sexual conflict and post-copulatory success. For example, male Acp's were identified as mediating a cost of mating in females (Chapman et al. 1995; section 1.5.4). Females also mate multiply both in the lab and in the field (Harshman and Clark 1998) thus creating the conditions for sperm competition and selection for adaptations in males to increase both pre- and post-copulatory success. In the context of the laboratory, *Drosophila* are easy and cheap to maintain and have a relatively short life cycle (~10 days egg-adult) making them ideal for experimental evolution and selection studies.

### 1.5.1 *Sperm competition in Drosophila melanogaster*

Because females mate multiply and can retain viable sperm of multiple males for at least two weeks after mating (Neubaum and Wolfner 1999), there is the opportunity for the ejaculates of different males to compete. The determinants of sperm competition are complex. Male genotypes show extensive variation in their sperm competitive success (Civetta and Clark 2000; Clark et al. 1995; Hughes 1997) and females differ in their tendency to exhibit last male sperm precedence (Clark and Begun 1998). In addition, Clark et al. (1999), and more recently Bjork et al. (2007), have shown that genotypic-specific female-male interactions determine success of sperm in competition.

### 1.5.2 *Accessory gland proteins (Acps) of Drosophila melanogaster*

Like many insects, males of *D. melanogaster* transfer a host of seminal fluid proteins as well as sperm when they successfully mate (e.g. Gillott 2003); these have been particularly well studied in *Drosophila*. Seminal fluid proteins are a major determinant of post-copulatory fitness, directly affecting female behaviour and physiology. As well as being diverse in nature, ranging from small peptides to large glycoproteins (Chen et al. 1988; Swanson et al. 2001; Wolfner 1997; Wolfner et al. 1997a), some Acps exhibit high rates of evolutionary change (Aguadé et al. 1992; Begun et al. 2000; Swanson et al. 2001; Tsaur and Wu 1997), consistent with the idea that they mediate sexually antagonistic coevolution between the sexes. There are around 80 Acps (Swanson et al. 2001), some of which remain in the genital tract (Heifetz et al. 2000; Lung and Wolfner 2001; Wolfner et al. 1997a) and others which enter the circulatory system via the posterior vaginal wall (Lung and Wolfner 1999) following mating. Ravi Ram et al. (2005) have shown that other Acps target the ovary and found Acps on mature oocyte and on laid eggs. The 8 Acps they examined all had multiple targets and localised in a unique patterns (Ravi Ram et al. 2005). This understanding of the fates and targets of Acps may allow the function of Acps to be predicted or determined. Functions are confirmed for relatively few Acps (Chapman 2001); many Acps contain motifs that suggest functional roles, but in many cases these remain to be confirmed. Acps act to stimulate and regulate reproductive processes in females following mating; for example they stimulate ovulation (Heifetz et al. 2000; Heifetz et al. 2001; Herndon and Wolfner 1995),

oogenesis (Aigaki et al. 1991; Chen et al. 1988; Soller et al. 1997; Soller et al. 1999), increase sperm storage (Neubaum and Wolfner 1999), decrease receptivity to mating (Aigaki et al. 1991; Chen et al. 1988; Manning 1967), increase success in sperm competition (Chapman et al. 2000) and are essential for mating plug formation (Lung and Wolfner 2001). One Acp is responsible for at least a large part of the cost of mating in females (Wigby and Chapman 2005; section 1.5.4). The accessory gland also produces antibacterial peptides, proteases, protease inhibitors, lectins, lipases and CRISPs (Chapman 2001; Lung et al. 2001; Lung et al. 2002; Swanson et al. 2001; Mueller et al. 2004, 2005).

The accessory glands are comprised of two types of cell: approximately 1000 main cells in the lumen of the gland and 40-50 secondary cells at the distal end (Bairati 1968; Bertram et al. 1992). Following mating, gene expression in the accessory glands increases to replenish the seminal fluid transferred (DiBenedetto et al. 1990; Wolfner 1997). This is important since successive matings result in depletion of the accessory gland and impairment of fertility (Hihara 1981; Lefevre and Jonsson 1962; Linklater et al. 2007). After mating the transferred Acps localise to specific areas of the female reproductive tract (Heifetz et al. 2000; Lung and Wolfner 2001; Wolfner 1997). The actions of Acps have important consequences for both male and female fitness; some are beneficial to both sexes but at least one is detrimental to females while being beneficial to males (Wigby and Chapman 2005). Some genes encoding Acps exhibit high rates of evolutionary change (Aguade et al. 1992; Begun et al. 2000; Swanson et al. 2001; Tsaur et al. 1998; Tsaur and Wu 1997) and this has made them a particularly intriguing subject for examining evolutionary dynamics between the sexes. In the following sections I describe in more detail some of the roles of Acps in *D. melanogaster*.

### *1.5.3 Role of Acps in Drosophila melanogaster*

#### *Decline in female attractiveness, receptivity to mating and increase in egg laying*

Acps induce a number of post-mating changes in females. Mated females are sexually less attractive to males for between 5 and 9 days after mating (Tram and Wolfner 1998). This reduction in attractiveness occurs in two stages, a initial short-term response, independent of the receipt of Acps and sperm (Tram and Wolfner

1998) and a longer-term reduction lasting beyond the first 24 hours post-mating. Females mated to males not transferring sperm or not transferring sperm and Acp3 do not exhibit the long term reduction in attractiveness suggesting that this is associated with the storage of sperm (Tram and Wolfner 1998), supporting the finding of (Manning 1962) that willingness to mate was associated with sperm supply.

Females also exhibit a reduction in sexually receptivity for approximately 11 days after mating, during which time females actively reject the attempts by males to mate with them (Chen et al. 1988). The reduction in sexual receptivity is caused by the action of sex peptide (SP, Acp70A), which is also responsible for a dramatic increase in egg laying immediately after mating (Liu and Kubli 2003). Acp26Aa also stimulates oogenesis, but for just 1 day following mating (Herndon and Wolfner 1995). Acp26Aa localises to the base of the ovaries and stimulates the release of mature eggs into the lateral oviducts (Herndon and Wolfner 1995).

#### *Sperm storage and displacement*

Sperm storage and displacement are also affected by Acp3. In particular, Acp36DE, which localises with stored sperm, has been identified as necessary for sperm storage (Neubaum and Wolfner 1999). Males lacking Acp36DE pass normal quantities of sperm but store only 15% of it (Neubaum and Wolfner 1999; Tram and Wolfner 1999) which significantly decreases a male's success in sperm competition (Chapman et al. 2000). As males lacking Acp36DE are less able to store sperm, they are also less able to displace the sperm of previous males (Chapman et al. 2000).

Two of the defining characteristics of males in the context of sperm competition are their ability to displace the sperm previous males (sperm offence) and/or to prevent the displacement of their own sperm (sperm defence) and both are affected by Acp3 (Clark et al. 1995; Gilchrist and Partridge 1995; Harshman and Prout 1994). Acp3 are known to be involved in sperm displacement, since males transferring Acp3 but not sperm reduce the number of resident sperm from a previous mating (Harshman and Prout 1994) though this action has not been attributed to individual Acp3. A correlation has also been reported between alleles at four Acp loci (Acp26Aa, Acp29AB, Acp36DE and Acp53Ea) and levels of sperm defence in lines carrying

chromosomal isolation from the wild (Clark et al. 1995). Fiumera et al. (2005) also found several significant associations between Acp alleles (polymorphisms in or near CG8137, CG17331, CG31872, Acp33A, Acp29AB and Acp26Aa) and male sperm competitive ability. The degree of sperm precedence achieved is affected by male-female genotype interactions (Clark et al. 1999) and the genotypes of the competing males (Clark et al. 2000).

#### Immunological properties of seminal fluid

Male and female reproductive tracts are in contact with the external environment and provide an opportunity for the entry of microorganisms that may affect fertility and/or survival. In addition to the structural and cellular barriers used to prevent and combat bacterial infection, some seminal fluid products have immunological properties and function in the protection of sperm, the female reproductive tract and newly laid eggs (Lung et al. 2001; Samakovlis et al. 1991; for a review of the interactions between mating and immunity in invertebrates see (Lawniczak et al. 2007, Appendix II). In *D. melanogaster*, the peptide andropin and two other seminal fluid proteins are transferred to females during mating (Lung et al. 2001; Samakovlis et al. 1991).

#### *1.5.4 Acps, the cost of mating and sexual conflict*

The most dramatic of the consequences of mating for females is the cost of mating. Females suffer reduced survival by mating multiply (Fowler and Partridge 1989). This cost is mediated by Acps (Chapman et al. 1995). Recently, the cost of mating has been attributed to the actions of Acp70A (SP) (Wigby and Chapman 2005). Double stranded RNA mediated gene silencing was used to knockdown the expression of SP (Chapman et al 2003a). As expected, females continuously housed with SP knockdown males mated significantly more (12 times more frequently) than females mated with control males (since SP causes a reduction in receptivity to mating). However, despite the differences observed in mating frequency, the survival of females mated to SP-lacking males was not lower than for females housed with control males, suggesting that SP is a major contributor to the cost of mating (Wigby and Chapman 2005). Other potential Acp candidates involved in

mediating mating costs are CG10433, CG8137, SP and Acp62F (see below) which are toxic upon ectopic expression (Lung et al. 2002; Mueller et al. 2007).

#### *Acp62F and the cost of mating in Drosophila melanogaster (Chapter 6)*

The protease inhibitor Acp62F can pass from the female reproductive tract into the haemolymph (Lung and Wolfner 1999; Wolfner 2002). Approximately 10% of the Acp62F protein transferred by the male enters the haemolymph via the posterior vagina and is detectable in the female within 5 minutes after the start of mating (Lung and Wolfner 1999). The remaining protein localises within the female sperm storage organs, the sperm receptacle and spermathecae (Lung et al. 2002), suggesting that it may have a role in storing or protecting sperm. The first suggestion that Acp62F might play a role in mediating a cost of mating came from apparent sequence similarities to a neurotoxin of the Brazilian armed spider *Phoneutria nigriventer* (Wolfner et al. 1997a). However, this high degree of similarity has been subsequently attributed to the high cysteine content of Acp62F (Lung et al. 2002). The putative function of Acp62F has subsequently been inferred from sequence similarities to serine protease and trypsin inhibitors and the *in vitro* inhibition by Acp62F of trypsin cleavage, suggesting involvement of Acp62F in the protection of seminal substances from proteolysis, seminal protein breakdown and in the prevention of semen coagulation (Lung et al. 2002).

Ectopic expression of Acp62F, and 3 more of 21 Acps tested (Mueller et al. 2007), was found to be toxic to adult flies (Lung et al. 2002), leading to the suggestion that Acp62F may interfere with 'essential protease-regulated processes' such as the prophenoloxidase activation, a cascade critical to immune function and wound repair (Lung et al. 2002). However, ectopic expression produced Acp62F at more than 50 times the levels found in female haemolymph after a single mating and because heat shock was used to drive ubiquitous, ectopic expression, Acp62F would have been delivered to sites not usually exposed following normal mating (Lung et al. 2002). It is therefore necessary to confirm whether the toxic effects reflect those incurred during normal mating.

Although SP is now known to contribute to at least a large part of the survival cost suffered by females, it is necessary to determine whether other Acps also have detrimental consequences for females. This is especially interesting if such factors

are beneficial to males whilst being detrimental to females as they may eventually reveal the nature and mechanisms of sexual conflict. In the case of Acp62F, several authors have speculated on the costs of Acp62F and the possibility that it is mediating a battle between the sexes, based on the finding of the ectopic expression studies (Lung et al. 2002; Swanson 2003; Wolfner 2002). The confirmation of the toxicity of Acp62F under normal delivery would hence pave the way for investigating its targets and potential coevolution between protein and receptors. Chapter 6 examines the effect of the protein on female survival and fecundity using males that lack full expression of Acp62F due to RNAi interference and using males that lack a functional Acp62F gene and which therefore do not express the Acp62F protein.

I have outlined some of the consequences female promiscuity has for males in generating sperm competition and sexual conflict. In *D. melanogaster*, particular attention has been paid to the role of Acps in determining male and female fitness. The following section outlines how this thesis will use *D. melanogaster* to ask how reproductive traits, strategies and morphologies have been shaped by sexual selection and sexual conflict.

## 1.6 Outline of Thesis

The work in this thesis was funded by the Natural Environment Research Council and was performed under the supervision of Tracey Chapman (Principal Supervisor) and Kevin Fowler (Second Supervisor). All experiments were conducted by the author. In Chapter 3, selection lines in which adult sex-ratios were manipulated were initiated by Tracey Chapman, then maintained by Stuart Wigby and then by me. These lines are described in Wigby and Chapman (2004). In Chapter 6, males carrying constructs that induced RNAi of Acp62F (*UAS- Acp62F-1R1b*, *UAS- Acp62F-1R3A* and *UAS- Acp62F-1R3C*) and the line carrying the *Gal4* driver (*Acp26Aa-P-Gal4*) were created by Oliver Lung and Mariana Wolfner (Cornell University). The Acp62F knock out lines in which the Acp62F gene was mutated were created by Jacob Mueller and Mariana Wolfner (Cornell University).

Chapter 2 describes the general materials and methods used in the thesis.

Chapter 3 uses experimental evolution to examine how male ejaculate allocation patterns respond to manipulation of adult sex-ratio. The work contributes to the understanding of the evolutionary consequences of selection on post-copulatory success of male *Drosophila melanogaster*. The work described in Chapter 3 has been published in the journal *Evolution* with co-authors Bregje Wertheim, Stuart Wigby and Tracey Chapman (Appendix I).

In Chapter 4, I describe an experiment in which I use artificial selection to generate males with large or small accessory glands. These males are used to examine the effects of accessory gland size on male and female fitness. The work demonstrates the importance of reproductive morphology, particularly accessory gland size, in determining male post-copulatory reproductive success.

Chapter 5 describes the use of the large, small and unselected accessory gland size males from the selection lines in Chapter 4 to investigate the contribution of accessory gland size to the cost of mating in females. This work contributes to the understanding of importance of reproductive traits contributing to the costs of mating.



Chapter 6 investigates the role of a single male seminal fluid protein, Acp62F, in determining female mating costs. I use males null for the Acp62F protein and males that lack Acp62F via RNAi mediated gene silencing, to examine the effect of Acp62F on female fitness. The work makes an important contribution to establishing which Acps mediate the cost of mating in female *Drosophila melanogaster*.

Chapter 7 is a general discussion of the findings of the thesis and their wider implications. I discuss future work that might be undertaken to increase our understanding of the mechanisms sperm competition and male adaptations to it. I include in this discussion, some hypotheses that link immunity, sperm competition and sexual conflict, and these ideas have been published as a review in *Trends in Ecology and Evolution* (2007, Vol. 22 (1), pages 48-55) with co-authors Mara Lawniczak, Andrew Barnes, James Boone, Stuart Wigby and Tracey Chapman.

## Chapter 2 General Materials and Methods

### 2.1 Fly Culturing

Stocks were maintained in cages, bottles or vials on either Sugar-Yeast (SY) or Maize-Yeast (ASG) medium (see below). All flies used in experiments were maintained in non-humidified rooms at  $25 \pm 0.5^{\circ}\text{C}$  on a 12:12 hour light: dark cycle.

#### *2.1.1 Bottles and vials (stocks and experiments)*

Experiments were carried out in glass vials (23x73mm) containing 7ml food medium. Stocks were maintained in either vials (as above), or glass bottles (189ml) containing 70ml food medium.

#### *2.1.2 Standard larval density culture*

The technique of standardising larval density is important because it minimises environmentally-derived differences in body size caused by competition between larvae for food, and by variation in food quality (i.e. differences in contamination level by larval faeces). Eggs for standard density cultures were collected on Petri dishes containing grape juice medium (section 2.2.3) with live yeast paste. Petri dishes were placed in fly cages for ~8-16 hours after which they were taken out and the yeast paste removed. Standard density culture was achieved in 2 ways:

(i) In instances where few flies were required, first instar larvae were picked off the Petri dishes using a mounted needle and 100 larvae were then put into each food vial.

(ii) Where large numbers of flies were required, larvae were raised in bottle cultures at standard density, using the method described by (Clancy and Kennington 2001). This involved collecting eggs as described above, and washing them off the Petri dishes using 1 x PBS solution (phosphate buffered saline) into a funnel placed in a plastic Universal tube (Clancy and Kennington 2001). Any remaining eggs were washed from the funnel sides, allowed to settle and most of the supernatant poured off to leave 5-10ml of solution (Clancy and Kennington 2001). More PBS was

added to rinse away any residual yeast paste if necessary. Eggs were then pipetted from this solution using a 100µl micropipette, broadened by cutting it 6mm from the end, and 18µl of this solution was dispensed into each SY bottle, (Clancy and Kennington 2001).

## **2.2 Media**

### *2.2.1 Sugar-Yeast medium*

Sugar-Yeast medium (SY) consisted of 100g autolysed yeast powder, 100g sugar, 20g agar, 30ml Nipagin (10% solution) and 3ml Propionic Acid in every 1 litre of distilled water. The yeast, sugar and agar were added to the water and brought to the boil and then left to simmer for several minutes. The food was removed from the heat and allowed to cool to 60°C after which the Nipagin and Propionic Acid (antifungal and antibacterial agents respectively) were added and the medium was then immediately dispensed into bottles or vials.

### *2.2.2 Maize-Yeast medium*

Maize-Yeast medium (ASG) consisted of 20g autolysed yeast powder, 85g sugar, 10g agar and 25ml Nipagin (10% solution) in every 1 litre of distilled water. The yeast, sugar and agar were added to the water, brought to the boil and then left to simmer for several minutes. The food was removed from the heat and allowed to cool to 60°C after which the Nipagin was added and the medium immediately dispensed into bottles or vials.

### *2.2.3 Grape juice medium*

Grape juice medium was used for collecting fly eggs for standard density cultures. The medium consisted of 1.1L distilled water, 50g agar, 600ml red grape-juice concentrate and 42.5ml Nipagin (10% solution). The agar was added to the water and brought to the boil. The red grape-juice was then added, the mixture brought to the boil once more and allowed to simmer for a few minutes. The remaining water was added and the medium allowed to cool to 60°C before the Nipagin was added.

The grape-juice medium was then dispensed into Petri dishes and allowed to set at room temperature.

## **2.3 Stocks**

### *2.3.1 Wild-type D. melanogaster*

Wild-type flies were collected from Dahomey, now Benin, in West Africa in 1970 and have been maintained in laboratory culture since. Dahomey flies were kept in four population cages (45 x 25 x 25cm) with overlapping generations. Each cage contained 12 bottles of SY medium. Each week the cages were supplied with three 189ml bottles containing 70ml SY medium and the three oldest bottles were removed.

### *2.3.2 Manipulating sperm competition by experimental evolution of sex ratio (Chapter 3)*

The intensities of sexual selection and sperm competition were varied by altering the adult sex ratio in replicated selection lines, as previously described by Wigby and Chapman (2004). Each generation, three replicate lines each of male-biased (MB, 70 males and 30 females) and female-biased (FB, 25 males and 75 females) sex ratio treatments were propagated. Each line was maintained in a plastic cage (220 x 140 x 85mm) with a gauze covered top. Flies were provided access to water and were fed ad libitum with two vials of SY food with added live yeast every two or three days. Nine days after the cages were set up eggs were collected. The majority of eggs were allowed to hatch before larvae were collected, thus minimizing selection on early egg hatchability. Larvae were raised at standard density of 100 larvae per vial (section 2.1.2). All adults were allowed to eclose over two days, minimizing selection on development time, before being allocated to the same sex-ratio treatment and replicate number as their parents for the next generation.

### 2.3.3 sparkling poliart stock (Chapter 4)

*sparkling poliart* flies, that had been backcrossed into a wild-type background, were used as competitor males in sperm competition experiments. They are homozygous for a recessive mutation and have a phenotype of small, smooth, glassy eyes, which is easily distinguishable from wild-type flies when viewed under a dissecting microscope. The stock was described in Partridge et al. (1994) and was maintained in bottle culture prior to experiments.

### 2.3.4 Flies lacking *Acp62F* – gene silencing of *Acp62F* by RNAi (Chapter 6)

In Chapter 6, the effects of *Acp62F* on the longevity and fecundity of Dahomey females were investigated in two replicate experiments by using targeted knockdown of the *Acp62F* protein, using RNA interference (RNAi). *Acp62F* RNAi transgenic stocks were created by Lung (2000). Figure 2.1a shows the crossing regime used to create experimental RNAi knockdown and control males. Transgenic flies were created which carried an Upstream Activating Sequence (UAS) upstream of the *Acp62F* coding region in the sense followed by antisense orientation (inverse repeat (IR), *UAS-Acp62F-IR*) and in addition carried a  $w^+$  marker (Lung 2000). A vector containing the construct was injected into a  $w'(d2,3)/TM2Sb$  background. The expression of this construct creates a double-stranded RNA hairpin loop, which knocks down expression of the protein by RNAi (Fire et al. 1998). Three different transgenic lines were produced and used to generate three homozygous transgenic stocks with differing efficiency of *Acp62F* knockdown (Lung 2000). *Acp62F* knockdown was driven by crossing RNAi flies to a stock containing the promoter for *Acp26Aa* fused to *GAL4* (*Acp26AaP-GAL4*). *Acp62F* levels in *Acp26AaP-GAL4; UAS-Acp62F-IR1b* males, containing two transgene insertions, and *Acp26AaP-GAL4; UAS-Acp62F-IR3A* males, containing a single insert, knock-downed *Acp62F* to 2-3% of control levels (Lung 2000). For an unknown reason, males with the *Acp26AaP-GAL4; UAS-Acp62F-IR3C* genotype, which also contained a single insertion, do not show significantly reduced levels of *Acp62F* (~80% of control levels, Lung 2000). However, as they have the same genetic background and transgenic insertion, this allowed them to be used instead as good controls for other two lines in which *Acp62F* did show significant knockdown.

### 2.3.5 Flies lacking *Acp62F* – null mutants (Chapter 6)

To complement the experiments using RNAi knockdown males, null mutants for the *Acp62F* coding region were also used to examine the effects of *Acp62F* on the survival, egg laying and fecundity of females.

*Acp62F* null flies were made by Jacob Mueller from Mariana Wolfner's lab (Cornell University). Precise deletion of the *Acp62F* coding region was achieved using an ends-in targeting approach (Rong and Golic 2000; Mueller 2006). An 800bp construct (*Acp62F<sup>del</sup>*), encompassing 145bp upstream of the *Acp62F* start codon to 316bp downstream of the stop codon, was inserted into *w<sup>1118</sup>* flies by P-element mediated transformation to produce a tandem duplication of the *Acp62F<sup>del</sup>* adjacent to the endogenous *Acp62F* allele. Males with the selected targeted allele were crossed to females carrying a 70I-*CreI* transgene and heat-shocked. This induced recombination between flanking regions of homology within the endogenous *Acp62F* allele and the *Acp62F<sup>del</sup>* allele left only the mutant copy of *Acp62F*. Independent resolutions of the reduction step yielded *Acp62F<sup>1b</sup>* and *Acp62F<sup>1c</sup>* lines. Males from these lines, containing both the targeted mutant copy of *Acp62F* and the 70I-*CreI* transgene, were crossed to *w<sup>1118</sup>;TM3,Sb* females to generate stocks. To construct a control line, one additional line was held back from the reduction stage, in order to retain the tandem gene duplication, the wild-type copy of which expresses the *Acp62F* protein (Mueller 2006).

To equalise both genetic background and the eye colour of all the males in the *Acp62F* null experiment, I conducted a crossing regime (Figure 2.2) to introduce a wild-type X chromosome to each of the null and control male genotypes described above. This was important since null males differed in eye colour from males with the tandem duplication, which is likely to cause significant differences in pre-mating male mating success and in mating frequency. In addition, *Acp62F<sup>1b</sup>*, *Acp62F<sup>1c</sup>* and *w<sup>1118</sup>* males had white eyes, rendering them blind and hence unsuitable for fitness assays since they mate at a low frequency. The crossing regime was successful and produced red-eyed males containing a wild-type X chromosome, homologous *w<sup>1118</sup>* 2<sup>nd</sup> chromosomes (i.e. the background in which the original deletion was generated), and a 3<sup>rd</sup> chromosome homologous for either the mutant *Acp62F* allele, the tandem duplication or *w<sup>1118</sup>* (Figure 2.2). To do this, a balancer stock (*Bar;Cy;Tb,Hu*) was

crossed to the original  $w^{1118}$  stock, and each of the transgenic lines and a double balanced stock ( $w^{1118}/Dp(1;Y)y+;CyO/nub1\ b1\ nocSco\ lt1\ stw3; MKRS/TM6B, Tb1$ ) crossed to the wild-type Dahomey stock (section 2.3.1), to yield males with a wild-type X chromosome,  $w^{1118}$  balanced over *Curly* on the 2<sup>nd</sup> chromosome and the insert on the 3<sup>rd</sup> chromosome balanced over *Stubble* and females with an additional Bar balancer over wild-type on the X chromosome. These males and females were crossed together to give experimental ( $w^+;w^{1118};Acp62F^{lb}$  (two lines,  $Acp62F^{lbB}$  and  $Acp62F^{lbC}$ ),  $w^+;w^{1118};Acp62F^{lc}$  ( $Acp62F^{lc}$ ) and control lines ( $w^+;w^{1118};Acp62F^{Tandem}$  ( $Acp62F^{Tandem}$ )). In addition, a further line,  $Acp62F^{lcCtrl}$ , was used as a control. These males were the result of the crosses expected to produce males with a  $w^+;w^{1118};Acp62F^{lc}$  genotype (i.e. Acp62F null), however they did, for an unknown reason, produce Acp62F protein (Figure 6.1). The failure of this line may have resulted from inefficient balancer chromosomes in early generations of the crossing regime or the failure of the insertions on the 3<sup>rd</sup> chromosome to align correctly in the final generation of the crossing regime.

## 2.4 General Methods

### 2.4.1 *Mating and courtship observations*

Mating and courtship observations were made on flies in vials placed on a viewing rack in the CT rooms, and usually began immediately after lights-on. Ten observations were made on each observation day, with at least 20 minutes between observations to ensure matings were not counted twice. Courtship was defined as wing display directed towards a female or an attempted mounting by males.

### 2.4.2 *Egg counts and egg to adult viability*

To measure female fecundity, females were placed in vials containing normal SY food with additional charcoal (4g/l). The addition of charcoal to the food created a greater contrast between the eggs and the food, making the eggs more visible and the egg counts more accurate. Females were allowed to lay for up to 24 hours (but for any given day the laying period was the same). Eggs counts were made using a dissection microscope at  $\times 25$  magnification. Vials were retained for  $\sim 13$  days to allow offspring to emerge after which offspring were counted immediately or frozen at  $-80^{\circ}\text{C}$  to be counted at a later date. Egg to adult viability was calculated as the proportion of eggs giving rise to adults.

### 2.4.3 *Morphological measurements of accessory glands, testis and body size*

All body size, testis size and accessory gland size measurements were made using the NIH Object Image program (version 1.62n3, by Norbert Vischer, available at <http://simon.bio.uva.nl/object-image.html>) on a Macintosh computer attached to a compound microscope ( $\times 100$  magnification). For body size, a measure of wing area was used (Gilchrist and Partridge 1999; Calboli 2004). Wings were mounted on glass slides using Propanol and Aquamount (as described in Calboli (2004)). Wing perimeter was measured as described in Gilchrist and Partridge (1999) by calculating the area within 6 landmark points around the edge of the wing. Testes and accessory glands were dissected in PBS (phosphate buffered saline) on a glass slide (Bangham et al. 2002). The polygon tool of the NIH Object Image program was used to measure the perimeter and area of testes and accessory glands. Where



possible, both accessory glands and testes from each individual were measured and the mean was used in subsequent analyses. If only one testis or accessory gland was measurable (e.g. if the other was damaged during dissection) then a single measurement was made.

#### *2.4.4 Western Blot analysis*

Western blotting was performed to confirm Acp62F levels in knockdown, null and control males (Chapter 6). Males were placed in groups of 10 into Eppendorf tubes with 40µl of homogenization buffer (25 mM Tris·HCl (pH 7.8), 10mM MgCl<sub>2</sub>, 15mM EDTA, 75mM NaCl, 1mM NaF, 0.5mM NaVO<sub>3</sub>, 15mM BGP, 15mM PNPP, 0.1% Nonidet P40, protease cocktail inhibitor and Antipain) and partially homogenized. 40µl of 2× sample buffer (125 mM Tris·HCl, pH 6.8/20% (vol/vol) glycerol/4% SDS/0.01% bromophenol blue/10% (vol/vol) 2-mercaptoethanol) was added, and the samples were fully homogenized, boiled for 4 min, transferred to ice for 2 min and centrifuged at 100 x g for 5 min at 4°C. An equal amount of protein extract for each line was loaded on an SDS/polyacrylamide gel (15% acrylamide/bisacrylamide) and subjected to electrophoresis at 120V for approximately 1 hour. The gel was equilibrated with Towbin buffer (25mM Tris Base, 192mM Glycine, 0.1% SDS (10%), 20% Methanol) and blotted on Hybond ECL nitrocellulose membrane (Amersham Pharmacia). The membrane was washed in blocking solution (5% low-fat dry milk in PBS/0.1% Tween 20 (PBS-T)) for 1 hour and incubated for 1.5 hours with the primary antibody for Acp62F (donated by Mariana Wolfner, Cornell University). After washing with PBS-T solution, the membrane was incubated with peroxidase-labeled anti-goat-anti-rabbit secondary antibody (Amersham Pharmacia) for 1 hour, and then treated with the ECL Western blotting detection system (Amersham Pharmacia), according to the manufacturer's instructions. The Western blots showed that Acp62F knockdown and null males produced no detectable Acp62F and control males produced Acp62F (Figure 6.1).

**Figure 2.1** The crosses used to produce Acp62F knockdown and control males. To induce RNA interference of Acp62F resulting in Acp62F knockdown, homozygous females carrying one of 3 inverted repeat (IR) constructs, *UAS-Acp62F-IR1b*, *UAS-Acp62F-IR3A*, and *UAS-Acp62F-IR3C*, were crossed to males carrying the X-linked transgene *Acp26AaP-Gal4*. The resulting male offspring possessed the *Gal4* driver and *UAS-Acp62F* transgenes. (i) *Acp26AaP-Gal4;UAS-Acp62F-IR1b* experimental males, (ii) *Acp26AaP-Gal4;UAS-Acp62F-IR3A* experimental males (iii) *Acp26AaP-Gal4;UAS-Acp62F-IR3C* control males. In the second RNAi experiment, as an additional control, non-transgenic females, with the same genetic background as those carrying *UAS-Acp62F*, were crossed to the *Acp26AaP-Gal4* stock. The male offspring resulting from this cross (iv) have the genotype *Acp26AaP-Gal4;+* and hence did produce Acp62F.

(i)

|   |
|---|
| <p>• <i>Y;UAS-Acp62F-1b UAS-Acp62F-1b</i> x <i>Acp26Aa-P-Gal4/Acp26Aa-P-Gal4</i><br/> <math>\Rightarrow</math> <i>Acp26Aa-P-Gal4 Y;UAS-Acp62F-IR-1b</i> +                      males with knocked-down levels of Acp62F</p> |
|---|

(ii)

|   |
|---|
| <p>• <i>Y;UAS-Acp62F-3A UAS-Acp62F-3A</i> x <i>Acp26Aa-P-Gal4/Acp26Aa-P-Gal4</i><br/> <math>\Rightarrow</math> <i>Acp26Aa-P-Gal4 Y;UAS-Acp62F-IR-3A</i> +                      males with knocked-down levels of Acp62F</p> |
|---|

(iii)

|   |
|---|
| <p>• <i>Y;UAS-Acp62F-3C UAS-Acp62F-3C</i> x <i>Acp26Aa-P-Gal4/Acp26Aa-P-Gal4</i><br/> <math>\Rightarrow</math> <i>Acp26Aa-P-Gal4 Y;UAS-Acp62F-IR-3C</i> +                      males with control levels of Acp62F (the 3C<br/> construct does not induce gene silencing)</p> |
|---|

(iv)

|   |
|---|
| <p><i>Non-transgenic sibling</i> x <i>Acp26Aa-P-Gal4/Acp26Aa-P-Gal4</i><br/> <math>\Rightarrow</math> <i>Acp26Aa-P-Gal4 Y; +</i> +                      males with control levels of Acp62F</p> |
|---|

**Figure 2.2** Construction of control and experimental males for the Acp62F null experiment. To equalize the genetic background and eye colour of all the males in the Acp62F null experiment, I conducted a crossing regime to introduce a wild-type X chromosome. This was necessary since null mutants were created in a  $w^{1118}$  background and had white eyes. In addition, males with the tandem duplication had non-white eyes. Since white-eyed males are blind, they do not mate at wild-type rates and are therefore unsuitable for use in tests of female fitness, particularly when in comparison to non-white males. As a result of the crossing regime below, Acp62F null and control flies with wild-type Dahomey X chromosome (red eyes), homozygous  $w^{1118}$  2nd chromosomes and homozygous null or control 3rd chromosomes were created.

|              |                                |                                |                                |                                |
|--------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
| Generation 1 |                                | (1) x (2) $\Rightarrow$ (3)    | (4) x (5) $\Rightarrow$ (6)    |                                |
| Generation 2 | (3) x (6) $\Rightarrow$ (7)    | (2) x (8) $\Rightarrow$ (12)   | (2) x (9) $\Rightarrow$ (13)   | (2) x (10) $\Rightarrow$ (14)  |
| Generation 3 | (12) x (7) $\Rightarrow$ (16)  | (13) x (7) $\Rightarrow$ (17)  | (14) x (7) $\Rightarrow$ (18)  | (15) x (7) $\Rightarrow$ (19)  |
| Generation 4 | (16) x (19) $\Rightarrow$ (20) | (17) x (19) $\Rightarrow$ (21) | (18) x (19) $\Rightarrow$ (22) | (19) x (19) $\Rightarrow$ (23) |

**KEY**

(1)  $w^{1118}; w^{1118}; w^{1118}$

(2)  $Bar; Cy; Tb, Hu$

(3)  $w^{1118}/Bar; w^{1118}/Cy; w^{1118}/Tb, Hu$

(4)  $w^+; w^+; w^+$

(5)  $Cy/Sco; Tb, Hm/Sb$

(6)  $w^+; Sco/w^+; Sb/w^+$

(7)  $w^+/Bar; Sco/w^{1118}; Sb/w^{1118}$

(8)  $Acp62F^{lb}/Acp62F^{lb}$

(9)  $Acp62F^{lc}/Acp62F^{lc}$

(10)  $Acp62F^{tandem}/Acp62F^{tandem}$

(11)  $w^{1118}$

(12)  $Bar; Acp62F^{lb}/Cy; Acp62F^{lb}/Tb, Hu$

(13)  $Bar; Acp62F^{lc}/Cy; Acp62F^{lc}/Tb, Hu$

(14)  $Bar; Acp62F^{tandem}/Cy;$

$Acp62F^{tandem}/Tb, Hu$

(15)  $Bar; w^{1118}/Cy; w^{1118}/Tb, Hu$

(16)  $w^+/Bar; w^{1118}/Cy; Sb/ Acp62F^{lb}$

(17)  $w^+/Bar; w^{1118}/Cy; Sb/ Acp62F^{lc}$

(18)  $w^+/Bar; w^{1118}/Cy; Sb/ Acp62F^{tandem}$

(19)  $w^+/Bar; w^{1118}/Cy; Sb/w^{1118}$

(20)  $w^+; w^{1118}; Acp62F^{lb}$

(21)  $w^+; w^{1118}; Acp62F^{lc}$

(22)  $w^+; w^{1118}; Acp62F^{tandem}$

(23)  $w^+; w^{1118}; w^{1118}$

$w^+$ =wild-type

$Cy$ =Curly

$Sb$ =Stubble

$Sco$ =Scutoid

$Tb, Hu$ =Tubby, Humoral

## **Chapter 3 Ejaculate depletion patterns evolve in response to experimental manipulation of sex ratio in *Drosophila melanogaster***

### **3.1 Abstract**

I assessed the extent to which traits related to ejaculate investment have evolved in lines of *Drosophila melanogaster* that had an evolutionary history of maintenance at biased sex ratios. Measures of ejaculate investment were made in males that had been maintained at male-biased (MB) and female-biased (FB) adult sex ratios, in which levels of sperm competition were high and low, respectively. Theory predicts that when the risk of sperm competition is high and mating opportunities are rare (as they are for males in the MB populations), males should increase investment in their few matings. I therefore predicted that males from the MB lines would (1) exhibit increased investment in their first mating opportunities and (2) deplete their ejaculates at a faster rate when mating multiply, in comparison to FB males. To investigate these predictions I measured the single mating productivity of males from 3 replicates each of MB and FB lines mated to 5 wild-type virgin females in succession. In contrast to the first prediction, there was no evidence for differences in investment between MB and FB line males in their first matings. The second prediction was upheld: males of MB and FB males suffered increasingly reduced fertility with successive matings, but the decline was significantly more pronounced for MB than for FB males. There was a significant reduction in the size of the accessory glands and testes of males of MB and FB regimes after 5 successive matings. However, the accessory glands, but not testes, of MB males became depleted at a significantly faster rate than those of FB males. The results show that male reproductive traits have evolved in response to the risk of sperm competition and suggest that the ability to maintain fertility over successive matings is associated more strongly with the rate of accessory gland than testis depletion.

This work has been accepted for publication in *Evolution*.

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### 3.2 Introduction

In species where females mate with more than one partner, males may have to compete in sperm competition. Numerous studies have documented the various ways in which males have adapted to the threat of sperm competition, including by making a greater investment in sperm production (Parker 1990a)(see section 1.3). Intra-specific studies have documented the evolution of plastic responses to the perceived threat of sperm competition (Wedell and Cook 1999), have investigated the ejaculate investment of different male morphs (Gage et al. 1995) or have measured evolved responses to artificial selection (Hosken and Ward 2001). When sperm competition is analogous to a fair raffle, high sperm number is advantageous in assuring paternity (Parker 1990a) and theory predicts that where sperm competition risk is high, males should increase sperm number (Parker 1990b). Although individual sperm are expected to be energetically cheap to produce (Parker 1970), spermatophores and whole ejaculates may be energetically expensive or even limiting in some cases (Dewsbury 1982; Wedell et al. 2002). In these situations, males are expected to evolve strategies to allocate their ejaculates (Parker 1990a; Parker 1990b) according to the level of sperm competition that they experience and the frequency of their access to mates (Parker 1970; Parker 1990a; Parker 1990b).

An important assumption underlying predictions about strategic ejaculate allocation is that ejaculates can be in limited supply, or that the rate of ejaculate replenishment may be slow (Dewsbury 1982; Wedell et al. 2002). For example, the reproductive success of the males of many species may be constrained by the production rate of sperm or ejaculate products such as the accessory gland proteins that constitute a major part of the seminal fluid in many species. Hihara (1981) observed that over 5 successive matings, the accessory glands of male *D. melanogaster* became depleted, which caused a decrease in the number of eggs laid by the fourth- and fifth-mated females, and in the fertility of fifth-mated females. The latter drop in fertility was associated with accessory gland depletion and not with sperm depletion, suggesting that accessory gland products were the main limiting factor in determining fertility (Hihara, 1981). Mating frequency will also influence the expected number and size of ejaculates a male has to deliver. Consistent with this idea, *D. nanoptera* males transfer consistently large ejaculates to an average of 2.3 females whereas *D. pachae*

transfer significantly smaller ejaculates to an average of 4.32 mates per day (Pitnick and Markow 1994). Together with the plastic responses to sperm competition described above, these findings suggest that the ejaculate allocation patterns of males will be shaped by the mating system and degree of sperm competition. However, to date there have been few explicit experimental tests of this idea.

In this chapter I use experimental evolution to examine the ejaculate allocation strategies of *Drosophila melanogaster* males evolving under differing levels of sperm competition. Mating opportunities and the risk and intensity of sperm competition were manipulated by using males from selected lines that varied in adult sex ratio. Three replicate lines of each male-biased (MB) and female-biased (FB) regimes were created (as described in Wigby and Chapman 2004). During the 9-day adult sex ratio biased period of each generation, mating opportunities for males in the MB lines were significantly fewer than for males in FB lines (a sample of 3 h of observations days gave an average of 0.22 matings per male for MB males and 0.69 matings per male for FB males, (Wigby and Chapman 2004)). Extrapolating these figures to give the total number of matings during the 9-day selection period gives an average for MB and FB males respectively of (i) 1.32 and 4.14 matings, assuming no mating during the dark, (ii) 1.98 and 6.21 matings if there is 50% mating during the dark (Willmund and Ewing 1982) and (iii) 2.64 and 8.28 matings if mating occurs irrespective of the dark (Fujii et al. 2007). Note that matings were sampled during peak mating periods and so in each case the extrapolated figures are likely to be overestimates.

Regardless of which estimate is used, MB males mate infrequently but face a high risk and intensity of sperm competition when they do mate (each MB female mates on average 3 times as often as each male and is therefore multiply inseminated). MB males should therefore be selected to increase their reproductive success by transferring large ejaculates, particularly in their first matings (Parker 1998). The first prediction was therefore that MB males would have higher productivity in their first matings than FB males. The mating rate estimates also reveal that selection for MB males to maintain fertility over rapid, successive matings should be weak, as opportunities for MB males to mate multiply are relatively rare during the selection procedure. The second prediction was therefore that MB males would show weaker partitioning of ejaculates (and hence more ejaculate exhaustion) with successive

partners than would FB males. To test these predictions, males were given the opportunity to mate with 5 wild-type females in succession in a series of single pair matings. To test whether any exhaustion was due to ejaculate depletion, I compared the effect of successive matings on the reduction in size of the testes and the accessory glands (i.e. the structures that produce the ejaculate).

### **3.3 Materials and Methods**

#### *3.3.1 Stocks and Cultures*

##### *(i) Wild-type Stock*

The Dahomey wild-type stock used in these experiments is described in section 2.3.1. Females were obtained by collecting eggs from population cages and placing those eggs at standard density (Clancy and Kennington 2001).

##### *(ii) Males with an evolutionary history of high and low sperm competition*

Males for the experiments were obtained from the selection lines described in Wigby and Chapman (2004) and section 2.3.2. Briefly, in these lines the intensity of sexual selection and sexual conflict were varied by altering the adult sex ratio. Each generation, three replicate lines each of male-biased (MB, 75 males and 25 females; 70 females: 30 males from generation 53 onwards) and female-biased (FB, 25 males and 75 females) sex ratio treatments were propagated. Nine days after the cages were set up eggs were collected. The majority of eggs were allowed to hatch before larvae were collected and raised at standard density to minimise environmentally-determined differences in adult body size. All adults were allowed to eclose over two days before being allocated to the same sex-ratio treatment and replicate number as their parents for the next generation.

### 3.3.2 *Experimental procedures*

#### (i) *Progeny sired by males from the MB and FB lines mated to 5 wild-type virgin females in series*

To test the ability of males to allocate ejaculates over successive matings, I mated males from the MB and FB lines to 5 wild type virgin females in succession and recorded the number of progeny produced from each of those matings. The experiments were performed in 3 blocks using males from different generations of selection; generation 60 (Block 1), 65 (Block 2) and 67 (Block 3). Progeny data were not recorded for replicate 3 of each regime in Block 2. SY vials seeded with live yeast were used throughout. Males used in experiments were housed in groups of 10 in vials and aged for 10 days after eclosion (the age at which eggs were taken from the lines to propagate the next generation during selection). Dahomey wild-type virgin females were anaesthetized on ice and aged for 5 days in groups of 10-12. Females were housed individually one day prior to experiments.

Individual males were randomly allocated to a wild-type female and allowed to mate once. Males that mated successfully were transferred to another vial containing a virgin female. This was repeated up to 5 times. Males failing to mate within one hour were transferred to a new vial containing another virgin female. A second consecutive failed mating was considered a 'fail' and such males were not given further opportunities to mate. The total number of progeny produced by each of the 5 females mated to each male were counted for 15 days following each mating. Females were transferred to fresh food vials on days 2, 3, 4, 6, 8, 10 (Block 1) and days 2, 4, 7, 10 (Blocks 2 and 3).

#### (ii) *The time to mating and mating duration of males from the MB and FB lines mated to 5 wild-type virgin females in series*

For each of the matings of MB and FB males described above, the time from the introduction of the male into each vial until the time when mating began (to the nearest minute) was recorded. I also recorded the duration of each mating (in minutes).



(iii) *The accessory gland and testis size of males from the MB and FB lines mated to 5 wild-type virgin females in series*

I examined the degree to which 5 successive matings reduced the size of the accessory glands and testes of the MB and FB males, using the protocol described above. Males were collected from generation 86 and after completing 5 successful matings, were immediately frozen at  $-80^{\circ}\text{C}$ . In addition, 20 control males per line were not mated, but were placed on the same observation rack for the duration of the experiment and were frozen at the end of that day. Males were dissected as in Bangham et al. (2002) in phosphate-buffered saline on a glass slide, and images of the accessory glands and testes were captured from a compound microscope using a video camera attached to a Macintosh computer. All dissections were coded blind. Images were analysed using the NIH Object Image program using the polygon tool to measure the area of the testes and accessory glands (section 2.4.3). To record body size, I used a measure of wing area (Gilchrist and Partridge 1999). The wings of males from all selection treatments were mounted on glass slides and were again measured using the NIH Object Image program. Analysis was performed on the average of both wings, accessory glands or testes of each male, or single measures if only one was available (wings, 6/111 were single measures, for accessory glands, 0/198 and for testes, 18/194).

### 3.3.3 *Statistical analysis*

Statistical analysis was performed using JMP statistical software (version 5, SAS Institute Inc.) and the nlme package of R software (Pinheiro et al. 2006).

All data were analysed with linear mixed effects ANOVA model (by REML), specifying selection regime (FB and MB) and the 5 wild-type virgin females in series as fixed effects. Females were treated as a factorial variable to explore when differences between the regimes occurred. To account for the repeated measurements on individual males and the blocking structure of the experiment, the individual males nested in replicate and block, were included as random effects. Data for zero progeny values were removed from the analysis. Different variance strata were applied to replicates within regime for the progeny data to account for non-constant variance (such weighting improved the models although it did not change the result). Both time to mating and mating duration were log-transformed in the analyses to remove skew.

### 3.4 Results

#### 3.4.1 *Progeny sired by males from the MB and FB lines mated to 5 wild-type virgin females in series*

The total number of progeny sired by males mated to 5 virgin females in series declined significantly for both MB and FB regimes over the series (factor(female),  $F_{4, 669}=36.1$ ,  $P<0.0001$ , Figure 3.1). There was also a significant interaction effect between female in the series and regime (factor(female) by regime,  $F_{4, 669}=3.7$ ,  $P=0.006$ ), showing that the decline in progeny produced by MB males over the series of females was significantly more rapid than the decline for FB males (Figure 3.1). There were no significant differences between the regimes in the number of offspring sired by males with females 1, 2 or 3, but FB males sired significantly more offspring than MB males with females 4 and 5 in the series (Table 3.1). There was no difference in the total number of progeny sired across the 5 females by males from the two regimes (linear mixed effects model (by REML) with different standard deviations per stratum, individual males nested in replicate and Block as random effects,  $F_{1, 12}=1.16$ ,  $P=0.30$ ). The number of males that failed to mate with 5 females in succession did not differ between regimes (all blocks combined,  $\chi^2=0.024$ ,  $df=6$ ,  $P>0.99$ ).

#### 3.4.2 *The time to mating and mating duration of males from the MB and FB lines mated to 5 wild-type virgin females in series*

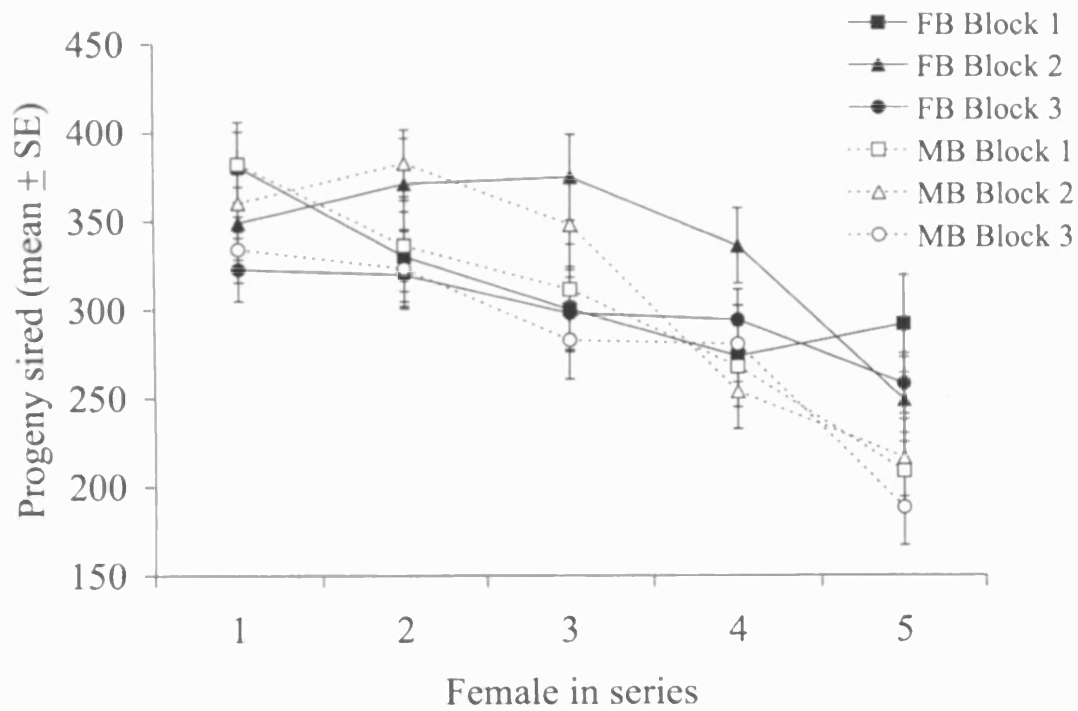
The time taken to initiate each mating did not significantly differ between females in the series and did not differ between regimes (ANOVA, factor(female),  $F_{4, 786}=0.76$ ,  $P=0.55$ , regime,  $F_{1, 12}=0.02$ ,  $P=0.8917$ , factor(female) by regime,  $F_{4, 786}=1.37$ ,  $P=0.24$ , Figure 3.2). There was a significant decline in mating duration over the series of matings for both FB and MB males but there was no significant difference between the regimes (Figure 3.3, Table 3.2).

#### 3.4.3 *The accessory gland and testes sizes of males from the MB and FB lines mated to 5 wild-type virgin females in series*

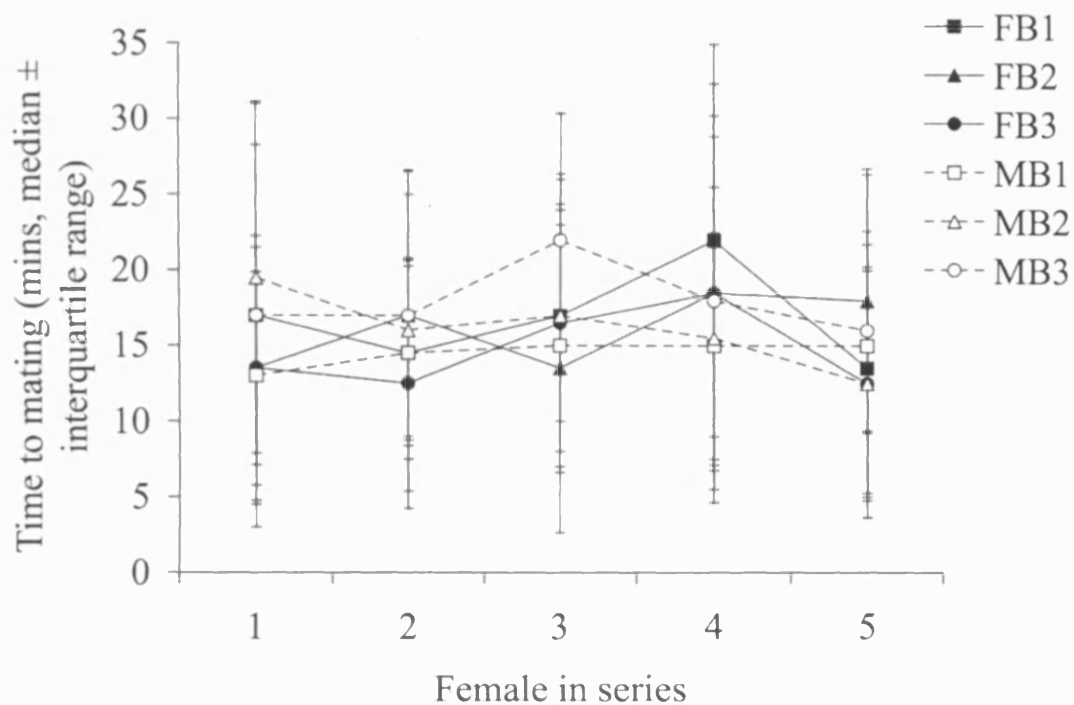
There were no significant differences between the non-mating males from MB and FB lines in body size or in relative and absolute accessory gland and testis size (Figure 3.4a, b, Table 3.3). Males from both regimes that had mated 5 times had significantly

smaller accessory glands and testes (absolute size) than males that did not mate (Figure 3.4, Table 3.4). However, there was a significant interaction between mating status and regime for accessory gland size (Table 3.4), and although there was a tendency for the same effect in testis size, it was not significant (Table 3.4). This analysis shows that the reduction in accessory gland size after 5 matings was significantly greater for MB males than for FB males.

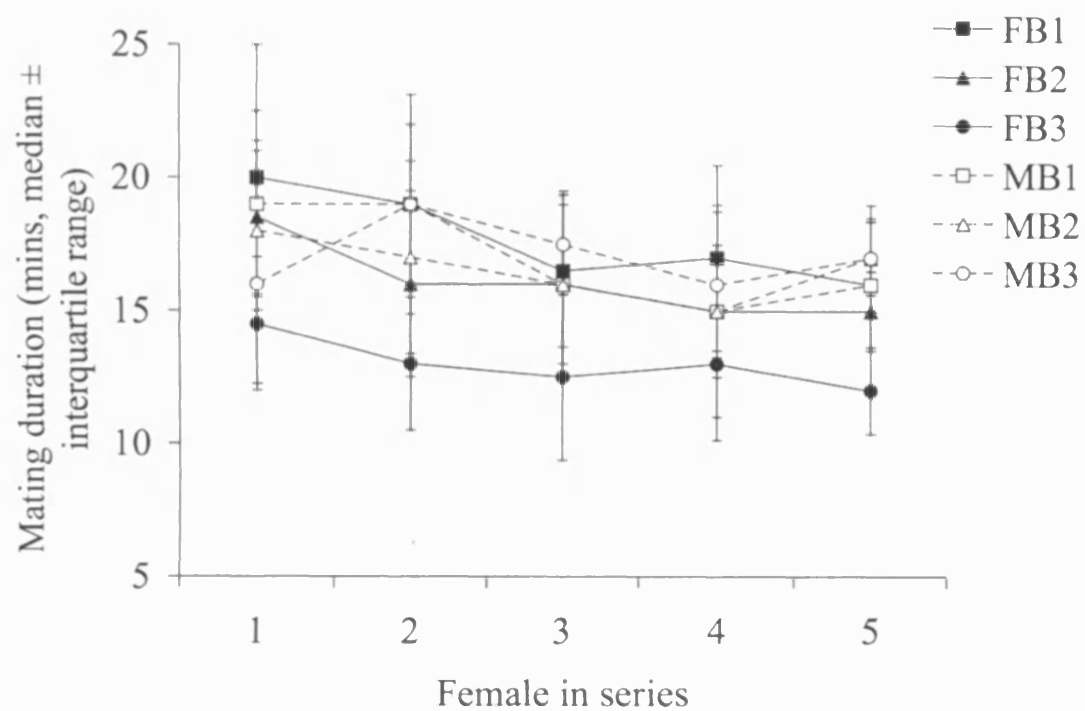
**Figure 3.1** Mean ( $\pm$  SE) total number of progeny produced by males from the MB and FB lines mated in series to 5 virgin wild type females. Replicates have been combined for both regimes. Progeny were collected for individual females for 15 days. The counts are standardised for female egg laying time.



**Figure 3.2** Median ( $\pm$  interquartile range) time to mating of each mating for females 1-5 mated to FB and MB males. Replicates have been combined for regimes within each block.

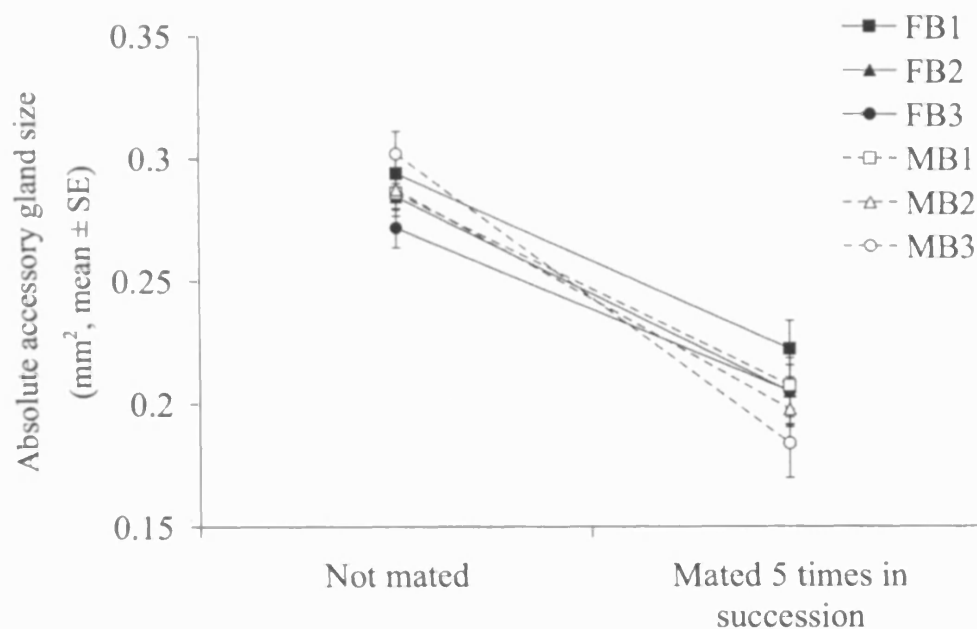


**Figure 3.3** Median ( $\pm$  interquartile range) mating duration of each mating for females 1-5 mating with MB and FB males. Replicates have been combined for regimes within each block.

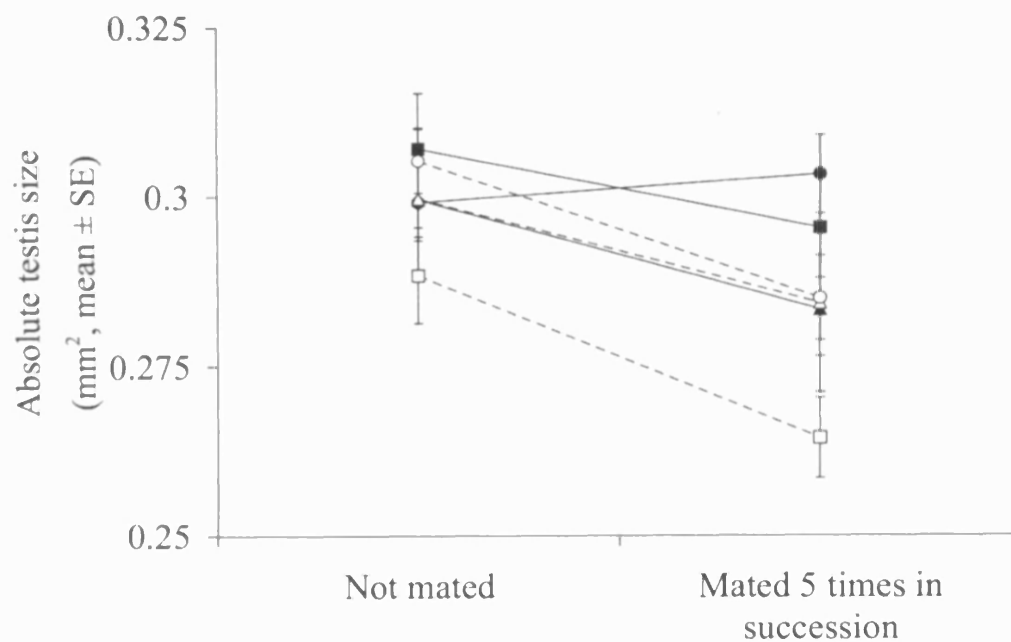


**Figure 3.4** (a) Mean ( $\pm$  SE) accessory gland size ( $\text{mm}^2$ ) and (b) testes size for males from FB and MB lines that were either not mated or mated to 5 females in series. Replicates have been combined for each Block.

(a)



(b)



**Table 3.1.** Effect sizes of linear mixed effects ANOVA model (by REML) on the total number of progeny sired by FB and MB males mated to 5 wild-type virgin females in series. The total number of progeny produced was recorded for 15 days following each mating. Female and regime were set as fixed effects, and individual males was nested in replicate and Block as random effects. Zero progeny values were removed from the analysis. Different variance strata were applied to account for differences in variation between replicates within regime (such weighting improved the model although it did not change the result).

| Factor                    | Value  | SE    | DF  | t     | P       |
|---------------------------|--------|-------|-----|-------|---------|
| Intercept                 | 355.65 | 14.60 | 669 | 24.35 | <0.0001 |
| Female 2                  | -8.02  | 13.89 | 669 | -0.58 | 0.5637  |
| Female 3                  | -37.90 | 14.36 | 669 | -2.64 | 0.0085  |
| Female 4                  | -45.84 | 14.68 | 669 | -3.12 | 0.0019  |
| Female 5                  | -84.87 | 15.70 | 669 | -5.41 | <0.0001 |
| Regime                    | 8.62   | 18.93 | 12  | 0.46  | 0.6571  |
| Female 2:Regime FB vs. MB | -4.47  | 20.25 | 669 | -0.22 | 0.8255  |
| Female 3:Regime FB vs. MB | -5.02  | 20.71 | 669 | -0.24 | 0.8085  |
| Female 4:Regime FB vs. MB | -44.45 | 21.34 | 669 | -2.08 | 0.0376  |
| Female 5:Regime FB vs. MB | -70.34 | 22.32 | 669 | -3.15 | 0.0017  |



**Table 3.2.** Effect sizes of linear mixed effects ANOVA model (by REML) on the mating duration (log transformed) of FB and MB males mated to 5 wild-type virgin females in series. Female and regime were set as fixed effects, and individual males was nested in replicate and Block as random effects.

| Factor                    | Value | SE   | DF  | t     | P       |
|---------------------------|-------|------|-----|-------|---------|
| Intercept                 | 2.89  | 0.05 | 658 | 60.45 | <0.001  |
| Female 2                  | -0.11 | 0.05 | 658 | -2.40 | 0.0166  |
| Female 3                  | -0.18 | 0.05 | 658 | -3.72 | 0.0002  |
| Female 4                  | -0.20 | 0.05 | 658 | -4.02 | 0.0001  |
| Female 5                  | -0.24 | 0.05 | 658 | -4.46 | <0.0001 |
| Regime                    | -0.02 | 0.07 | 12  | -0.35 | 0.7332  |
| Female 2:Regime FB vs. MB | 0.07  | 0.07 | 658 | 1.10  | 0.2711  |
| Female 3:Regime FB vs. MB | 0.11  | 0.07 | 658 | 1.64  | 0.1006  |
| Female 4:Regime FB vs. MB | 0.03  | 0.07 | 658 | 0.43  | 0.6694  |
| Female 5:Regime FB vs. MB | 0.14  | 0.08 | 658 | 1.90  | 0.0575  |

**Table 3.3.** Mixed effects ANOVA of (a) body (wing), (b) relative testis and (c) relative accessory gland size of unmated males from replicate MB and FB lines, with selection regime (fixed effect) and replicate line nested within selection regime (random effect).

| Source of variation  | DF  | SS     | MS     | F      | P      |
|--|-----|--------|--------|--------|--------|
| <i>(a) Body size</i>   |     |        |        |        |        |
| Selection regime   | 1   | 0.015  | 0.015  | 0.431  | 0.5475 |
| Replicate line within selection regime <sup>1</sup>                      | 4   | 0.143  | 0.036  | .      | .      |
| Error <sup>2</sup>   | 111 | 0.367  | 0.003  |        |        |
| <i>(b) Relative testis size</i>  |     |        |        |        |        |
| Selection regime   | 1   | 0.002  | 0.002  | 1.4988 | 0.288  |
| Replicate line within selection regime <sup>1</sup>                      | 4   | 0.006  | 0.0015 | .      | .      |
| Error <sup>2</sup>   | 108 | 0.091  | 0.0008 |        |        |
| <i>(c) Relative accessory gland size</i>                                 |     |        |        |        |        |
| Selection regime   | 1   | 0.0002 | 0.0002 | 0.297  | 0.6144 |
| Replicate line within selection regime <sup>1</sup>                      | 4   | 0.003  | 0.0007 | .      | .      |
| Error <sup>2</sup>   | 111 | 0.118  | 0.0012 |        |        |
| <sup>1</sup> Error term for MS <sup>selection regime</sup>               |     |        |        |        |        |
| <sup>2</sup> Error term for MS <sup>regime within selection regime</sup> |     |        |        |        |        |

**Table 3.4.** Effect sizes of mixed effects ANOVA of (a) absolute accessory gland size and (b) absolute testis size (log transformed) of males from replicate MB and FB lines that were not mated or that were mated to 5 females in series, with status (not mated versus mated 5 times in succession without recovery), selection regime and status x regime (fixed effects) and replicate line nested within selection regime (random effect).

| Source of variation             | Value  | SE    | DF  | t      | P       |
|---------------------------------|--------|-------|-----|--------|---------|
| <i>(a) Accessory Gland Size</i> |        |       |     |        |         |
| Intercept                       | 0.25   | 0.003 | 190 | 78.34  | <0.0001 |
| Status                          | 0.04   | 0.003 | 190 | 14.82  | <0.0001 |
| Regime                          | 0.001  | 0.003 | 4   | 0.43   | 0.6685  |
| Status*Regime                   | -0.006 | 0.003 | 190 | -2.01  | 0.0457  |
| <i>(b) Testis Size</i>          |        |       |     |        |         |
| Intercept                       | -1.23  | 0.01  | 183 | -105.5 | <0.0001 |
| Status                          | 0.02   | 0.008 | 183 | 2.95   | 0.0036  |
| Regime                          | 0.02   | 0.01  | 4   | 1.51   | 0.1319  |
| Status*Regime                   | -0.01  | 0.01  | 183 | -1.65  | 0.0997  |

### 3.5 Discussion

Males that had different evolutionary histories of sperm competition risk/intensity and mating opportunities differed significantly in their ability to maintain the fertility of their mates across successive matings. There was no evidence to support the first prediction that males from the MB and FB regimes differed in first mating investment. This could mean that the prediction is wrong, or that in these experiments males from both regimes transferred sufficient sperm and seminal fluid to maximally inseminate their first mates, masking any such effect. However, the second prediction, that MB and FB males would differ in their rate of ejaculate depletion, was supported: although males from both selection regimes suffered reduced fertility when mating with 5 virgin females in succession, the mates of MB males, that had evolved under a high level of sperm competition, declined in progeny production at a significantly faster rate than did mates of FB males. This resulted in significantly more offspring being sired in matings 4 and 5 by FB as compared to MB males. There were significant reductions in accessory gland size after 5 matings for males of both regimes. However, the degree of accessory gland reduction was significantly greater for MB than FB males. The results suggest that MB males decline in fertility faster than FB males because they exhibit faster rates of ejaculate, and particularly accessory gland, depletion.

The failure of the first prediction to be upheld, suggests alternative explanations. Theory predicts that when multiple male ejaculates compete, there may be selection for decreased ejaculate investment (Ball and Parker 1997; Parker et al. 1996). MB line females are estimated to mate on average 4 times and FB females 1.4 times during the selection period (data from Wigby and Chapman 2004). Hence, although individual MB males mate infrequently, when they do their ejaculates are in competition. Therefore MB males could have decreased their ejaculate expenditure, which is consistent with the lack of differences in first mating productivity followed by lower productivity in subsequent matings. However, this strategy is expected to increase fitness only with access to multiple mating opportunities (Ball and Parker 1997; Parker et al. 1996), which is not the case for MB males. An alternative possibility is that the low productivity of MB males across successive mates reflects a cost of sperm competition. For example, MB males may transfer ejaculate at a greater rate and

become ejaculate depleted sooner than FB males, but might show increased fitness in a competitive environment, a possibility that would be interesting to test.

There was no evidence that differences in sperm competition levels selected for differences in accessory gland or testis size, in contrast to findings in the yellow dung fly, *S. stercoraria* (Hosken and Ward 2001). This could indicate that the two types of experimental protocol produced differences in the intensity versus risk of sperm competition. MB and FB males differed in the degree to which their accessory gland size decreased following 5 matings, suggesting that the failure of MB males to maintain productivity was associated with accessory gland protein depletion. This interpretation is consistent with previous observations of the loss of fertility in serially mated males (Hihara 1981). However, testis size was also reduced as a consequence of consecutive matings, and the interaction with selection regime was only just non-significant (Table 3.4). Hence a lesser contribution of sperm depletion to the lower productivity of MB males in matings 4-5 is likely. Reductions in testis size with consecutive matings have been reported in *S. stercoraria* (Ward and Simmons 1991) but not *Cyrtodiopsis dalmanni* (Rogers et al. 2005).

Virgin females were used throughout these experiments. Under certain circumstances, males would be expected to allocate more ejaculate to virgin than to mated females (Parker and Ball 2007), but it is unclear whether, if this were the case, it would alter the pattern of the results here. This could be tested in the future by asking whether there is any significant interaction in ejaculate allocation between female mating status and male line.

Mating duration showed a tendency to decline over successive matings but it did not explain variation in progeny number and did not differ significantly between regimes. The observed decline in mating duration could be adaptive for females if they were able to reduce mating costs by disengaging earlier with males that are unable to provide sufficient ejaculate. However, it is unclear whether either sex has predominant control of mating duration (Lefranc and Bundgaard 2000; MacBean and Parsons 1967). Ejaculate transfer could also occur earlier or be shorter in successive matings because less ejaculate remains to be transferred. Since mating duration showed no difference between regimes, and given that sperm displacement ability is independent

of copulation duration (Gilchrist and Partridge 2000), the results indicate that selection has had no effect on this trait. There were also no significant differences in time to mating. MB males did not increase the interval between matings to compensate for reduced ability to fertilize females and males from both regimes did not differ, or show an increase, in mating failure rate (i.e. matings that produced no progeny) with successive matings. These results suggest that males did not lose their willingness or ability to mate multiply.

Previous work on various *Drosophila* species has also documented variation in fertility across multiple matings. For example, *D. melanogaster* males have been observed to mate 3 to 5 times before showing reduced fertility (Hihara 1981; Lefevre and Jonsson 1962). Observations suggested to be consistent with ejaculate partitioning have also been observed. For example, males of *D. hydei* and *D. buzzati* are reported to sire few offspring per copulation but to maintain high fertility in up to six matings (Markow 1985). In *D. hydei* this pattern could arise because this species produces few, large sperm that may itself constrain how many sperm can be transferred. Inter-specific variation in ejaculate allocation is also reported by Pitnick and Markow (1994), who suggested that selection may favour a bet-hedging strategy whereby males that partition ejaculates amongst females can realise greater reproductive success.

In summary, I found evidence for differences in ejaculate delivery patterns in lines selected under high and low levels of sperm competition. In serial matings, males with an evolutionary history of exposure to high levels of sperm competition exhibited faster declines in fertility than did males exposed to lower levels of sperm competition. The evidence suggests that this effect was due to differences in rates of ejaculate, particularly accessory gland, depletion.

## **Chapter 4 Accessory gland size responds to artificial selection and is associated with male reproductive success**

### **4.1 Abstract**

In species in which females mate with multiple males, the ability of males to be successful in post-copulatory competition is an important part of overall male reproductive success. In *D. melanogaster*, as in many other species, ejaculate proteins produced by the male accessory glands (Acps) transferred together with sperm during mating have a significant influence on male post-copulatory success. Individual Acps are known, for example, to reduce female receptivity, increase egg production and increase sperm storage. However, less is known of the functional significance of the size of the accessory glands themselves. To test whether accessory gland size is significantly associated with male post-copulatory success, I performed replicated bi-directional artificial selection on male accessory gland size. Accessory gland size showed a clear and significant response to selection with males in 'large' lines having significantly larger accessory glands than males in 'small' lines. These changes were independent of body size, which did not differ significantly between the regimes. Testis size showed significant within regime differences between replicates, but did not differ significantly between regimes. When in competition with other males, males with larger accessory glands sired significantly more offspring than did males from the small or unselected lines, in the absence of any significant differences in mating frequency. No significant differences between regime differences in sperm displacement ability in a non-competitive situation were found, but both accessory gland and testis size significantly determined the ability of males to displace the sperm of rival males. The results show that accessory gland size plays a significant role in the post-copulatory success of *D. melanogaster* males.

## 4.2 Introduction

In species in which females mate with multiple males, overall male reproductive success is influenced both by the acquisition of mates and by a male's effectiveness in effecting post-copulatory processes and his success in post-copulatory male-male competition (Parker 1970). Females of many insect species mate multiply and store sperm. In doing so, they facilitate male-male competition that extends beyond the point of copulation (Parker 1970). Selection is expected to act upon morphological and behavioural traits in males that maximize success in post-copulatory competition. These traits will be those that determine the ability of a male to displace the sperm of a previous male from his mate(s) and prevent his own sperm from being displaced from storage during subsequent matings. For instance, post-copulatory success is associated with large body size (Bissoondath and Wiklund 1997) and reproductive morphology (Arnqvist and Danielsson 1999; Danielsson and Askenmo 1999). There may also be significant interactions between body size and reproductive morphology. For example, large males of the yellow dung fly, *Scatophaga stercoraria*, transfer ejaculate more rapidly than do small males (Simmons et al. 1996), whereas small males tend to copulate for longer. Hence, both large and small males realize a similar overall post-copulatory success (Parker and Simmons 1994; Simmons and Parker 1992).

Sperm competition, the competition between sperm of two more rival males for the fertilisation of a given set of eggs (Parker 1970; Parker 1998), is a powerful selective force acting on many of the adaptations associated with increased post-copulatory success. In terms of adaptations in reproductive morphology, attention has principally focused on variation in testis size (Pitnick 1996), since sperm competition theory has primarily made predictions regarding the relationship between the level of sperm competition and investment in spermatogenesis. Very little theoretical or empirical attention has been focussed on the potential associations between ejaculate (Acp) volume and the level of sperm competition. The prediction that a positive relationship should exist between investment in spermatogenesis and levels of sperm competition is supported by comparative studies across diverse taxa, with the finding that males in polyandrous mating systems have relatively larger testes (e.g. in primates, Harcourt et al. 1981; birds, Møller and Ninni 1998; amphibians, Jennions and Passmore 1993;



butterflies, Gage 1994; Karlsson 1995; bats, Hosken 1997; and fish, Stockley et al. 1997), and are hence capable of producing more sperm (Gage 1994; Møller 1988; Møller 1989). In addition, the reasons for the huge variation in sperm morphology within and between species (e.g. Gage 1994) have been subject to intense investigation. In the genus *Drosophila*, for example, sperm flagellum and thus testis lengths vary by a factor of more than 180 (Pitnick et al. 1995).

In *D. melanogaster*, as in other insects, in addition to associations with sperm-related traits, male post-copulatory success is also influenced by the actions of ejaculate proteins that are transferred with sperm during mating (Wolfner 1997). Approximately 80-100 accessory gland proteins (Acps) are produced in the male accessory glands and they have striking effects on female physiology and behaviour (Wolfner 1997). For example, Acp26Aa and Acp70A (SP) cause an increase in egg laying following mating (Chen et al. 1988; Herndon and Wolfner 1995) and SP also decreases female receptivity to mating (Chen et al. 1988). Studies with transgenic males lacking Acps have demonstrated that accessory gland proteins are necessary for sperm storage, which is likely to be a key component of sperm competition (Kalb et al. 1993; Tram and Wolfner 1999). In particular, males lacking Acp36DE are less effective in storing sperm (Neubaum and Wolfner 1999) and consequently have significantly reduced effectiveness in sperm competition (Chapman et al. 2000). Associations also exist between alleles in or near Acp26Aa, Acp29AB, Acp36DE, Acp53E and a male's ability in sperm defence (i.e. prevention of the removal of a male's sperm by a later mating male, (Clark et al. 1995). Fiumera et al. (2005) also found several significant associations between Acp alleles (polymorphisms in or near CG8137, CG17331, CG31872, Acp33A, Acp29AB and Acp26Aa) and male sperm competitive ability. In addition, there may remain as yet unidentified Acps that contribute to post-mating success via processes such as sperm displacement (Harshman and Prout 1994).

The quantity of Acps transferred during mating is an important determinant of post-copulatory success (Bangham et al. 2002; Hihara 1981; Kalb et al. 1993). Accessory glands become depleted after 4-5 successive matings, leading to decreased fertility (Hihara 1981; Chapter 3) even though motile sperm remain in the seminal vesicles (Hihara 1981), suggesting that secretions of the accessory gland are responsible for fertility maintenance. In addition, fertility was restored following Acp delivery. The

necessity to replenish the contents of the accessory glands may also affect pre-copulatory success, since this may constrain male mating frequency. Consistent with this idea, it has been found that males with larger accessory glands mate more often than males with smaller glands (regardless of body size, Bangham et al. 2002).

To examine the contribution of male reproductive morphology to male reproductive success, I performed replicated bi-directional artificial selection on male accessory gland size in *D. melanogaster* and assayed direct and two correlated responses to selection, after 16 and 38 generations. The role of accessory gland size in determining post-copulatory success was examined in two ways. Firstly, selection line males were mated to sparkling females in a wild-type background that had previously mated with a competitor (*sparkling*) male in a test for sperm displacement ability in a non-competitive mating environment. Secondly, selection line males were placed together in competition with sparkling males for 10 days with sparkling females in a wild-type background, to establish the effects of accessory gland size on overall male competitive ability.

### **4.3 Materials and Methods**

#### *4.3.1 Artificial selection for large and small accessory glands*

##### *Generation 1:*

Flies for the first generation of selection were obtained by collecting eggs from population cages. Two replicate lines each selected for large or small male accessory gland size were initiated from the wild-type Dahomey population. 50 individual males for each replicate were each housed with two females in ASG vials (section 2.1.3) seeded with yeast balls for 1 day. The males were then removed and housed individually for 3-7 days to allow the accessory glands to replenish their contents. Females were discarded after the laying period. The accessory glands from these 50 males of each replicate were then dissected as in Bangham et al. (2002) in phosphate-buffered saline on a glass slide. Images of the accessory glands and testes were captured using the NIH Object Image program and measured using the polygon tool (section 2.4.3). Males were coded for dissection and image measurement so that the

investigator was blind as to male identity. Males were ranked according to accessory gland area and then uncoded. Virgin progeny were collected from the largest and smallest 8 families respectively to propagate the next generation. A further 8 vials per replicate containing the progeny of males that had not been dissected were chosen at random to create an unselected group.

#### *Subsequent generations:*

To propagate the next generation, virgin males and females from the highest and lowest ranked families were anaesthetized on ice and housed in family groups of ~12 and aged between 3-10 days. Females from families ranked 1, 3, 5 and 7 were mated to males from families 2, 4, 6, 8 and vice versa, to ensure that there were no matings between full siblings. Individual males were housed with two females, allowed to mate for one day and then maintained alone to replenish accessory glands, as above. 25 males per group per replicate line were successfully dissected for each generation and all subsequent generations were propagated in the same way. No selection was imposed in generation 20, 21, 23, 26-29 or 31-37. In these generations matings groups were set up as described but families for the next generation were chosen at random.

#### *Accessory gland measurements:*

To establish the robustness of measuring the accessory glands, 19 accessory glands were mounted and measured 3 times each. The glands were then lifted from the PBS and returned and measured a further 3 times. The average measure of the first and second 3 measures was then compared. There was a less than 4% difference between in the size of the accessory glands showing this to be a reliable measure

#### *4.3.2 Stocks*

The Dahomey wild-type stock used in these experiments is described in section 2.3.1. Larvae were raised using a standard density culturing method (Clancy and Kennington 2001). Hence, any variation in the accessory gland size of sparkling males was minimised and, in addition, males were randomly assigned across experimental treatments so there was not predicted to be any systematic bias between treatments.

Competitor males were from a *sparkling poliart* stock that had been backcrossed into a wild-type background (section 2.3.3) and that had been kept in bottle culture.

*Sparkling* flies are homozygous for a recessive mutation that results in a phenotype of small, smooth, glassy eyes, and which is hence easily distinguishable from the wild-type, when viewed under a dissecting microscope. *Sparkling* flies were raised at standard density (Clancy and Kennington 2001) and virgin males and females anaesthetized on ice and aged for 2 days in single sex vials in groups of ~10. One day prior to the start of the experiment females were housed in pairs (for the competition experiment) or individually (for the sperm displacement ability experiment).

#### *4.3.3 Direct and correlated responses to selection on male accessory gland size*

The direct response of accessory gland size to selection was measured in every generation in which selection was applied. 25 males were dissected and measured, as in section 4.3.1, for each replicate of the large and small lines.

To test for correlated responses, I measured body size and testis size on males that were the offspring of females from generations 16 and 38. Females were allowed to lay on grape-juice medium in population cages that had been coded blind as to identity. The resulting larvae were reared at standard density (Clancy and Kennington 2001) in SY bottles. Males were aged 10 days and then frozen at  $-80^{\circ}\text{C}$ . Males were later transferred from the freezer on ice and accessory glands and testes dissected and measured. To record body size, I used a measure of wing area (Gilchrist and Partridge 1999). The wings of males from all selection treatments were mounted on glass slides and were again measured using the NIH Object Image program. Analysis was performed on the average measure of both wings, accessory glands or testes of each male or the single measure when only one was available.

#### *4.3.4 The effects of selection for accessory gland size on male post-copulatory success*

##### *(i) The effect of selection for accessory gland size on pre and post-mating competitive male reproductive success:*

To investigate the effect of accessory gland size on male competitive mating ability, both pre- and post-mating, males from the selection lines were placed in competition

with rival *sparkling* males for fertilisations. Males from the selection lines were the offspring of generation 16 (as described for correlated responses) and housed in groups of ~10 in SY vials one day after emerging as adults. Experimental SY vials were then set up, containing two each of selection line males together with two *sparkling* males and two *sparkling* females. These groups of flies were maintained in this way for 10 days, and were transferred onto fresh food on days 1, 4 and 8. The identity of mating males was recorded on days 2, 4, 7 and 9 by scoring the eye marker of mating males using a dissecting microscope. Mating observations were made ~every 20 mins over a 3 hour period. Paternity was scored by recording the eye colour of the progeny; those fathered by accessory gland selection males had wild-type eyes, and progeny fathered by *sparkling* males had the *sparkling* eye colour phenotype. The number of progeny produced by each male during the 10 days in which they were in competition was counted. The number of matings obtained and progeny sired in competition provide measures of pre- and post-mating competitive ability.

*(ii) The effects of selection on post-mating sperm displacement ability:*

Controlled, two-mating experiments were performed to examine whether accessory gland size affected the ability of males to displace the sperm of rival males. Males were the offspring of generation 16. Single *sparkling* females were placed with single *sparkling* males, observed to mate once and the males then removed (Day 1). The following day (Day 2), single selection line males, aged 2 days, were introduced to those females and observed until mating occurred. Females were transferred to new food vials the following day (Day 3) and on days 4, 6, 8, 10, 13, 15 and 17. The number of progeny produced from eggs laid between day 3 and 17 was counted and scored by eye colour. The proportion of wild type to *sparkling* progeny therefore gives a measure of each male's ability to displace already stored sperm.

## 4.4 Results

### 4.4.1 Direct and correlated responses to selection on male accessory gland size

#### (i) Accessory gland size

In both replicates, selection produced a striking divergence in the size of the male accessory glands (Figure 4.1). Analysis of each generation revealed significant differences (at  $P < 0.05$ ) between large and small lines at generations 2-8, 10-13, 16-19, 22-25, 30-31 in replicate 1 and at generations 2-7, 10-13, 16-19, 22-25, 30-31 in replicate 2 (Wilcoxon tests).

As expected, the additional measures of accessory gland size at generations 16 and 38 also revealed significant differences, with males selected for large accessory glands having significantly larger accessory glands than males selected for small accessory glands and unselected males (Table 4.1, Figure 4.2).

#### (ii) Testis size

The response in testis size at generation 16 and 38 is shown in Figure 4.3. Both absolute and relative testis size in generation 16 and 38 had significant heterogeneity of variance between regimes. In generation 16, because of the high degree of heterogeneity of variances, the means for each replicate were averaged. This revealed no significant differences in testis size between regimes ( $F = 1.83$ ,  $df = 2$ ,  $P = 0.3$ ). Nested ANOVA also revealed that there were significant differences within but not between regimes in absolute (regime (fixed effect),  $F_{2,3} = 1.26$ ,  $P = 0.4004$ , replicate within regime (random effect),  $F_{3,93} = 24.41$ ,  $P < 0.001$ ) and relative testis size (regime (fixed effect),  $F_{2,3} = 0.79$ ,  $P = 0.53$ , replicate within regime (random effect),  $F_{3,90} = 24.81$ ,  $P < 0.001$ ).

Absolute and relative testis measurements in generation 38 were Box-Cox transformed (absolute,  $((\text{testis size}^2 - 1)/0.5298)$ ; relative,  $((\text{relative testis size}^{1.8} - 1)/0.5676)$ ) to homogenize variance between lines for both absolute (Bartlett test,  $F = 1.74$ ,  $df = 5$ ,  $P = 0.1225$ ) and relative (Bartlett test,  $F = 2.12$ ,  $df = 5$ ,  $P = 0.0598$ ) measures. Such transformation resulted in normal residuals for the absolute measurements (Shapiro-Wilk test,  $W = 0.98$ ,  $P = 0.1607$ ), although relative

measurements remained non-normal (Shapiro-Wilk test,  $W=0.96$ ,  $P=0.0072$ ).

However, the ANOVA procedure is sufficiently robust to such deviations and I proceeded to conduct nested ANOVAs on actual and derived testis sizes. Selection did not result in significant differences between regimes in either absolute (regime (fixed effect),  $F_{2,3}=1.36$ ,  $P=0.3806$ , replicate within regime (random effect),  $F_{3,93}=20.91$ ,  $P<0.0001$ ) or relative (regime (fixed effect),  $F_{2,3}=0.77$ ,  $P=0.5375$ , replicate within regime (random effect),  $F_{3,90}=22.03$ ,  $P<0.0001$ ) testis size.

### *(iii) Body size*

There was no significant effect of accessory gland selection on body size in either generation 16 or 38, although there were significant differences between replicates (Table 4.2, Figure 4.4).

## *4.4.2 The effects of selection for accessory gland size on male post-copulatory success*

### *(i) The effect of selection for accessory gland size on the pre and post-mating competitive male reproductive success:*

There were no significant differences in the number of matings obtained by competitor males versus any of the selection line males (Likelihood Ratio  $\chi^2=4.2$ ,  $df=5$ ,  $P=0.5194$ , Table 4.3). However there were significant differences in the number of progeny sired by accessory gland selection line males (Table 4.4a). Replicate did not significantly vary within regime (Table 4.4b). Males with large accessory glands sired significantly more offspring than unselected males and males with small accessory glands (Table 4.4a, b). Unselected males also sired significantly more progeny than males with small accessory glands (Table 4.4a).

### *(ii) The effects of selection on post-mating sperm displacement ability:*

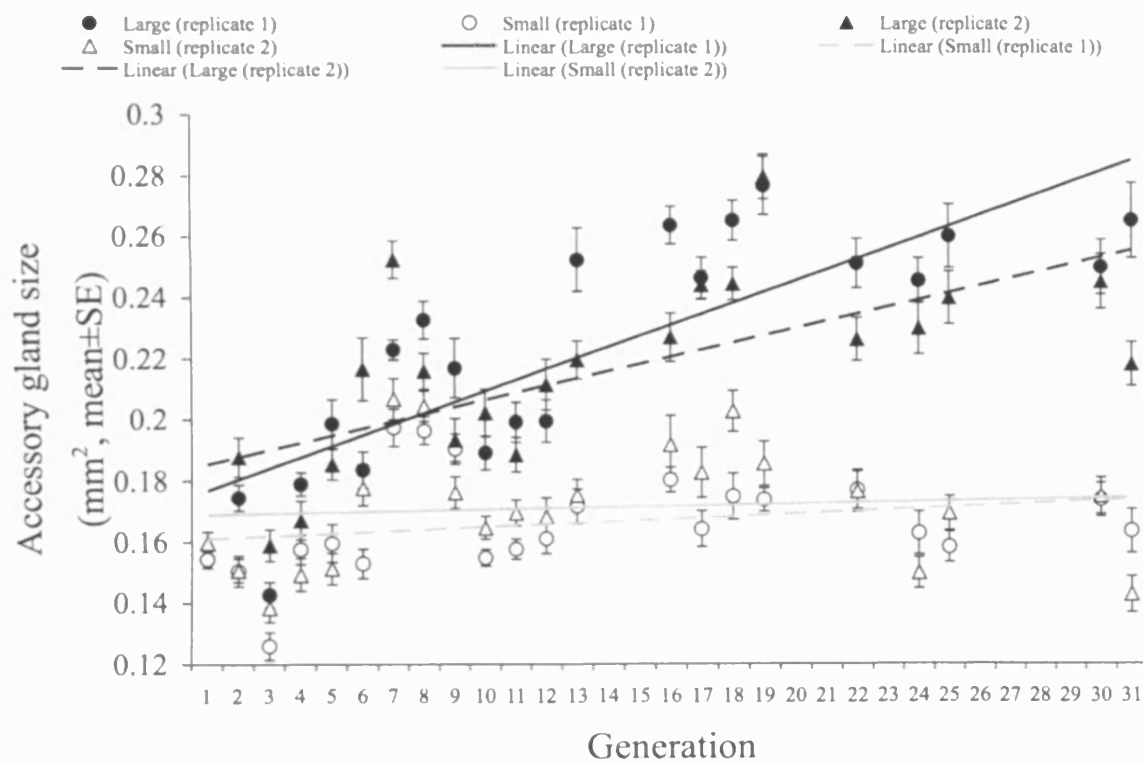
To analyse the sperm displacement ability of males from large, unselected and small accessory gland lines, a General Linear Model was run with a quasipoisson distribution, with regime and replicate nested within regime as factors (Table 4.5). The model shows that males with large accessory glands had significantly higher sperm displacement ability than unselected or small gland males (Table 4.5). Within

the large but not un selected or small treatments, SDA varied significantly between the two replicates.. There was no difference between the progeny sired by unselected or small gland males (Table 4.5).

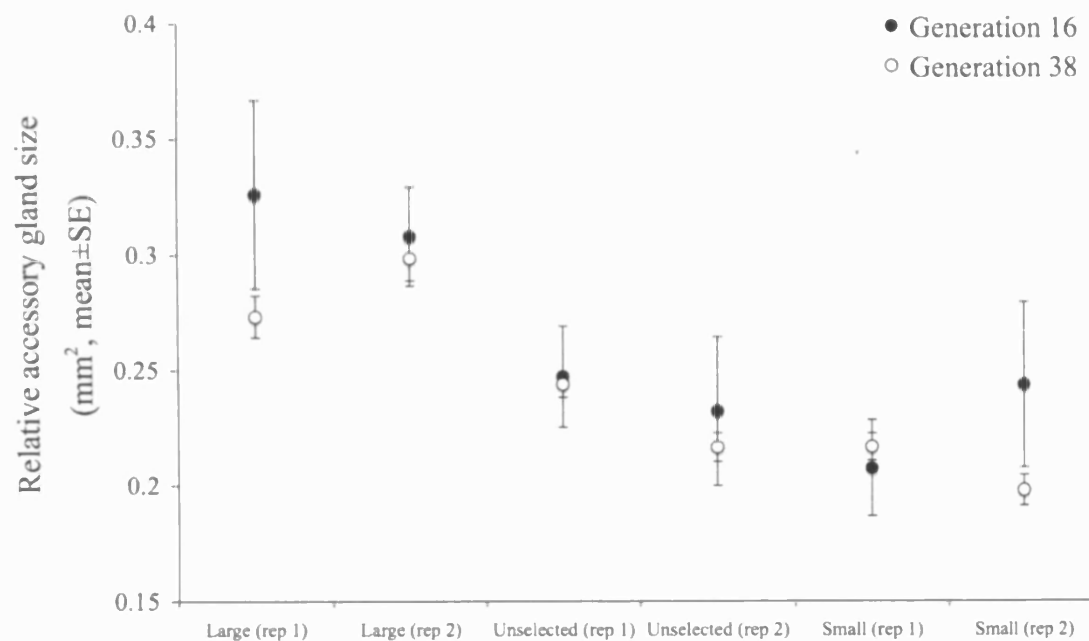
In order to explore further the size of male reproductive morphology and sperm displacement ability, I ran an ANOVA model (multiple regression) containing accessory gland, testis and body size of all males. The model provided a significant explanation of variance in sperm displacement ability (model,  $F_{3,70}=3.37$ ,  $P=0.0232$ ; accessory gland size,  $F=2.49$ ,  $df=1$ ,  $P=0.1193$ , testis size,  $F=3.73$ ,  $df=1$ ,  $P=0.0576$ , body size,  $F=0.17$ ,  $df=1$ ,  $P=0.6854$ ). The removal of body size from the model resulted in both accessory gland and testis size explaining a significant proportion of the variance in sperm displacement ability (model,  $F_{2,85}=7.16$ ,  $P=0.0013$ ; accessory gland size,  $F=5.17$ ,  $df=1$ ,  $P=0.0254$ , testis size,  $F=4.17$ ,  $df=1$ ,  $P=0.0443$ ) suggesting that the size of both characters strongly influences the ability of males to displace the sperm of rival males under this experimental protocol.



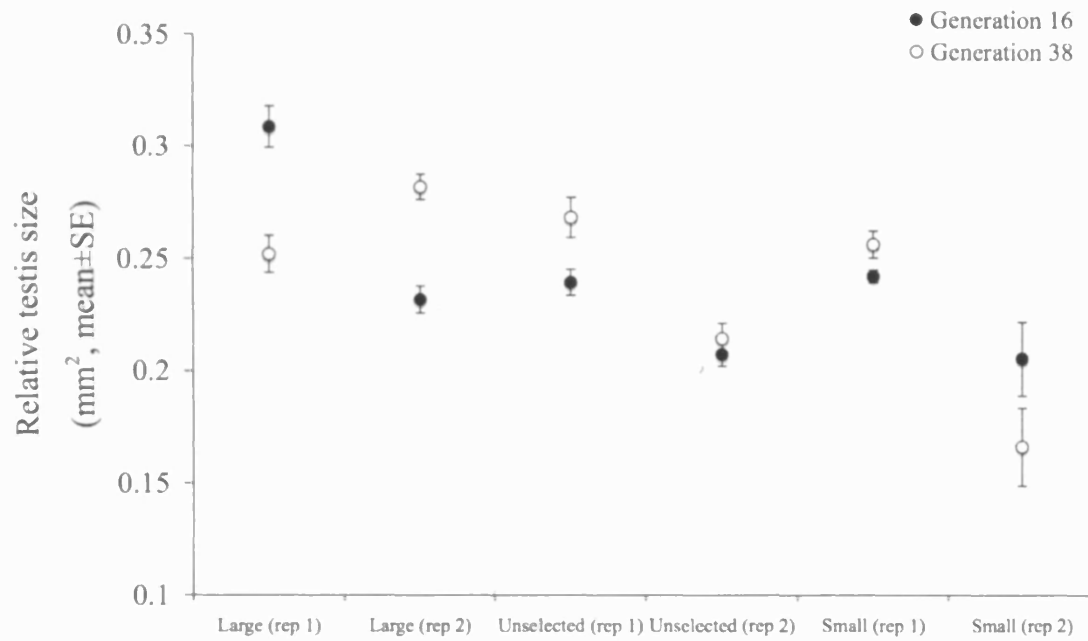
**Figure 4.1** Direct response to bi-directional selection on male accessory gland size plotted as mean accessory gland size ( $\text{mm}^2$ ) against generation of selection. Lines on the graph represent the line of best fit for each selection line. N is approximately 25 for each line at each generation.



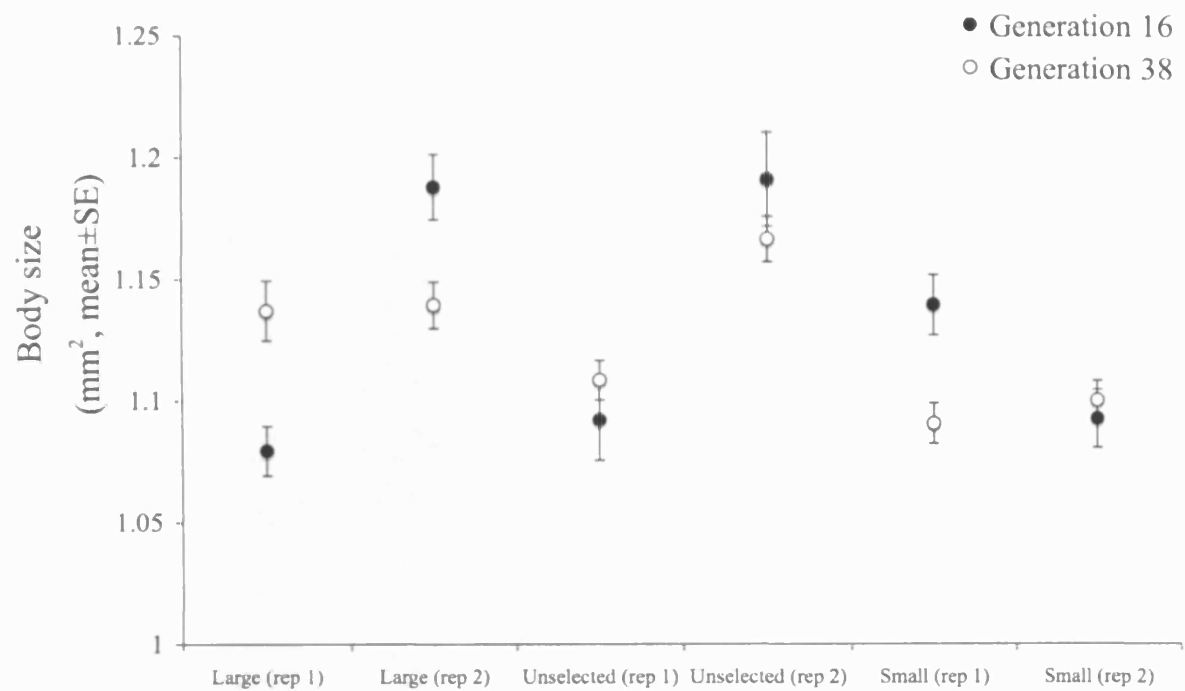
**Figure 4.2** Relative accessory gland size (mean  $\pm$  SE) of large, small and unselected accessory gland selection lines at generation 16 and 38, plotted as relative accessory gland size (accessory gland size/body size).



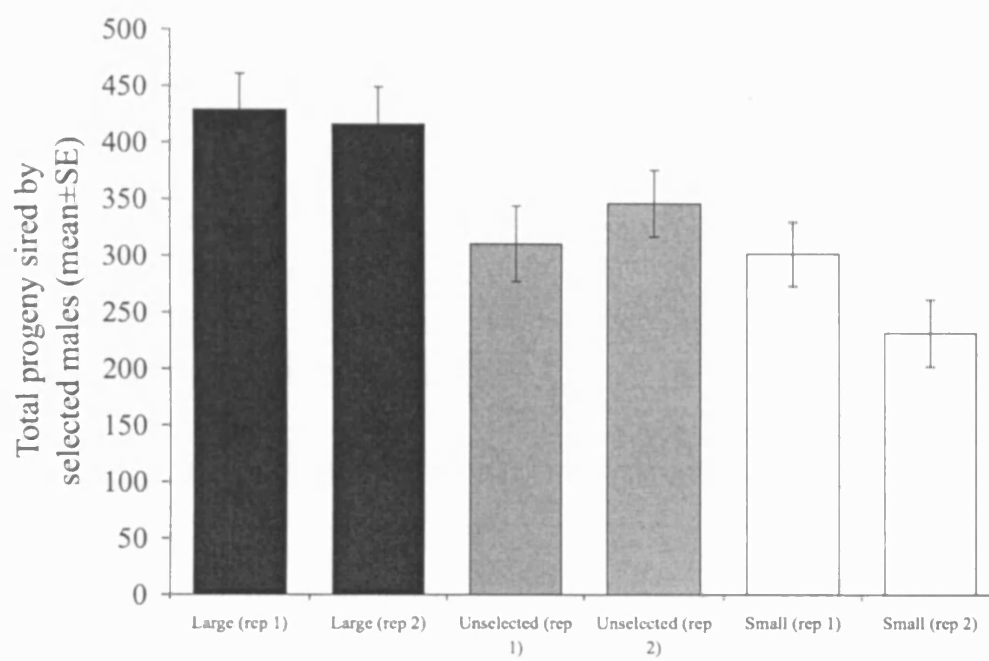
**Figure 4.3** Relative testis size (mean  $\pm$  SE) of large, small and unselected accessory gland selection lines at generation 16 and 38, plotted as relative testis size (testis size/body size).



**Figure 4.4** Body size (mean  $\pm$  SE) of large, small and unselected accessory gland selection lines at generation 16 and 38.



**Figure 4.5** Total progeny (mean  $\pm$  SE) sired by large, unselected or small accessory gland selection line males in competition with *sparkling* males. N = 23, 22, 22, 28, 30, 27 respectively.



**Table 4.1** Nested ANOVA of relative accessory gland size from the large, small and unselected lines at (a) generation 16 and (b) generation 38, with selection regime (fixed effect) and replicate line nested within selection regime (random effect).

| Source of variation                                 | <i>df</i> | <i>MS</i> | <i>F</i> | <i>P</i> |
|---|-----------|-----------|----------|----------|
| (a)   |           |           |          |          |
| Selection regime                                    | 2         | 0.069     | 15.20    | 0.0269   |
| Replicate line within selection regime <sup>1</sup> | 3         | 0.005     | 5.11     | 0.0027   |
| Error <sup>2</sup>                                  | 84        | 0.001     |          |          |
| (b)   |           |           |          |          |
| Selection regime                                    | 2         | 0.059     | 11.18    | 0.0406   |
| Replicate line within selection regime <sup>1</sup> | 3         | 0.005     | 5.45     | 0.0016   |
| Error <sup>2</sup>                                  | 105       | 0.001     |          |          |

<sup>1</sup>Error term for MS<sup>selection regime</sup>

<sup>2</sup>Error term for MS<sup>regime within selection regime</sup>

**Table 4.2** Nested ANOVA of body size of males from the large, small and unselected lines artificially selected for male accessory gland size at (a) generation 16 and (b) generation 38, with selection regime (fixed effect) and replicate line nested within selection regime (random effect).

| Source of variation                                 | <i>df</i> | <i>MS</i> | <i>F</i> | <i>P</i> |
|---|-----------|-----------|----------|----------|
| (a)   |           |           |          |          |
| Selection regime                                    | 2         | 0.005     | 0.09     | 0.92     |
| Replicate line within selection regime <sup>1</sup> | 3         | 0.060     | 18.40    | <0.0001  |
| Error <sup>2</sup>                                  | 86        | 0.003     |          |          |
| (b)   |           |           |          |          |
| Selection regime                                    | 2         | 0.023     | 2.06     | 0.2729   |
| Replicate line within selection regime <sup>1</sup> | 3         | 0.113     | 6.86     | 0.0003   |
| Error <sup>2</sup>                                  | 109       | 0.002     |          |          |

<sup>1</sup>Error term for  $MS^{\text{selection regime}}$

<sup>2</sup>Error term for  $MS^{\text{regime within selection regime}}$

**Table 4.3** Mating frequencies of *sparkling* females mating with selection line and *sparkling* males. Numbers in brackets represent percentage of total mating opportunities.

| Regime            | Replicate | Mating opportunities taken            |                                   | Mating opportunities not taken |
|-------------------|-----------|---------------------------------------|-----------------------------------|--------------------------------|
|                   |           | <i>Selection line</i><br><i>males</i> | <i>Competitor</i><br><i>males</i> |                                |
| <i>Large</i>      | 1         | 10 (0.43)                             | 1 (0.04)                          | 2309                           |
| <i>Unselected</i> | 1         | 12 (0.59)                             | 2 (0.09)                          | 2026                           |
| <i>Small</i>      | 1         | 12 (0.52)                             | 4 (0.17)                          | 2284                           |
| <i>Large</i>      | 2         | 6 (0.25)                              | 1 (0.04)                          | 2413                           |
| <i>Unselected</i> | 2         | 13 (0.52)                             | 0 (0)                             | 2487                           |
| <i>Small</i>      | 2         | 10 (0.38)                             | 0 (0)                             | 2590                           |



**Table 4.4a** General Linear Model of progeny sired by males from large, unselected and small males in competition with *sparkling* males with regime set as a factor.

| Factor                 | Estimate | SE    | t      | P      |
|------------------------|----------|-------|--------|--------|
| (Intercept)            | 330.80   | 22.03 | 15.014 | <0.000 |
| Factor(regime) - large | 91.89    | 32.01 | 2.870  | 0.005  |
| Factor(regime) - small | -61.68   | 30.19 | -2.043 | 0.043  |

**Table 4.4b** Nested ANOVA of total progeny sired by males from the large, small and unselected lines, with selection regime (fixed effect) and replicate line nested within selection regime (random effect).

| Factor    | DF  | Sum SQ  | Mean SQ | F     | P      |
|-----------|-----|---------|---------|-------|--------|
| Regime    | 2   | 594269  | 297134  | 12.29 | <0.000 |
| Replicate | 3   | 86726   | 28909   | 1.20  | 0.3136 |
| Residuals | 146 | 3529616 | 24175   |       |        |

**Table 4.5** Effect sizes of General Linear Model with quasipoisson distribution on the sperm displacement ability of males from large, unselected and small accessory gland lines with regime and replicate nested with regime.

| Factor                               | Estimate | SE    | t      | P      |
|--------------------------------------|----------|-------|--------|--------|
| Intercept                            | 3.445    | 0.544 | 6.327  | <0.000 |
| Regime - Large                       | 1.863    | 0.718 | 2.595  | 0.011  |
| Regime - Small                       | -0.793   | 0.912 | -0.87  | 0.387  |
| Replicate within Regime (unselected) | 0.218    | 0.346 | 0.631  | 0.529  |
| Replicate within Regime (large)      | -1.021   | 0.353 | -2.896 | 0.004  |
| Replicate within Regime (small)      | 0.226    | 0.451 | 0.501  | 0.617  |

## 4.5 Discussion

Artificial selection on male accessory gland size produced a strong and significant response. Males in large lines had significantly larger accessory glands than males in small and unselected lines. An asymmetric response to selection is suggested by the finding that males from the large lines had significantly larger accessory glands than both males in small and unselected lines. Furthermore, accessory gland size did not differ significantly between unselected and small line males. Selection for increased and decreased accessory gland size did not produce a correlated response in testis size. The response in accessory gland size was not associated with any correlated response in body size, although there remained a significant positive relationship between body size and accessory gland size when comparing across all lines together ( $F_{1,88}=4.9$ ,  $P=0.0294$ ), which is consistent with previous reports (Bangham et al. 2002). When in competition for matings, males with larger accessory glands sired significantly more offspring than did males from the small or unselected lines, in the absence of any significant differences in mating frequency. This suggests that the per-mating competitive reproductive success of males with large accessory glands was significantly higher than that for males from the other lines. In the absence of competition for matings, there were no significant differences between regime differences in sperm displacement ability, though there was an overall association between both accessory gland and testis size and sperm displacement ability. The results therefore suggest that accessory gland and testis size have a significant influence on sperm displacement ability as measured in a non-competitive situation. Taken together, the results show that males with large accessory glands have significantly higher reproductive success.

Testis size did not consistently correlate with the response in accessory gland size, suggesting a weak or no genetic correlation between the two traits. Hence, differing selection pressures may shape the morphology of testis and accessory gland size. The response also indicates that increased investment in accessory gland tissue is not at the expense of investment in testis tissue. Interestingly, in *D. melanogaster*, artificial selection experiments that increased mating rate while lowering sperm competition levels, resulted in males with significantly larger testes, while at the same time slightly smaller accessory glands (Reuter et al. in press). Similarly, selection on mating

frequency in *C. dalmanni* resulted in an increase in accessory gland but not testis length (Rogers et al., 2005).

In contrast to previous studies in both *D. melanogaster* (Bangham et al. 2002) and the stalk-eyed fly, *C. dalmanni* (Rogers et al., 2005), males with larger accessory glands did not mate at a higher rate than males from other lines. However, the nature of the competition assay may not have afforded sufficient mating opportunities. Previous assays of the association between mating frequency and accessory gland size allowed potentially higher male mating rates by exposing males to many females (Bangham et al. 2002; Rogers et al. 2005). In the competition assay described in this chapter, flies were housed together with 2 other males and 2 females, which may have provided few opportunities to mate, relative to how many times males could potentially have mated. Despite the similarities in mating frequencies, males from lines selected for large accessory glands sired significantly more offspring than males from small and unselected lines in this competitive environment. One explanation for this is that larger accessory glands may contain greater quantities of Acps and, if these are transferred in greater volume during mating and act in a dose dependent way, males with larger accessory glands can realize greater post-copulatory success. Acp36DE for example acts to increase the efficiency of sperm storage (Neubaum and Wolfner 1999). Males transferring more of Acp36DE could therefore have a greater proportion of their sperm stored, which could lead to increased success in sperm competition. Larger quantities of Acps might be transferred because more of them are available to transfer or because they are transferred at a faster rate.

There was no correlated response in the sperm displacement ability of males selected for large or small accessory glands in a non-competitive mating situation when both first and second matings with single males were observed. Males with large accessory glands could achieve greater sperm displacement ability if the Acps affecting this trait acted in a dose dependent way, but this appeared not to be the case under this experimental paradigm. However, it was apparent from the subsequent analysis that, overall, both testis and accessory gland size were significant determinants of sperm displacement ability. The lack of differences in sperm displacement between selection lines may therefore infer that the differences in accessory gland size between those lines were not sufficient to cause detectable differences in sperm displacement ability

in a single mating. Moreover, however, sperm precedence and sperm displacement were high and also highly variable, a common trait of these assays (e.g. *D. melanogaster*, Garcia-Gonzalez 2004; *Teleopsis dalmanni*, Corley et al. 2006) perhaps obscuring the detection of differences in sperm displacement ability between lines.

Males with larger accessory glands have greater pre- and post-copulatory success. For example, the drop in fertility suffered by *D. melanogaster* males over successive matings is associated with accessory gland depletion and not with sperm depletion, suggesting that accessory gland products are a potential limiting factor in determining fertility (Hihara 1981; Chapter 3). Accessory gland size has also been found to correlate with mating frequency. Bangham et al. (2002) found a significant, positive association between accessory gland size and mating frequency in *D. melanogaster*, and Rogers et al. (2005) found accessory gland length to be a correlate of selection on male mating frequency in the stalk-eyed fly, *C. dalmanni*. In addition, as demonstrated here and elsewhere (Bangham et al. 2002), males with larger accessory glands have higher post-copulatory success. There are presumably costs that constrain the evolution of accessory gland size. For example, accessory gland size may be constrained by development time or the time taken to reach sexual maturity (e.g. as in stalk eyed flies, Baker et al. 2002). The data seem to suggest a constraint to the evolution of small accessory glands, because, after selection, there was no difference between the size of males from the small and unselected lines, when tested in generation 16 and 38. This may indicate that a minimum size and quantity of accessory gland products are necessary to successfully fertilize a female.

In conclusion, replicated artificial selection on accessory gland size produced a strong response in males selected for large accessory glands, but did not result in consistent correlated responses in testis or body size. The response to selection indicates that there is significant standing and additive genetic variance for increased accessory gland size and suggests that costs exist to accessory gland size. I demonstrate that accessory gland size is an important determinant of post-copulatory success, presumably because large accessory gland males transfer a greater quantity of Acps. In a new collaboration in which I am involved, an ELISA technique developed by Laura Sirot from the Wolfner laboratory (Sirot & Wolfner, unpublished) is currently being used to test whether males from the large and small accessory gland selected

lines differ significantly in the amount, or rate of transfer, of Acps to females during single matings in competitive and non-competitive situations. Future work could also investigate correlated responses to selection in females, such as in reproductive morphology fitness and the ability of such females to store sperm.

## **Chapter 5 The effect of accessory gland size on the survival and fitness of female *Drosophila melanogaster***

### **5.1 Abstract**

Male *Drosophila* transfer more than 80 accessory gland proteins during mating and these have striking effects on male and female fitness. Acp's act to stimulate and regulate reproductive processes in females following mating and at least one Acp, SP, mediates the cost of mating in females. While the functions of several individual Acp's have been determined, the effects of the quantity of Acp's on the degree of phenotype expression are less clear. If males with larger accessory glands transfer more Acp's, and if those Acp's act in a dose-dependent manner, then matings with such males may cause greater stimulation of Acp-controlled reproductive traits such as egg production, and cause a greater cost of mating in females. In chapter 5, I use males selected for large and small accessory gland size to examine the effects of accessory gland size on female survival, egg laying and egg to adult viability. I tested whether females continuously exposed to males selected for large accessory glands suffered greater survival costs and laid more eggs than females mated to males with small accessory glands. The results were somewhat inconsistent: although females housed with large accessory gland males had significantly shorter lifespans than those housed with small males in both replicates, the survival of females housed with unselected males was variable, being significantly shorter lived than either of the other groups in replicate 1, but not significantly different from females housed with small males in replicate 2. There was no consistent pattern of differences in egg laying, egg to adult viability and mating frequencies between females housed with large, small or unselected accessory glands, but females housed with males with large glands were courted more frequently than females housed with males with small or unselected accessory glands.

## 5.1 Introduction

During mating, males transfer seminal proteins and peptides, along with sperm, to their mates. Seminal fluid comprises molecules from the accessory glands, ejaculatory duct and ejaculatory bulb and these have striking effects of male and females fitness. The majority of seminal fluid proteins are the accessory gland proteins (Acps) synthesised by the paired accessory glands. Acps act to stimulate and regulate reproductive processes in females following mating, including ovulation (Heifetz et al. 2000; Heifetz et al. 2001; Herndon and Wolfner 1995), oogenesis (Aigaki et al. 1991; Chen et al. 1988; Soller et al. 1997; Soller et al. 1999), sperm storage (Neubaum and Wolfner 1999), receptivity to mating (Aigaki et al. 1991; Chen et al. 1988; Manning 1967), sperm competition (Chapman et al. 2000) and mating plug formation (Lung and Wolfner 2001). Among the most dramatic effects of Acps are those involved in mediating the cost of mating suffered by females (Chapman et al. 1995). In addition to the evidence that SP is responsible for this cost, another 3 other Acps (CG10433, CG8137, SP and Acp62F) have been found to be toxic upon ectopic expression (but see Chapter 6). Here, males are apparently maximising their reproductive fitness despite the reduction in longer term fitness that this may cause in their mates.

Acps also influence egg production, ovulation and oviposition. Mates of males lacking Acp26Aa (ovulin) lay fewer eggs for one day following mating (Herndon and Wolfner 1995). Acp26Aa stimulates the immediate release of mature oocytes accumulated in the ovary (Heifetz et al. 2001). SP also causes an increase in egg laying after mating (Aigaki et al. 1991; Chen et al. 1988), acting at an earlier stage, namely oogenesis (Aigaki et al. 1991; Chen et al. 1988; Soller et al. 1997; Soller et al. 1999) and SP also induces refractoriness to mating (Chapman et al. 2003a; Chen et al. 1988; Liu and Kubli 2003). Another Acp, Acp36DE, acts to increase the efficiency of sperm storage (Neubaum and Wolfner 1999). Males lacking Acp36DE, despite making and transferring normal numbers of sperm, store only 15% of those sperm, perhaps because fewer sperm enter or remain in storage (Neubaum and Wolfner 1999).



Phenotypic testing of individual Acps as described above has provided great insight into their effects on female physiology, behaviour and male and female fitness. However, it remains unclear whether any or all Acps act in a dose dependent way, and hence whether, assuming accessory gland size is proportional to the amount of Acps transferred, accessory gland size itself plays an important role in determining fitness. There is evidence to suggest that accessory gland size plays a role in both pre and post-copulatory success. For example, *D. melanogaster* (Bangham et al. 2002) and *C. dalmanni* (Rogers et al. 2005) males with large accessory glands have a higher mating frequency capacity than males with smaller accessory glands. Moreover, males selected to have larger accessory glands have higher fitness when in competition with other males (Chapter 4). If greater quantities of seminal fluid are passed during mating, it follows that more SP will be transferred and hence more will be available to bind with target sites. Similarly, greater quantities of Acp36DE may result in greater sperm utilisation and higher female fecundity. If SP and Acp26Aa act in a dose dependent manner, it is predicted that females mated to males with larger accessory glands will have higher early egg laying, higher fertility and suffer a greater cost of mating, unless that is, males with small accessory glands transfer sufficient Acps to maximise each of these measures.

Here, I test whether females mated to males with large accessory glands suffer greater costs in terms of survival relative to females mating with males with smaller accessory glands, and whether egg laying, and the viability of those eggs, is affected by male accessory gland size. In chapter 4, I described the results of artificial selection on accessory gland size that resulted in males with large or small accessory glands. In chapter 5, I use males from those selection lines to examine the effects of exposure to males with large and small accessory glands on female survival, egg laying and egg to adult viability. If the Acps involved in mediating costs of mating, initiation of ovulation, egg laying, sperm storage or sperm use act in dose-dependent ways, females mated to males selected to have large accessory glands may suffer greater survival costs and lay more eggs than females mated to males selected to have small accessory glands.

## 5.2 Materials and Methods

### 5.2.1 Females

The Dahomey wild-type stock used in these experiments is described in section 2.3.1. Experimental wild-type females were obtained by collecting eggs from population cages and raising the larvae at standard density (Clancy and Kennington 2001). Females were collected as virgins and aged 2 days prior to exposure to males.

### 5.2.2 Males

Males were the progeny of females from generation 16 of the selection regime described in Chapter 4 (section 4.3.1, see direct responses in Figure 4.2, 4.3 and 4.4. Males for the experiments described in this Chapter were housed in groups of ~12 and aged for 2 days prior to the start of the experiment.

### 5.2.3 *The effect of accessory gland size on female survival*

120 virgin wild-type Dahomey females were continuously housed in groups of five together with two replicates each of large, small or unselected accessory gland size males. Flies were housed in SY vials seeded with live yeast balls. Any males that died were replaced and the sex-ratio was maintained at 1:1. All males were replaced on day 22 with new 2-3 day old males, to encourage high rates of mating throughout the experiment. Female deaths were scored every 1-2 days and females were transferred to fresh vials every 2-3 days.

### 5.2.4 *The effect of accessory gland size on female fecundity and egg to adult viability*

Fecundity and fertility tests on the above females were carried out on days 3, 5, 8, 11, 16, 19, 24 and 26 (except that fertility was not recorded on day 5 or for females housed with unselected males in replicate 1 on day 8). Flies were transferred to fresh SY charcoal vials for between 4 and 24 hours (for the same duration for any given day) before being transferred back to fresh SY vials. Eggs were counted within 24 hours of the latter transfer and then allowed to develop at 25°C. Fecundity and fertility measures were corrected for the number of laying females in a vial and the laying

time. Measures of egg number and egg to adult viability per female per 24 hours were therefore calculated.

#### *5.2.5 Mating and courtship frequency*

Courtship and mating frequencies for the females described above were recorded on days 2, 4, 7, 10, 12, 16, 20, 23 and 26. Vials were scanned for matings and courtship behaviour 10 times, with observations not closer than 20 minutes (section 2.4.1), starting at lights on.

## 5.3 Results

### 5.3.1 *The effect of accessory gland size on female survival*

The survival plots for females mated to large, small or unselected males (Figure 5.1) show a significant effect of male line on female survival (Log Rank test,  $\chi^2=36.65$ ,  $df=5$ ,  $P<0.0001$ ). Testing within replicates revealed that females mated to males selected for large accessory glands had significantly shorter survival than females mated to males selected for small accessory glands in each replicate (correction for multiple comparisons,  $P=0.0167$ ; Replicate 1,  $\chi^2=5.61$ ,  $df=1$ ,  $P=0.0178$ , Replicate 2,  $\chi^2=11.15$ ,  $df=1$ ,  $P=0.0008$ ).

However, while females selected for large accessory glands did have lower survival than females mated to unselected males in replicate 2 ( $P=0.0167$ ;  $\chi^2=4.08$ ,  $df=1$ ,  $P=0.0434$ ), the pattern was very different in replicate 1, with females mated to unselected males having significantly lower survival than those mated to large ( $\chi^2=10.15$ ,  $df=1$ ,  $P=0.0014$ ) or small accessory gland males ( $\chi^2=18.53$ ,  $df=1$ ,  $P<0.0001$ ). In replicate 2, there was no significant difference between the survival of females mated to small or unselected males ( $\chi^2=2.4$ ,  $df=1$ ,  $P=0.1212$ ).

### 5.3.2 *The effect of accessory gland size on female fecundity and egg to adult viability*

Age-specific egg laying and egg to adult viability significantly declined as females aged (Figure 5.2a, b). GLM analysis showed that the number of eggs laid by females housed with males from large, unselected and small accessory gland lines did not differ, nor was there any significant interaction between line and day (Table 5.1a), suggesting that accessory gland size had no detectable effect on egg output during the experiment.

GLM analysis also showed that egg to adult viability declined over the course of the experiment (Table 5.1b). The egg to adult viability of females housed with males with large accessory glands in replicate 1 and males with small accessory glands in replicate 2 differed significantly from the other groups. In addition, all the lines showed significant variation in the decline in egg to adult viability. However, this

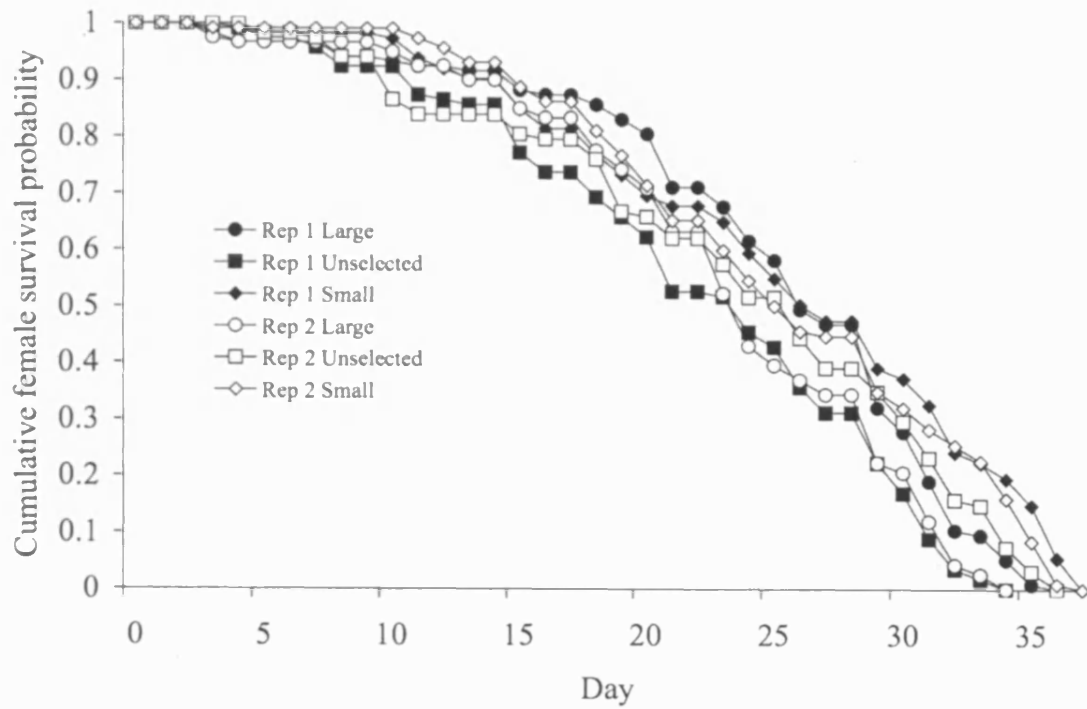
variation did not match with the pattern expected if accessory gland size affected egg to adult viability (Table 5.1b).

The results show that, overall, there was no consistent effect of accessory gland size on egg laying or egg to adult viability.

### *5.3.3 Mating and courtship frequencies*

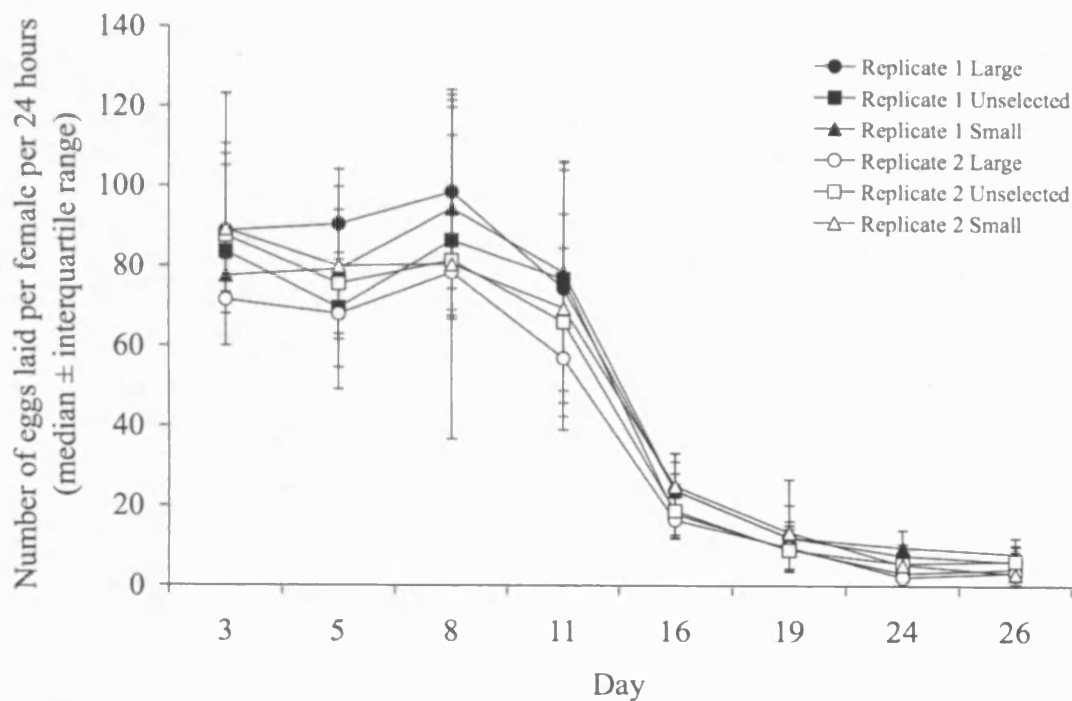
Mating and courtship frequencies were analysed in contingency tables using Chi-square tests (Zar, 1999)(Table 5.1). Mating frequency was analysed by comparing the total number of matings taken in each group to the total number of mating opportunities not taken. Mating opportunities were defined as the number of observation days multiplied by the number of females present on each sampling day. Courtship opportunities were defined as the total number of observations of courting events summed over all sampling days multiplied by the number of females present on each sampling day. The mating and courtship frequency between lines (females mated to males selected for large or small accessory glands or unselected males) did not significantly differ ( $P>0.05$ ). However, in replicate 2, females mated to males selected for small accessory glands had higher mating and lower courtship frequencies than females mated to males selected for large accessory glands or unselected males (Table 5.1).

**Figure 5.1** The effect of exposure to large, small and unselected accessory gland line males on female lifespan. Female cumulative survival probability against time (days).

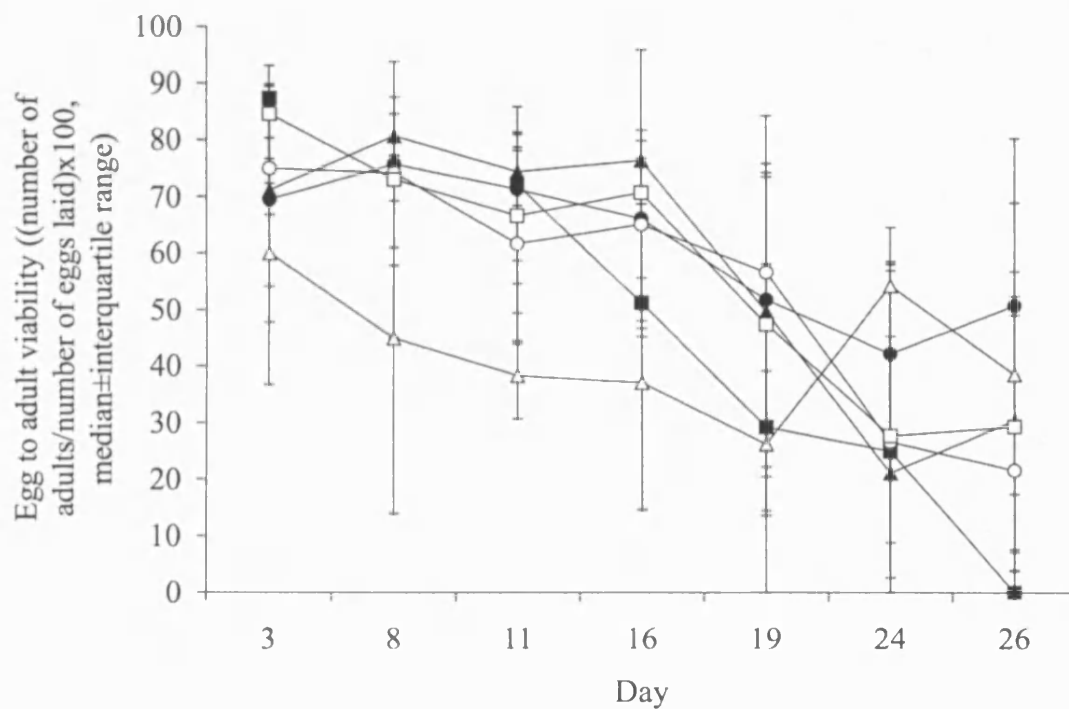


**Figure 5.2** Age-specific egg production and viability of eggs of wild-type females continuously exposed to large, small or unselected accessory gland males.

(a)



(b)



**Table 5.1** General Linear Model analysis on (a) the number of eggs laid by females mated to males from large, unselected or small accessory gland lines and (b) the egg to adult viability of those eggs, with replicate, Day and the interaction between replicate and Day as factors.

**(a)**

| Source of variation   | Estimate | SE     | t       | P      |
|-----------------------|----------|--------|---------|--------|
| Intercept             | 107.121  | 4.05   | 26.45   | <0.000 |
| Unselected (rep 2)    | -7.107   | 5.7243 | -1.242  | 0.215  |
| Large (rep 1)         | 9.167    | 5.658  | 1.62    | 0.106  |
| Large (rep 2)         | -10.286  | 5.712  | -1.801  | 0.072  |
| Small (rep 1)         | -3.968   | 5.713  | -0.695  | 0.488  |
| Small (rep 2)         | 4.077    | 5.682  | 0.717   | 0.473  |
| Day                   | -4.413   | 0.29   | -15.201 | <0.000 |
| Unselected rep2 x Day | 0.2575   | 0.4105 | 0.627   | 0.531  |
| Large rep1 x Day      | -0.263   | 0.3938 | -0.667  | 0.505  |
| Large rep2 x Day      | 0.5203   | 0.4066 | 1.280   | 0.201  |
| Small rep1 x Day      | 0.5179   | 0.4021 | 1.288   | 0.198  |
| Small rep2 x Day      | -0.1399  | 0.4009 | -0.349  | 0.727  |

**(b)**

| Source of variation   | Estimate | SE     | t      | P      |
|-----------------------|----------|--------|--------|--------|
| Intercept             | 0.955    | 0.0527 | 18.136 | <0.000 |
| Unselected (rep 2)    | -0.080   | 0.071  | -1.117 | 0.264  |
| Large (rep 1)         | -0.185   | 0.074  | -2.504 | 0.013  |
| Large (rep 2)         | -0.128   | 0.074  | -1.738 | 0.083  |
| Small (rep 1)         | -0.137   | 0.077  | -1.766 | 0.078  |
| Small (rep 2)         | -0.442   | 0.072  | -6.173 | <0.000 |
| Day                   | -0.030   | 0.003  | -9.283 | <0.000 |
| Unselected rep2 x Day | 0.011    | 0.005  | 2.436  | 0.015  |
| Large rep1 x Day      | 0.018    | 0.004  | 4.110  | <0.000 |
| Large rep2 x Day      | 0.014    | 0.005  | 2.899  | 0.004  |
| Small rep1 x Day      | 0.014    | 0.005  | 3.011  | 0.003  |
| Small rep2 x Day      | 0.024    | 0.005  | 5.307  | <0.000 |



**Table 5.2** Mating and courtship frequencies between females continuously housed with males selected for large, small or unselected accessory gland size.

| Males to which females mated  | Observed mating/<br>courting | Observed not mating/not courting | Percentage mating of total observations (%) | Df | Pearson Chi square | P       |
|-------------------------------|------------------------------|----------------------------------|---|----|--------------------|---------|
| <b>(a) Mating</b>             |                              |                                  |   |    |                    |         |
| <i>Replicate 1 Large</i>      | 49                           | 9021                             | 0.54  | 2  | 4.9                | 0.0863  |
| <i>Replicate 1 Unselected</i> | 66                           | 8164                             | 0.80  |    |                    |         |
| <i>Replicate 1 Small</i>      | 51                           | 8429                             | 0.60  |    |                    |         |
| <b>(b) Courtship</b>          |                              |                                  |   |    |                    |         |
| <i>Replicate 1 Large</i>      | 1114                         | 7956                             | 12.28                                       | 2  | 4.97               | 0.0833  |
| <i>Replicate 1 Unselected</i> | 973                          | 7237                             | 11.85                                       |    |                    |         |
| <i>Replicate 1 Small</i>      | 950                          | 7530                             | 11.20                                       |    |                    |         |
| <b>(c) Mating</b>             |                              |                                  |   |    |                    |         |
| <i>Replicate 2 Large</i>      | 45                           | 8605                             | 0.52  | 2  | 8.15               | 0.0170  |
| <i>Replicate 2 Unselected</i> | 52                           | 8128                             | 0.64  |    |                    |         |
| <i>Replicate 2 Small</i>      | 77                           | 8773                             | 0.87  |    |                    |         |
| <b>(d) Courtship</b>          |                              |                                  |   |    |                    |         |
| <i>Replicate 2 Large</i>      | 1178                         | 7472                             | 13.62                                       | 2  | 129.45             | <0.0001 |
| <i>Replicate 2 Unselected</i> | 1069                         | 7111                             | 13.07                                       |    |                    |         |
| <i>Replicate 2 Small</i>      | 767                          | 8083                             | 8.67  |    |                    |         |

## 5.4 Discussion

There was no evidence that the magnitude of the female cost of mating was positively associated with male accessory gland size. Although females housed with males with large accessory glands suffered reduced survival relative to females housed with males with small accessory glands (and relative to unselected males in replicate 2), in replicate 1, females mated to unselected males had significantly lower survival compared to females mated to large and small accessory gland males. There was therefore unexplained variability in the survival of females exposed to the two different replicates of unselected line males. Egg laying and egg to adult viability did not consistently differ between lines and there were also no consistent differences between the mating and courtship frequencies between females and males with large, small or unselected accessory glands. This suggests that there was also no positive association between male accessory gland size and the expression of at least some female post-mating responses.

In contrast to expectations, therefore, there was no unequivocal support for decreased survival in females mated to males with large accessory glands. Males with larger accessory glands were expected to transfer greater quantities of Acps. If more SP were bound to sperm and subsequently released within the female reproductive tract, or was transferred in the seminal fluid, this could have increased survival costs of mating suffered by females, if the cost of receiving SP was dose dependent. However, it is not clear yet whether large accessory gland males do transfer more Acps, including SP. The mechanisms(s) by which SP mediates the cost of mating are not known and nor is it clear whether SP alone is responsible for the totality of the cost of mating. SP causes the release of juvenile hormone from *corpora allata* (Moshitzky et al. 1996) that in turn stimulates oocyte progression in the ovary (Soller et al. 1999). The induced release of juvenile hormone may result in immunity suppression (Rolff and Siva-Jothy 2002).

One potential explanation for the variation in the survival of females exposed to unselected males was that the mating frequency of females mating with replicate 1 unselected males was approximately 30% higher than in other lines, which may have contributed to the significantly lower survival, since females that mate at high

frequencies suffer survival costs (Fowler and Partridge 1989). Interestingly in replicate 2, despite the fact that females housed with small accessory gland males mated at a significantly higher mating frequency than the other lines, those females had greater survival than females housed with unselected males or males with large accessory glands. This could indicate that small accessory gland males transfer less accessory gland fluid, including SP, during each mating, leading to lower mating costs than expected. The potentially confounding effect of variable mating frequencies should be removed in future experiments, by observing all matings and controlling mating frequency. In such a design it would then be possible to test for increased or decreased per mating costs in females.

There were no consistent differences in egg laying or egg to adult viability of females mated to large, small or unselected accessory gland males, suggesting that the Acps influencing these traits either do not act in a dose dependent manner or that the differences in the quantities transferred were not sufficient to detect an effect. The number of eggs laid, and the fertility of those eggs, is also influenced by numbers of sperm transferred. Since no differences were observed in testis size, similar numbers of sperm were expected to be transferred during each mating. Another possibility is that multiple mating by females in all lines was sufficient to maximise egg laying and egg to adult viability. It is intriguing that early in the experiment, egg to adult viability was lower in females mated to males with small accessory glands in replicate 2 (despite their relatively high mating frequency), but this was not mirrored in replicate 1. Therefore, it is unclear whether accessory gland size influences egg laying and egg to adult viability. Along with measurement of whether large and small gland males differ in the amount of Acps that they transfer to females, single or controlled numbers of matings are required to assess whether accessory gland size does indeed influence fecundity and fertility.

Although differences in mating frequencies existed between the lines, these were inconsistent. Males with larger accessory glands courted females more frequently than did males in small and unselected lines, indicating an increased willingness and capacity of males with large accessory glands to mate. However, this might not necessarily translate into increased mating frequency if the potentially increased amount of Acps they transfer induce longer refractoriness in females. Similarly, while

males with smaller accessory glands may have a lower capacity to mate, as indicated by their lower courtship frequencies in both replicates, because of lower Acp transfer, females may have been willing to remate sooner, thus providing small gland males with more opportunities to mate. This explanation holds for replicate 2, but there was no difference in the mating rate of males with large or small accessory glands in replicate 1. To explore the mating capacity of males with large or small accessory glands further it would be necessary to provide males with sequential mating opportunities as employed elsewhere (Bangham et al 2002; Rogers et al 2005). It would also be interesting to examine female refractoriness following one, or a controlled number of matings.

In conclusion, this chapter provides no equivocal evidence that female survival, egg laying or egg to adult viability were affected by exposure to males with different accessory gland sizes. Males with larger accessory glands courted females much more frequently, but this did not translate into higher mating frequencies, which may indicate that longer refractoriness to mating was induced in these females. Experiments are currently being designed to measure the relative quantities of particular Acps transferred, including SP, using ELISA technique developed in the Wolfner laboratory (Sirot & Wolfner, unpublished). These experiments will test whether males from the large and small accessory glands differ significantly in the amount, or rate of transfer, of Acps to females during single mating which, with additional complementary fitness assays, may reveal further the importance of accessory gland size as a trait influencing male and female fitness, and the mechanisms by which Acps influence females reproductive physiology and behaviour.

## **Chapter 6 The effect of Acp62F delivery on female lifespan and reproductive success**

### **6.1 Abstract**

In *Drosophila* the cost of mating suffered by females in terms of reduced longevity and lifetime reproductive success, is mediated by the action of accessory gland proteins (Acp) that are transferred along with sperm by males at mating. Recently, a large part of this cost has been shown to be mediated by Acp70A, the sex peptide (SP). However, another Acp, Acp62F, has also been implicated in causing the cost of mating and hence may play an additional role. Previous experiments showed that ectopic expression of Acp62F and 3 other loci out of a total of 21 loci studied is toxic to adult flies. To extend this investigation and test the effect on the cost of mating of the delivery of physiological levels of Acp62F via the normal route (i.e. mating), I used Acp62F RNAi knockdown and Acp62F knock-out null males. I examined the effect of presence or absence of Acp62F on female survival, egg laying and egg to adult viability. The results showed that females mated to males that did and did not transfer Acp62F did not show a consistent pattern of differences in either survival or fecundity and egg to adult viability. Therefore the results do not support the assertion that Acp62F contributes to the cost of mating in females.

Part of this work has recently been accepted for publication in the journal *Genetics*. Mueller, J., L., J. R. Linklater, K. R. Ram, T. Chapman and M. F. Wolfner. Targeted gene deletion and phenotypic analysis of the *Drosophila melanogaster* seminal fluid protease inhibitor Acp62F. In preparation.

## 6.2 Introduction

The disparity in male and female interests over reproductive roles can lead to the evolution of traits that confer an advantage to one sex but not the other (Parker 1979; Rice 2000). Males can benefit from the expression of adaptive traits that increase their own reproductive success (e.g. through increased sperm competition success or decreased female receptivity to subsequent mating) even if such traits are costly to their partners (Parker 1979). When such adaptations spread, counter-adaptations are expected to evolve in females to reduce the costs of male-induced harm. These in turn are expected to be countered by new mutations in males that tend to increase male, but not necessarily female, fitness. Hence, a cycle of sexually antagonistic co-evolution can occur (Morrow and Arnqvist 2003; Rice 2000; Rice and Holland 1997). This sexual antagonism can be manifest as a negative genetic correlation between adult male and female reproductive success (Chippindale et al. 2001) and is expected to generate rapid evolution in physiological and morphological traits, and may even lead to reproductive isolation, for example, if co-evolution proceeds differently in allopatric populations (Parker and Partridge 1998).

In *D. melanogaster*, the cost of mating suffered by females is seen as a reduction in survival and reproductive success (Fowler and Partridge 1989) and is mediated by the action of accessory gland proteins (Acps) (Chapman et al. 1995) which are transferred along with sperm during mating. Recently, Wigby and Chapman (2005) provided evidence that SP is responsible for at least a large part of this cost. In that study, females were continuously housed with either males deficient for SP or genetically matched controls. Despite mating more than 12 times as frequently, females continuously exposed to SP deficient males lived at least as long as those housed with control males and had higher fitness (Wigby and Chapman 2005).

A further candidate that has been proposed to contribute to the cost of mating is the protease inhibitor Acp62F (section 1.5.4) which, along with other seminal proteins, can pass from the female reproductive tract into the haemolymph (Lung and Wolfner 1999; Wolfner 2002) and when ectopically expressed, was found to be toxic to adult flies (Lung et al. 2002). However, because ectopic expression produced Acp62F at far greater quantities than would be delivered through mating and to sites not usually

exposed to Acp62F, it is necessary to confirm whether the toxic effects reported also occur during normal matings, and this is the aim of the work presented in this Chapter.

An alternative technique to ectopic expression for examining the affects of Acp62F *in vivo* is to silence or knock out the Acp62F protein in males and then to examine the effects of presence or absence of Acp62F on females following normal matings.

Acp62F silencing was achieved here by RNA interference (RNAi). In this technique, the transgenic males produced transfer normal seminal fluid except for the targeted Acp, the level of which is significantly reduced. The RNAi system I used restricted Acp62F knockdown to the accessory glands, by using an accessory gland-specific gene promotor to drive the gene silencing (as described in Chapman et al. 2003a). Bangham (2003) previously used this approach in an attempt to determine the effect of Acp62F delivery on female lifespan and reproductive success. The results did not support the contention that Acp62F contributes to the cost of mating in females (Bangham, 2003). The protocol used, however, may not have offered the best opportunity to detect costs of mating for two reasons. Firstly, females were only housed with males for approximately 8 hours every 2-3 days, limiting their opportunities for matings and hence the transfer of Acp62F. Secondly, live yeast was not added to vials in which flies were housed. It is thought that higher levels of nutrition are required to stimulate higher mating rates in order to detect costs of mating in terms of both survival and fecundity (Chapman and Partridge 1996).

However, a potential drawback of using RNAi is that the gene silencing may not be totally effective and that small amounts of the targeted protein can still be produced (Lung 2000). The RNAi gene silencing technique may not therefore produce true null phenotypes. A more accurate representation of functional significance may be achieved using knock out males in which the wild-type allele is removed and replaced with a non-functioning sequence via homologous recombination (Rong and Golic 2000). To comprehensively test the effects of Acp62F on wild-type female longevity, fecundity and fertility, I therefore used males in which Acp62F had been silenced by RNAi (Acp62F knockdown males) and in which the Acp62F gene had been mutated (Acp62F knockout males, Muller 2006). An increase in the longevity of females mated to Acp62F knockdown or knockout males, relative to their respective controls,

would be consistent with the idea that Acp62F was, at least in part, responsible for the cost of mating.

### 6.3 Materials and Methods

#### 6.3.1 Generation of Acp62F RNAi knockdown and control males

Two experiments were conducted using the stocks described in section 2.3.4, i.e. Acp62F RNAi experiment 1 and 2. In Acp62F RNAi experiment 1, I initially planned to use genetically matched control males that were the result of reciprocal crosses of the experimental male-generating crosses (i.e. males of the following genotypes:  $+/+; UAS-Acp62F-IR1b$ ,  $+/+; UAS-Acp62F-IR3A$ , or  $+/+; UAS-Acp62F-IR3C$  genotypes (Figure 2.1). However, because the mating frequencies of wild-type females with these control males was significantly lower than for females kept with their respective RNAi experimental males, these were not appropriate controls for the purpose of this experiment (confounding differing levels of delivery of other Acps). Instead, I used as controls  $Acp26Aa-P-GAL4; UAS-Acp62F-IR3C$  males, which contained the same transgenic insertion in the same genetic background, but in which, for an unknown reason, Acp62F was not silenced (Lung 2000; see Western Blot result, Figure 6.1). To generate the experimental and control males for the experiment, mating groups of  $Acp26AaP-GAL4$  virgin females and RNAi transgenic males (2 females: 2 males) were established (Figure 2.1) and females allowed to lay for 2 days to ensure low larval density.

To increase the efficiency of Acp62F silencing, male offspring from experimental ( $Acp26AaP-GAL4; UAS-Acp62F-IR1b$ ,  $Acp26AaP-GAL4; UAS-Acp62F-IR3A$ ) and control ( $Acp26Aa-P-GAL4; UAS-Acp62F-IR3C$ ) crosses were allowed to mate with sibling females and aged for 3-4 days. This opportunity for mating prior to the experiment gave increased opportunity for Acp62F knockdown in the experimental lines since Acp expression, and hence expression of the transgene, is induced after mating (DiBenedetto et al. 1990; Wolfner et al. 1997b) and this has been shown to increase knockdown efficiency (Lung 2000).

In Acp62F RNAi experiment 2, an additional control line was also used, to control for the position of the Gal4 driver insert ( $Acp26Aa-P-GAL4; +$ ). This was generated by



establishing mating groups of *Acp26AaP-GAL4* virgin females and non-transgenic males with the same genetic background as the RNAi stocks (Figure 2.1d).

### 6.3.2 *Generation of Acp62F null mutants*

Null flies and controls were constructed by Jacob Muller (Muller 2006) and obtained from Mariana Wolfner's Lab (Cornell University), see section 2.3.5. To generate experimental and control males for the null experiment that had very similar genetic backgrounds and the same eye colour, I took null and control males through an additional crossing regime (Figure 2.2). This resulted in the generation of a red-eyed stocks containing a wild-type X chromosome, homologous *w<sup>1118</sup>* 2<sup>nd</sup> chromosomes (the background in which the original deletion was generated), and a 3<sup>rd</sup> chromosome homologous for either the mutant *Acp62F* allele, the tandem duplication or *w<sup>1118</sup>*.

### 6.3.3 *Acp62F levels in RNAi knockdown, null and control males*

Western blots were performed to confirm levels of *Acp62F* in knockdown, null and their respective control males (Figure 6.1). *Acp62F* levels were tested in subsets of males frozen on the first day of *Acp62F* RNAi experiment 1 and from the null experiment. Levels of *Acp62F* were also tested in the *Acp26AaP-GAL4*;+ control line of RNAi experiment 2. Subsets of males were isolated and frozen at -80°C before protein extraction and testing (section 2.2.6). Westerns were performed with the help of Dr Su Davies.

### 6.3.4 *Acp62F RNAi experiment 1: Effect of Acp62F on female fitness*

100 virgin wild-type Dahomey females per group were continuously housed in groups of five with either five *Acp62F* knockdown (*Acp26AaP-GAL4*; *UAS-Acp62F-IR1b*, *Acp26AaP-GAL4*; *UAS-Acp62F-IR3A*) or control (*Acp26AaP-GAL4*; *UAS-Acp62F-IR3C*) males, in SY vials seeded with live yeast balls. Any males that died during the experiment were replaced and the sex-ratio was maintained at 1:1. All males were replaced on day 16 with new 2-3 day old males, to encourage mating throughout the experiment. Female deaths were scored every 1-2 days and females were transferred to fresh vials every 2-3 days.

Fecundity and fertility tests were carried out on days 2, 5, 7, 11, 15, 20, 25 and 27. For these, flies were transferred to fresh SY charcoal vials for between 4 and 24 hours (for the same duration for any given day) before being transferred back to fresh SY vials. Fecundity in each of these vials was recorded within 24 hours of the latter transfer and eggs were then allowed to develop at 25°C. Fecundity and fertility measures were corrected for the number of laying females in a vial and the number of hours of egg laying. I also used these measures to calculate egg to adult viability per female per 24 hours.

Courtship and mating frequencies were recorded (section 2.4.1) on days 4, 6, 8, 12, 14, 19, 21 and 26. Vials were scanned for matings and courtship behaviour 10 times on each day observation day, with observations not closer than 20 minutes, starting at lights on.

#### *6.3.5 Acp62F RNAi experiment 2: Effect of Acp62F on female fitness*

A second experiment using Acp62F RNAi males was conducted to establish the repeatability of the results in RNAi experiment 1 with an additional control. The experiment ran as described above (section 6.3.4) except that females were housed continuously with either Acp62F knockdown (*Acp26AaP-GAL4; UAS-Acp62F-IR1b*, *Acp26AaP-GAL4; UAS-Acp62F-IR3A*) or control (*Acp26Aa-P-GAL4; UAS-Acp62F-IR3C*, *Acp26AaP-GAL4; +*) males. All males were replaced on day 19.

The fecundity of females mated to experimental (*Acp26AaP-GAL4; UAS-Acp62F-IR1b*, *Acp26AaP-GAL4; UAS-Acp62F-IR3A*) and control (*Acp26Aa-P-GAL4; UAS-Acp62F-IR3C*, *Acp26AaP-GAL4; +*) males was tested (as section 6.3.4) on days 3, 6, 9, 15, 20, 22, 26, 29 and 34. Egg-adult viability was not recorded in this experiment.

Courtship and mating frequencies were recorded (as section 6.3.4) on days 1, 5, 8, 13, 21, 25, 28, 30 and 34.

### 6.3.6 *Acp62F* null experiment: Effect of *Acp62F* on female fitness

The experiment ran as described above (section 6.3.4) except that 120 virgin wild-type Dahomey females per line were continuously housed in groups of five with five *Acp62F* null (*Acp62F<sup>lhb</sup>*, *Acp62F<sup>lhc</sup>*, *Acp62F<sup>lc</sup>*) or control (*Acp62F<sup>Tandem</sup>*, *Acp62F<sup>lcc<sup>trl</sup></sup>*) males. All males were replaced on days 11 and 25.

Fecundity and fertility tests were carried out (as section 6.3.4) on days 2, 5, 7, 9, 13, 15, 18, 21, 24, 27, 31 and 34.

Courtship and mating frequencies were recorded (as section 6.3.4) on days 1, 4, 6, 8, 12, 15, 18, 21, 24, 27, 30 and 33.

## 6.4 Results

### 6.4.1 *Acp62F* levels in RNAi knockdown, null and control lines

The level of *Acp62F* expression in males carrying the *Acp26AaP-GAL4;UAS-Acp62F-IR* RNAi constructs was determined by Western blot analysis (Figure 6.1a). The results confirm that *Acp62F* was knocked down to undetectable levels in *Acp26AaP-GAL4;UAS-Acp62F-IR1b* and *Acp26AaP-GAL4;UAS-Acp62F-IR3A* males. In *Acp26AaP-GAL4;UAS-Acp62F-IR3C* control males, *Acp62F* was detected, demonstrating that *Acp62F* was, as expected based on previous results (Lung 2000), present in this line, and therefore that it could serve as a good control. The second Western blot confirmed that *Acp26AaP-GAL4;+* control males also expressed *Acp62F* as expected (Figure 6.1a).

The levels of *Acp62F* in males null for *Acp62F* are shown in Figure 6.1b. As expected, *Acp62F* was detected in *Acp62F<sup>Tandem</sup>* control males but not in *Acp62F<sup>lhb</sup>*, *Acp62F<sup>lhc</sup>* and *Acp62F<sup>lc</sup>* experimental males. As discussed in section 2.3.5, *Acp62F<sup>lcc<sup>trl</sup></sup>* males were expected to have a null genotype, but instead did produce *Acp62F*. They were hence subsequently designated as an additional control line.

#### 6.4.2 *Acp62F RNAi experiment 1: Effect of Acp62F on female fitness*

The survival plot for RNAi experiment 1 is shown in Figure 6.2. Log-rank tests were performed to determine differences in survival of females mated to either Acp62F knockdown (*Acp26AaP-GAL4;UAS-Acp62F-IR1b*, *Acp26AaP-GAL4;UAS-Acp62F-IR3A*) or control (*Acp26AaP-GAL4;UAS-Acp62F-IR3C*) males. The survival of females housed with Acp62F knockdown males (*Acp26AaP-GAL4;UAS-Acp62F-IR1b*, *Acp26AaP-GAL4;UAS-Acp62F-IR3A*) was significantly reduced compared to survival of females mated housed with control males (*Acp26AaP-GAL4;UAS-Acp62F-IR3C*; Log Rank,  $\chi^2=8.77$ ,  $df=2$ ,  $P=0.0125$ ). Comparisons between the survival of females in individual lines (corrected for multiple comparisons) revealed that females housed with each of the knockdown male lines had significantly reduced lifespan compared to the control line (P value corrected for multiple comparisons at 95% confidence=0.0167; *Acp26AaP-GAL4;UAS-Acp62F-IR1b* vs. *Acp26AaP-GAL4;UAS-Acp62F-IR3C*,  $\chi^2=7.45$ ,  $df=1$ ,  $P=0.0064$ ; *Acp26AaP-GAL4;UAS-Acp62F-IR3A* vs. *Acp26AaP-GAL4;UAS-Acp62F-IR3C*,  $\chi^2=5.71$ ,  $df=1$ ,  $P=0.0168$ ). Females exposed to the two Acp62F knockdown lines did not differ in survival from one another (*Acp26AaP-GAL4;UAS-Acp62F-IR1b* vs. *Acp26AaP-GAL4;UAS-Acp62F-IR3A*,  $\chi^2=0.27$ ,  $df=1$ ,  $P=0.6025$ ). These results suggest that female survival was not reduced as a result of mating with males transferring Acp62F, and in fact the presence of Acp62F seemed to confer a longevity-protecting effect on females.

Age-specific egg laying and egg to adult viability showed a tendency to decline as females aged (Figure 6.3a, b, Table 6.1a, b). General Linear Model analysis showed that there were no differences in the number of eggs laid by females mated to either control or knockdown RNAi males, nor were there differences in the interaction between the number of eggs laid over time (Table 6.1a). Similarly there were no differences in egg to adult viability of eggs laid by females mated to control or knockdown males (Table 6.1b).

Mating and courtship frequencies were analysed in contingency tables using Chi-square tests (Zar, 1999). Mating frequency was analysed by comparing the total number of matings taken in each group to the total number of mating opportunities not taken. Mating opportunities were defined as the number of observation days multiplied by the number of females present on each sampling day. Courtship

opportunities were defined as the total number of observations of courting events summed over all sampling days multiplied by the number of females present on each sampling day.

There were no differences in mating and courtship frequencies between RNAi lines (Table 6.4); hence the survival, fecundity and fertility of females housed with males that lacked or did not lack Acp62F was not confounded by any such differences.

Together, the results show that females continuously housed with males lacking Acp62F had reduced survival compared to females housed with males that transferred Acp62F. There is no evidence from this experiment therefore that receipt of Acp62F is toxic to females, and hence responsible for the cost of mating; the results instead suggested that receipt of Acp62F significantly prolonged female lifespan.

#### 6.4.3 *Acp62F RNAi experiment 2: Effect of Acp62F on female fitness*

In the second of the RNAi experiments, there was also a significant effect of male genotype on female survival (Log-Rank,  $\chi^2=10.84$ ,  $df=3$ ,  $P=0.0126$ , Figure 6.4). However, in this experiment there was no difference in survival between females housed with males that did and did not transfer Acp62F during mating. Females housed with *Acp26AaP-GAL4; UAS-Acp62F-IR1b* males did have significantly greater survival than females housed with either *Acp26AaP-GAL4; UAS-Acp62F-IR3A* ( $\chi^2=8.08$ ,  $df=1$ ,  $P=0.0045$ ) or *Acp26AaP-GAL4; +* ( $\chi^2=7.40$ ,  $df=1$ ,  $P=0.0065$ ) males, but did not differ significantly in survival from females housed with *Acp26AaP-GAL4; UAS-Acp62F-IR3C* males ( $\chi^2=4.74$ ,  $df=1$ ,  $P=0.0293$ ,  $P$  value corrected for multiple comparisons at 95% confidence=0.0083). There were no significant differences in the survival of females housed with *Acp26AaP-GAL4; UAS-Acp62F-IR3A* and either *Acp26AaP-GAL4; UAS-Acp62F-IR3C* ( $\chi^2=0.59$ ,  $df=1$ ,  $P=0.4517$ ) or *Acp26AaP-GAL4; +* ( $\chi^2=0.02$ ,  $df=1$ ,  $P=0.88$ ) males, or between females housed with *Acp26AaP-GAL4; UAS-Acp62F-IR3C* and *Acp26AaP-GAL4; +* males ( $\chi^2=0.50$ ,  $df=1$ ,  $P=0.4779$ ). These results are consistent with those of RNAi experiment 1 (section 6.3.4) in suggesting that receipt of Acp62F does not significantly decrease female survival. In contrast to the first RNAi experiment, there was no suggestion here of an increase in lifespan of females exposed to Acp62F.

General Linear Model analysis showed that there were no consistent differences in the number of eggs laid by females mated to control or knockdown males. Females mated to males from the second control line (C2) differed significantly from other lines both in the number of eggs laid and in the interaction with egg laid over time, but this was not seen in the other control line (Table 6.2).

There were significant differences in mating and courtship frequencies between all groups considered together (mating,  $\chi^2=9.49$ ,  $df=1$ ,  $P=0.0234$ ; courtship,  $\chi^2=17.62$ ,  $df=1$ ,  $P=0.0005$ ; Table 6.5). There was no significant difference in the mating frequency of *Acp26AaP-GAL4; UAS-Acp62F-IR1b* males compared to *Acp26AaP-GAL4; +* lines ( $\chi^2=0.09$ ,  $df=1$ ,  $P=0.7613$ ), but courtship frequency did significantly differ between these groups ( $\chi^2=12.7$ ,  $df=1$ ,  $P=0.0004$ ). There were no significant differences in the mating or courtship frequencies of *Acp26AaP-GAL4; UAS-Acp62F-IR3A* and *Acp26AaP-GAL4; UAS-Acp62F-IR3C* lines (mating,  $\chi^2=0.05$ ,  $df=1$ ,  $P=0.8199$ ; courtship,  $\chi^2=1.94$ ,  $df=1$ ,  $P=0.1640$ ).

In light of the potentially confounding differences in mating frequencies (Table 6.5), I conducted further survival analyses between lines that did not differ significantly in mating frequency. This was to avoid confounding effects due to differences in Acp62F levels versus levels of other Acps that would result when mating frequency differed. These additional comparisons revealed that survival of females housed with *Acp26AaP-GAL4; +* control lines was significantly reduced relative to *Acp26AaP-GAL4; UAS-Acp62F-IR1b* knockdown males ( $\chi^2=7.40$ ,  $df=1$ ,  $P=0.0065$ ) whereas the survival of females housed with *Acp26AaP-GAL4; UAS-Acp62F-IR3A* males did not significantly differ from that of females housed with *Acp26AaP-GAL4; UAS-Acp62F-IR3C* males ( $\chi^2=1.05$ ,  $df=1$ ,  $P=0.3048$ ). Hence any suggestion from the first of these comparisons that Acp62F was associated with decreased female lifespan was not supported by the second comparison. Hence overall, the results are consistent in providing no evidence that Acp62F contributes to the female cost of mating.

#### 6.4.4 *Acp62F* null experiment: Effect of *Acp62F* on female fitness

There were significant differences in survival between females exposed to the different male genotypes ( $\chi^2=14.87$ ,  $df=4$ ,  $P=0.0050$ , Figure 6.6). However, these

were mainly attributable to significantly reduced survival of females mated to *Acp62F<sup>lbc</sup>* males and not to the overall receipt or non-receipt of Acp62F during mating. The reduced survival of *Acp62F<sup>lbc</sup>* females may have been due to their significantly higher mating frequency ( $\chi^2=14.29$ ,  $df=4$ ,  $P=0.0064$ ) as compared to other groups, which did not differ in mating frequency from one another ( $\chi^2=5.70$ ,  $df=3$ ,  $P=0.1272$ ). This could have had the confounding effect of increasing the receipt of other potentially costly Acps (e.g. SP, Wigby and Chapman 2005) in this group relative to the other groups. Removal of the *Acp62F<sup>lbc</sup>* group from the survival analysis revealed that there were no significant differences in the survival of females mated to males that transferred or did not transfer Acp62F ( $\chi^2=1.90$ ,  $df=3$ ,  $P=0.5942$ ), suggesting that Acp62F has no effect on female survival.

There were significant differences in the age-specific fecundity of females mated to the different male genotypes (Figure 6.7a); however, these differences were not associated with receipt of Acp62F by females. GLM analysis revealed that females mated to *Acp62F<sup>ΔA</sup>* and *Acp62F<sup>ΔE</sup>* males differed significantly in egg production over time (Table 6.3) but that other groups did not significantly vary.

Age-specific egg to adult viability was variable between groups (Figure 6.7b, Table 6.3b). However, differences in egg to adult viability were not related to the presence or absence of Acp62F and were consistently attributable to lower egg-adult viability for females mated to *Acp62F<sup>lc</sup>* null and *Acp62F<sup>lcC<sup>tr</sup>l</sup>* control males (Table 6.3b). The *Acp62F<sup>lc</sup>* null and *Acp62F<sup>lcC<sup>tr</sup>l</sup>* control lines therefore seemed to exhibit, for an unknown reason not related to the presence or absence of Acp62F, lower egg fertility (and lower late fecundity, see above) than the other groups.

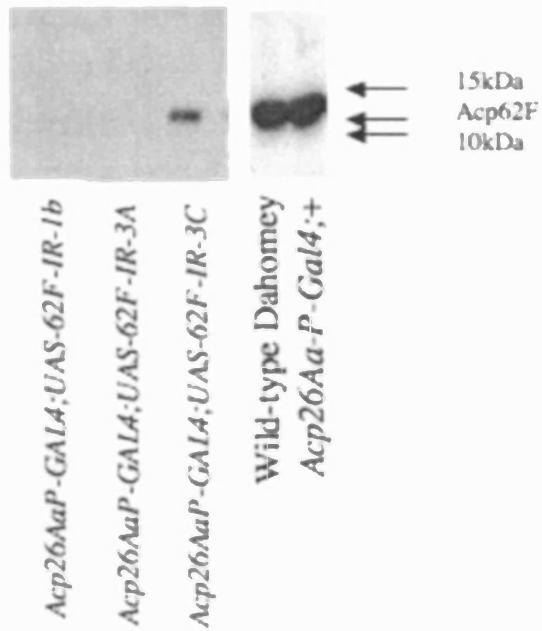
When all groups were analysed together, there were significant differences in both mating and courtship frequencies (Table 6.6). The difference observed in mating frequency was attributable to a higher mating frequency of *Acp62F<sup>lbc</sup>* males (comparison between lines excluding *Acp62F<sup>lbc</sup>*,  $\chi^2=5.7$ ,  $df=3$ ,  $P=0.1272$ ). There was also a significant difference between the lines in courtship frequency resulting from higher courtship in *Acp62F<sup>lcC<sup>tr</sup>l</sup>* group (Table 6.6b). There were no differences in the courtship frequency of females by males from the other lines ( $\chi^2=4.3$ ,  $df=3$ ,  $P=0.23$ ).

In summary, the results of the Acp62F null experiment provided no evidence that Acp62F is associated with decreased lifespan in females, or that the presence or absence of Acp62F had any consistent effect on fecundity or egg-adult viability.

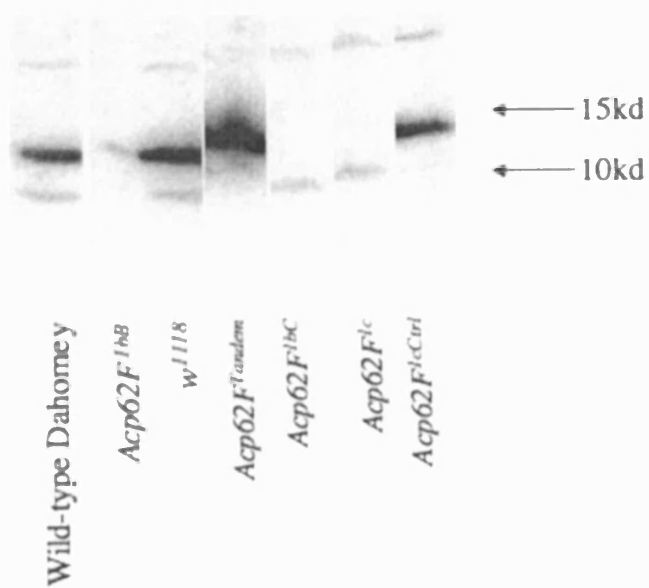


**Figure 6.1** Western Blot analysis of Acp62F levels in (a) RNAi knockdown (*Acp26AaP-Gal4;UAS-Acp62F-IR1b*, *Acp26AaP-Gal4;UAS-Acp62F-IR3A*) and control males (*Acp26AaP-Gal4;UAS-Acp62F-IR3C*, *Acp26AaP-Gal4;+*) and (b) *Acp62F* null and control males.

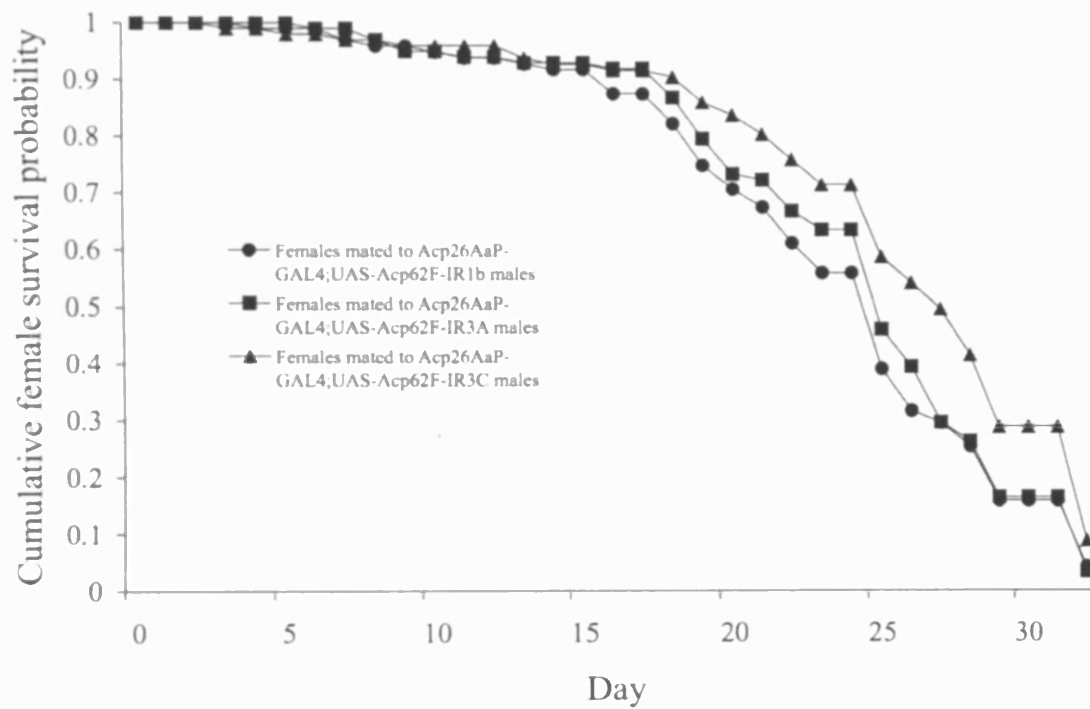
(a)



(b)

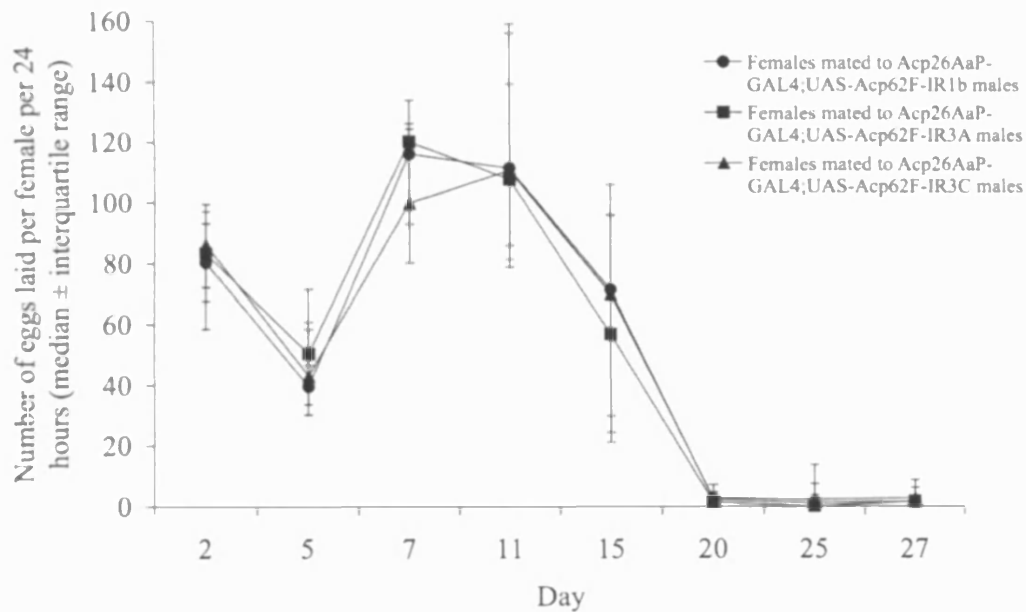


**Figure 6.2** Effect of Acp62F on female lifespan (RNAi experiment 1). Cumulative survival probability of females mated to Acp62F knockdown (*Acp26AaP-GAL4;UAS-Acp62F-IR1b* and *Acp26AaP-GAL4;UAS-Acp62F-IR3A*) or control (*Acp26AaP-GAL4;UAS-Acp62F-IR3C*) males, against time (days).

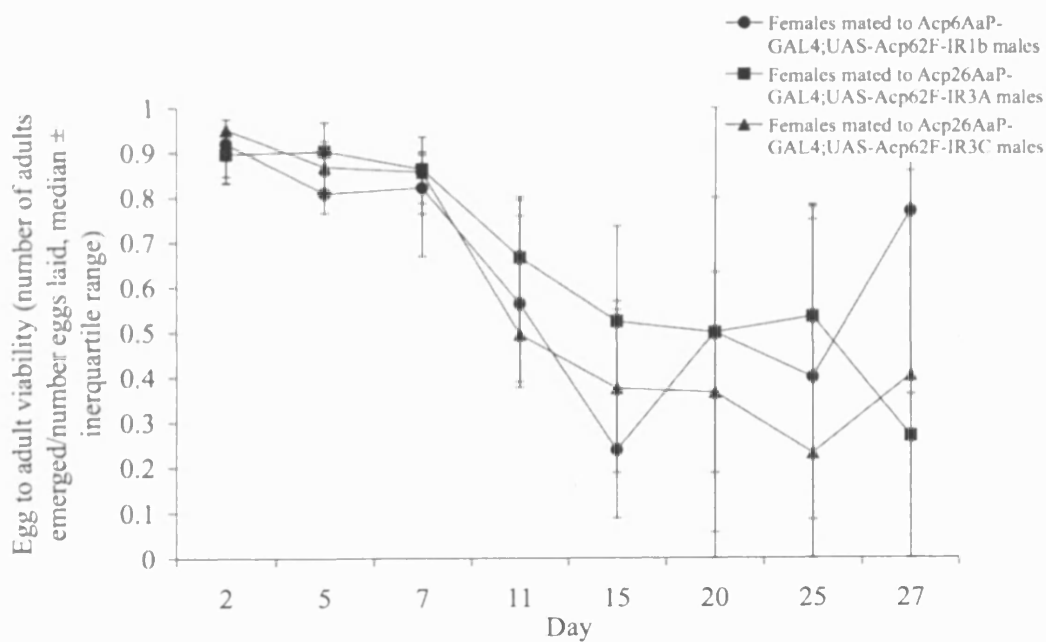


**Figure 6.3** Age-specific egg production (a) and viability (b) of eggs laid by wild-type females continuously exposed to Acp62F knockdown or control males (RNAi experiment 1. (a) median ( $\pm$  inter-quartile range) number of eggs laid per 24 hours by females continuously exposed to knockdown (*Acp26AaP-GAL4;UAS-Acp62F-IR1b* and *Acp26AaP-GAL4;UAS-Acp62F-IR3A*) and control (*Acp26AaP-GAL4;UAS-Acp62F-IR3C*) males (b) median ( $\pm$  inter-quartile range) egg-adult viability for the eggs laid by the females shown in (a).

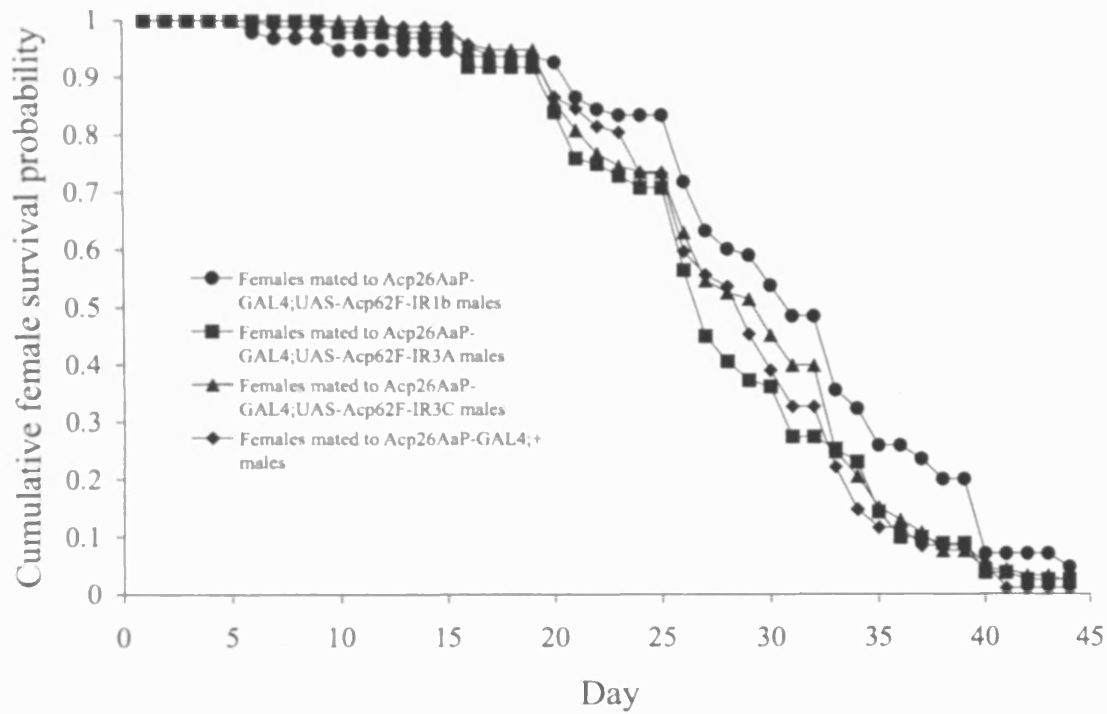
(a)



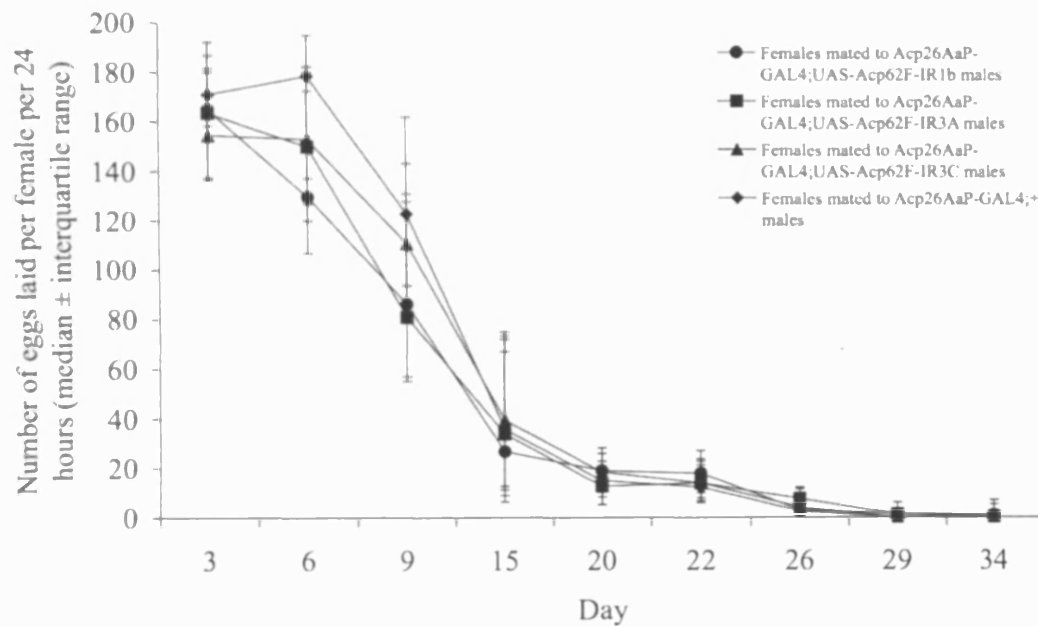
(b)



**Figure 6.4** Effect of Acp62F on female lifespan (RNAi experiment 2). Cumulative survival probability of females mated to Acp62F knockdown (*Acp26AaP-GAL4;UAS-Acp62F-IR1b* and *Acp26AaP-GAL4;UAS-Acp62F-IR3A*) and control (*Acp26AaP-GAL4;UAS-Acp62F-IR3C* and *Acp26AaP-GAL4;+*) males, against time (days).

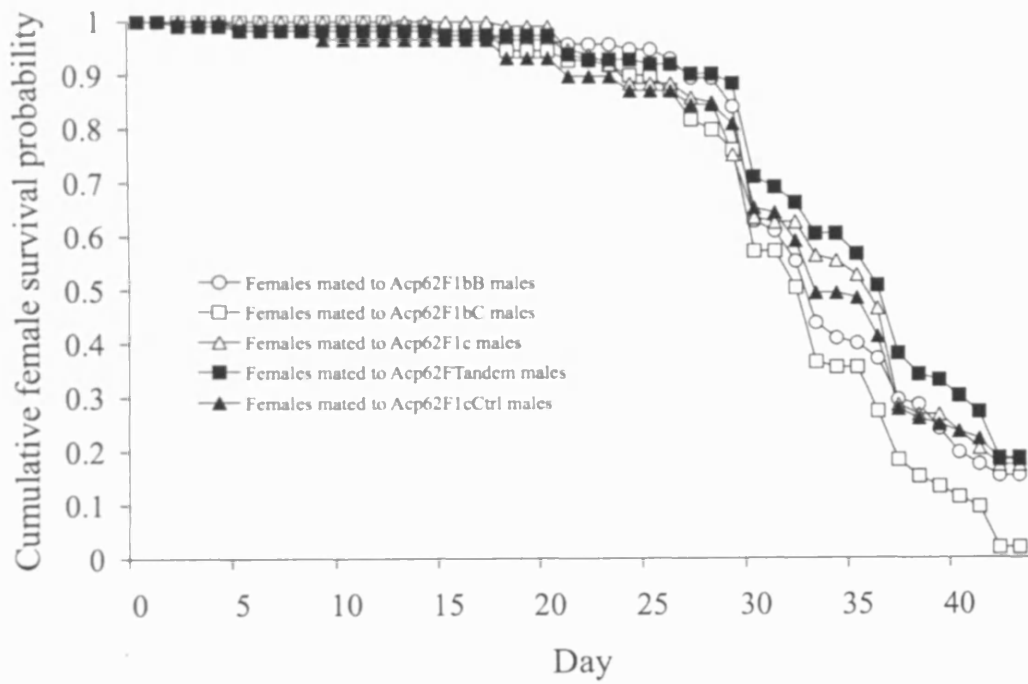


**Figure 6.5** Age-specific production of eggs laid by females continuously exposed to Acp62F knockdown (*Acp26AaP-GAL4;UAS-Acp62F-IR1b* and *Acp26AaP-GAL4;UAS-Acp62F-IR3A*) or control (*Acp26AaP-GAL4;UAS-Acp62F-IR3C* and *Acp26Aa-P-GAL4;+*) males (RNAi experiment 2) (median  $\pm$  inter-quartile range number of eggs laid per 24 hours).



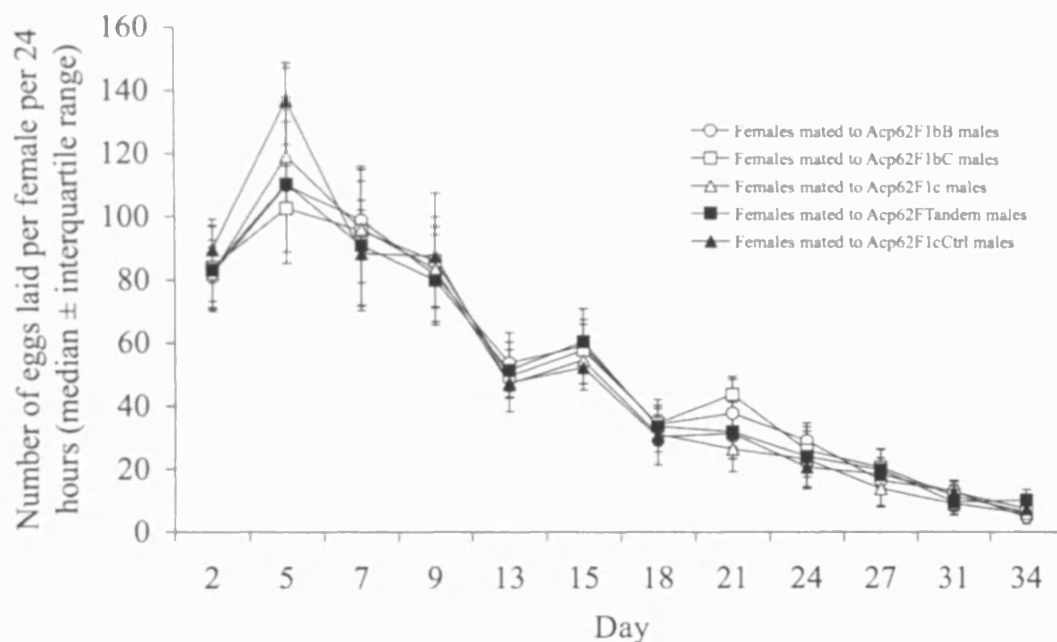
**Figure 6.6** Effect of Acp62F on female lifespan (Acp62F Null experiment).

Cumulative survival probability of females mated to Acp62F knockout ( $Acp62F^{lbb}$ ,  $Acp62F^{lbc}$ ,  $Acp62F^{lc}$ ) and control ( $Acp62F^{Tandem}$ ,  $Acp62F^{lcCtrl}$ ) males, against time (days).

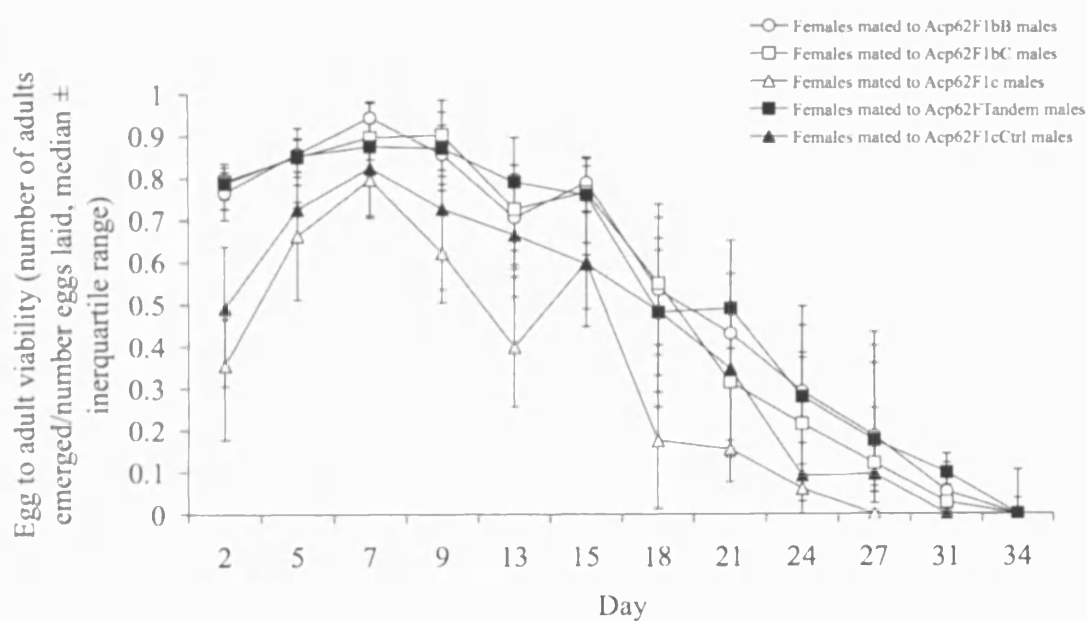


**Figure 6.7** Age-specific egg production and viability of eggs of wild-type females continuously exposed to Acp62F knockout or control males (Acp62F null experiment). (a) median ( $\pm$  inter-quartile range) number of eggs laid per 24 hours by females continuously exposed to Acp62F null (*Acp62F<sup>lbb</sup>*, *Acp62F<sup>lbc</sup>*, *Acp62F<sup>lc</sup>*) or control (*Acp62F<sup>Tandem</sup>*, *Acp62F<sup>lcCtrl</sup>*) males, (b) median ( $\pm$  inter-quartile range, error bars) egg-adult viability for the eggs laid by the females shown in (a).

(a)



(b)



**Table 6.1** General Linear Model analysis of (a) the number of eggs laid by males mated to males with knocked down or control Acp62F levels and (b) the egg to adult viability of those eggs (RNAi experiment 1).

**(a)**

| <b>Source of variation</b> | <b>Estimate</b> | <b>SE</b> | <b>t</b> | <b>P</b> |
|----------------------------|-----------------|-----------|----------|----------|
| Intercept                  | 100.317         | 6.934     | 14.468   | <0.000   |
| 3A-                        | 10.179          | 9.785     | 1.040    | 0.299    |
| 3C                         | 6.119           | 9.692     | 0.631    | 0.528    |
| Day                        | -3.178          | 0.512     | -6.203   | <0.000   |
| 3A- x Day                  | -0.730          | 0.718     | -1.016   | 0.310    |
| 3C x Day                   | -0.340          | 0.695     | -0.489   | 0.625    |

**(b)**

| <b>Source of variation</b> | <b>Estimate</b> | <b>SE</b> | <b>t</b> | <b>P</b> |
|----------------------------|-----------------|-----------|----------|----------|
| Intercept                  | 0.844           | 0.042     | 20.265   | <0.000   |
| 3A-                        | 0.106           | 0.059     | 1.788    | 0.075    |
| 3C                         | 0.077           | 0.058     | 1.335    | 0.183    |
| Day                        | -0.020          | 0.003     | -6.202   | <0.000   |
| 3A- x Day                  | -0.005          | 0.005     | -1.037   | 0.301    |
| 3C x Day                   | -0.005          | 0.004     | -0.969   | 0.333    |



**Table 6.2** General Linear Model analysis of the number of eggs laid by males mated to males with knocked down or control Acp62F levels in RNAi experiment 2.

| Source of variation | Estimate | SE    | t       | P      |
|---------------------|----------|-------|---------|--------|
| Intercept           | 161.605  | 5.493 | 29.418  | <0.000 |
| 3A-                 | 3.692    | 7.826 | 0.472   | 0.637  |
| 3C                  | 7.331    | 7.789 | 0.941   | 0.347  |
| C2                  | 26.208   | 7.820 | 3.352   | <0.001 |
| Day                 | -6.183   | 0.308 | -20.087 | <0.000 |
| 3A- x Day           | -0.401   | 0.454 | -0.882  | 0.378  |
| 3C x Day            | -0.374   | 0.446 | -0.837  | 0.403  |
| C2 x Day            | -1.369   | 0.451 | -3.037  | 0.002  |

**Table 6.3** General Linear Model analysis on (a) the number of eggs laid by females mated to each of the groups on the null experiment and (b) the egg to adult viability of those eggs. Group, and the interaction between group and day, are set as factors.

**(a)**

| Source of variation         | Estimate | SE    | t       | P      |
|-----------------------------|----------|-------|---------|--------|
| Intercept                   | 4.913    | 0.035 | 142.251 | <0.000 |
| Acp62F <sup>1bc</sup>       | -0.081   | 0.050 | -1.627  | 0.104  |
| Acp62F <sup>3C</sup>        | 0.030    | 0.049 | 0.616   | 0.538  |
| Acp62F <sup>4A</sup>        | 0.085    | 0.049 | 1.745   | 0.081  |
| Acp62F <sup>4E</sup>        | 0.026    | 0.049 | 0.519   | 0.604  |
| Day                         | -0.067   | 0.003 | -25.234 | <0.000 |
| Acp62F <sup>1bc</sup> x Day | 0.003    | 0.004 | 0.903   | 0.366  |
| Acp62F <sup>3C</sup> x Day  | -0.003   | 0.004 | -0.879  | 0.379  |
| Acp62F <sup>4A</sup> x Day  | -0.011   | 0.004 | -2.867  | 0.004  |
| Acp62F <sup>4E</sup> x Day  | -0.010   | 0.004 | -2.557  | 0.011  |

**(b)**

| Source of variation         | Estimate | SE    | t       | P      |
|-----------------------------|----------|-------|---------|--------|
| Intercept                   | 2.662    | 0.163 | 16.382  | <0.000 |
| Acp62F <sup>1bc</sup>       | 0.131    | 0.238 | 0.552   | 0.581  |
| Acp62F <sup>3C</sup>        | -0.229   | 0.226 | -1.014  | 0.311  |
| Acp62F <sup>4A</sup>        | -1.124   | 0.210 | -5.346  | <0.000 |
| Acp62F <sup>4E</sup>        | -1.529   | 0.207 | -7.373  | <0.000 |
| Day                         | -0.141   | 0.009 | -15.949 | <0.000 |
| Acp62F <sup>1bc</sup> x Day | -0.013   | 0.013 | -1.026  | 0.305  |
| Acp62F <sup>3C</sup> x Day  | 0.014    | 0.012 | 1.184   | 0.237  |
| Acp62F <sup>4A</sup> x Day  | 0.030    | 0.012 | 2.544   | 0.011  |
| Acp62F <sup>4E</sup> x Day  | 0.026    | 0.012 | 2.167   | 0.031  |

**Table 6.4** Courtship and mating frequencies of females continuously housed with Acp62F RNAi knockdown and control males (RNAi experiment 1). Chi-square tests were used to test for differences in (a) total mating and (b) total courtship frequencies for females mated to Acp62F knockdown (*Acp26AaP-GAL4;UAS-Acp62F-IR1b*, *Acp26AaP-GAL4;UAS-Acp62F-IR3A*) and control (*Acp26AaP-GAL4;UAS-Acp62F-IR3C*) males.

| Males with which females were housed | Number of matings / courtships observed | Number of matings / courtships not observed | Percentage mating/courting out of the total number of observations | Df | Pearson Chi square test statistic | P      |
|--------------------------------------|---|---|--|----|-----------------------------------|--------|
| (a) Mating                           |   |   |  |    |                                   |        |
| <i>Acp26AaP-GAL4;UAS-Acp62F-IR1b</i> | 36                                      | 6214  | 0.58   | 2  | 1                                 | 0.6069 |
| <i>Acp26AaP-GAL4;UAS-Acp62F-IR3A</i> | 45                                      | 6505  | 0.69   |    |                                   |        |
| <i>Acp26AaP-GAL4;UAS-Acp62F-IR3C</i> | 37                                      | 6533  | 0.56   |    |                                   |        |
| (b) Courtship                        |   |   |  |    |                                   |        |
| <i>Acp26AaP-GAL4;UAS-Acp62F-IR1b</i> | 368                                     | 5882  | 5.88   | 2  | 4.13                              | 0.1268 |
| <i>Acp26AaP-GAL4;UAS-Acp62F-IR3A</i> | 432                                     | 6118  | 6.60   |    |                                   |        |
| <i>Acp26AaP-GAL4;UAS-Acp62F-IR3C</i> | 383                                     | 6187  | 5.83   |    |                                   |        |

**Table 6.5** Courtship and mating frequencies of females continuously housed with Acp62F RNAi knockdown or control males (RNAi experiment 2). Chi-square tests were used to test for differences in (a) total mating and (b) courtship frequencies for females housed with *Acp26AaP-GAL4;UAS-Acp62F-IR1b* or *Acp26AaP-GAL4;+* males; or for differences in (c) total mating and (d) courtship frequencies for females housed with *Acp26AaP-GAL4;UAS-Acp62F-IR3A* or *Acp26AaP-GAL4;UAS-Acp62F-IR3C* males.

| Males with which females were housed | Number of matings / courtships observed | Number of matings / courtships not observed | Percentage mating/courting out of the total number of observations | Df | Pearson Chi square test statistic | P      |
|--------------------------------------|---|---|--|----|-----------------------------------|--------|
| (a) Mating                           |   |   |  |    |                                   |        |
| <i>Acp26AaP-GAL4;UAS-Acp62F-IR1b</i> | 44                                      | 6516  | 0.67   | 1  | 0.09                              | 0.7613 |
| <i>Acp26AaP-GAL4;+</i>               | 44                                      | 6106  | 0.72   |    |                                   |        |
| (b) Courtship                        |   |   |  |    |                                   |        |
| <i>Acp26AaP-GAL4;UAS-Acp62F-IR1b</i> | 313                                     | 6247  | 4.77   | 1  | 12.7                              | 0.0004 |
| <i>Acp26AaP-GAL4;+</i>               | 216                                     | 5934  | 3.51   |    |                                   |        |
| (c) Mating                           |   |   |  |    |                                   |        |
| <i>Acp26AaP-GAL4;UAS-Acp62F-IR3A</i> | 64                                      | 5906  | 1.07   | 1  | 0.05                              | 0.8199 |
| <i>Acp26AaP-GAL4;UAS-Acp62F-IR3C</i> | 65                                      | 6245  | 1.03   |    |                                   |        |
| (d) Courtship                        |   |   |  |    |                                   |        |
| <i>Acp26AaP-GAL4;UAS-Acp62F-IR3A</i> | 368                                     | 5882  | 5.89   | 1  | 1.94                              | 0.1646 |
| <i>Acp26AaP-GAL4;UAS-Acp62F-IR3C</i> | 432                                     | 6118  | 6.60   |    |                                   |        |

**Table 6.6** Courtship and mating frequencies of females continuously housed with Acp62F null and control males. Chi-square tests were used to test for differences in (a) total mating and (b) total courtship frequencies for females mated to Acp62F null (*Acp62F<sup>lbbB</sup>*, *Acp62F<sup>lbc</sup>*, *Acp62F<sup>lc</sup>*) or control (*Acp62F<sup>Tandem</sup>*, *Acp62F<sup>lcCtrl</sup>*) males.

| Males with which females were housed | Number of matings / courtships observed | Number of matings / courtships not observed | Percentage mating/courting out of the total number of observations | Df | Pearson Chi square test statistic | P       |
|--------------------------------------|---|---|--|----|-----------------------------------|---------|
| (a) Mating                           |   |   |  |    |                                   |         |
| <i>Acp62F<sup>lbbB</sup></i>         | 76                                      | 12394                                       | 0.61   | 4  | 14.29                             | 0.0064  |
| <i>Acp62F<sup>lbc</sup></i>          | 106                                     | 11664                                       | 0.91   |    |                                   |         |
| <i>Acp62F<sup>lc</sup></i>           | 73                                      | 12517                                       | 0.58   |    |                                   |         |
| <i>Acp62F<sup>Tandem</sup></i>       | 98                                      | 12242                                       | 0.8  |    |                                   |         |
| <i>Acp62F<sup>lcCtrl</sup></i>       | 73                                      | 12297                                       | 0.59   |    |                                   |         |
| (b) Courtship                        |   |   |  |    |                                   |         |
| <i>Acp62F<sup>lbbB</sup></i>         | 1278                                    | 11192                                       | 11.42  | 4  | 80.03                             | <0.0001 |
| <i>Acp62F<sup>lbc</sup></i>          | 1212                                    | 10558                                       | 11.48  |    |                                   |         |
| <i>Acp62F<sup>lc</sup></i>           | 1681                                    | 10909                                       | 15.41  |    |                                   |         |
| <i>Acp62F<sup>Tandem</sup></i>       | 1342                                    | 10998                                       | 12.2   |    |                                   |         |
| <i>Acp62F<sup>lcCtrl</sup></i>       | 1338                                    | 11032                                       | 12.13  |    |                                   |         |

## 6.5 Discussion

Taken together, analyses of the longevity, egg laying and egg to adult viability of females mated to males that transfer or did not transfer Acp62F provides no evidence in any of the 3 experiments that Acp62F contributes to the cost of mating in *D. melanogaster* females. In fact, females mated to males lacking Acp62F lived significantly less long than those mated to control males in RNAi experiment 1, which might suggest increased survival in females that received Acp62F. However, this effect was not consistent, because any differences in the survival of females housed with males transferring or not transferring Acp62F in RNAi experiment 2 and the null experiment that did occur were not associated with the transfer of Acp62F. There were also no consistent significant differences in either egg laying or egg to adult viability between females mated to males that did or did not transfer Acp62F.

The results presented here contrast with the suggestion that Acp62F might contribute to the cost of mating suffered by *D. melanogaster* females. Lung et al. (2002) found that ectopic expression of Acp62F, but not the expression of 7 other Acps tested, was toxic to adult flies leading to the suggestion that Acp62F may mediate the cost of mating. However, in that study, ectopic expression of Acp62F introduced more than 50 times the normal mating levels of Acp62F, and in addition, introduced it to novel sites in the female. It is also possible that the process of ectopic expression itself causes disruption of pathways during development that adversely affect survival independently of any effects of the protein under examination. Rather, the results of the experiments presented in this Chapter are consistent with those of Bangham (2003) who found no significant differences in the survival and fecundity of females mated to the same knockdown and control males as I used here in RNAi experiment 2. Therefore, both when mating rates were high (i.e. with added yeast in these experiment) and low (without added yeast, Bangham 2003), no costs of mating could be detected, using Acp62F RNAi knockdown males. Although expression of Acp62F was not detected in knockdown lines in Western analysis, the expression in the *Acp26AaP-GAL4;UAS-Acp62F-IR1b* and *Acp26AaP-GAL4;UAS-Acp62F-IR3A* lines was previously reported as 2-3% of control lines (Lung et al. 2002). This residual expression of Acp62F might have obscured any potential effects of Acp62F on either survival or fecundity. However, this suggestion

is countered by the results from the Acp62F null experiment in which no Acp62F was produced, which shows that, although variation existed between lines, the patterns of survival, egg laying and egg to adult viability did not match those expected under the hypothesis that Acp62F mediates a cost of mating in females. A subsequent expression study found that a further 3 Acps in addition to Acp62F (Acp70A, CG10433, CG8137) but not 18 others tested, were toxic to adults (Mueller et al. 2007). SP has been previously implicated in mediating the cost of mating in fitness assays (Wigby and Chapman, 2005). It will be interesting to establish the mechanism by which these Acps (Acp62F, Acp70A, CG10433, CG8137) cause toxicity upon ectopic expression and examine what effects CG10433 and CG8137 have by normal delivery in fitness assays.

There was some variability in the survival of females between the three experiments. In RNAi experiment 1, females mated to males not transferring Acp62F suffered reduced survival compared to the control line. In this experiment, males in the 3 lines differed in the location of the transgenic insert. These same males, differing in the position of transgenic insert, and an additional control line containing no inserts, were used in RNAi experiment 2. In RNAi experiment 2, no consistent differences were observed in the survival of females mated with males transferring or lacking Acp62F suggesting that Acp62F did not affect female survival. This was also true in the null experiment which, as well as being the best controlled experiment, used experimental males that had the Acp62F gene precisely deleted, and hence provides the strongest evidence that Acp62F is not responsible for the cost of mating.

There was a high degree of variance in egg to adult viability. This variance was mostly attributable to low egg to adult viability of females mated to *Acp62F<sup>1cCtrl</sup>*, but was not attributable to Acp62F levels since females mated to other males not producing Acp62F had egg to adult viability comparable with that of control lines. Overall however, and as reported elsewhere (Mueller 2006), there were no consistent differences in the number of eggs laid or the egg to adult viability of females mated to males that transferred or did not transfer Acp62F.

Differences in the mating frequencies in the Acp62F null experiment were due to an elevated frequency of matings between females mating with *Acp62F<sup>1bC</sup>* males compared to mating frequencies in the other groups. Although females mated to

*Acp62F<sup>lbc</sup>* had reduced survival relative to females mated to other males, it does not alter the conclusion that Acp62F does not affect female survival, since females mated to males from *Acp62F<sup>lhb</sup>*, *Acp62F<sup>Tandem</sup>*, *Acp62F<sup>lcc'ir1</sup>* and *Acp62F<sup>lc</sup>* did not significantly differ from one another in survival.

The function of Acp62F remains unclear. It has been hypothesised that Acp62F could regulate processing of other seminal fluids, such as Acp26Aa and SP, upon transfer to the female (Mueller 2006). However, since no effects of Acp62F have been found in reproductive traits related to these Acps, i.e. receptivity to mating, egg laying or fertility (this work; Mueller 2006), this hypothesis may be incorrect. An alternative explanation is that other Acps, e.g. other serine protease inhibitors, may act in the same pathway as Acp62F. Hence functional redundancy could obscure any phenotype normally attributable to Acp62F. For example, CG8137 is a predicted protease inhibitor that exhibits the same localisation patterns as Acp62F (sperm storage organs, Lung et al. 2002; Ravi Ram et al., 2005). Since they are both toxic upon ectopic expression, show similar location and have the same predicted function (e.g. seminal fluid proteolysis) they may have similar functions (Mueller et al. 2007). Acp62F function may therefore be revealed when double knockout mutants, combining Acp62F and other serine protease inhibitors, are created (Mueller 2006). Interestingly, in the only other work attempting to assign function to Acp62F, using the Acp62F null lines (in their original, unbackcrossed, form), it was found that sperm from males which do not transfer Acp62F were significantly better at resisting displacement from subsequent males to mate than males that did transfer Acp62F (Mueller 2006). In mammals, seminal protease inhibitors are involved in sperm motility and capacitation (references in Mueller 2006) and it might be expected that those of *D. melanogaster* may also play a similar role in affecting sperm storage and sperm competition. This potential role may be an exciting and fruitful line for future enquiry. Future experiments might also explore the possibility of using double deletion mutations to examine the possibility of functional redundancy between protease inhibitors.



## Chapter 7 General discussion of the thesis and future research directions

### 7.1 Introduction – summary of the thesis work

This thesis investigates how selection pressures arising from sexual selection and sexual conflict have shaped the evolution of the *D. melanogaster* mating system. Females of *D. melanogaster* mate with multiple males and store the sperm of those males, thus creating the conditions for sperm competition. Males of many species are known to tailor their ejaculate investment to the level of sperm competition (e.g. Cook and Wedell 1996). The results described in Chapter 3 show that males maintained at different sex ratios, and thus experiencing different levels of sperm competition, evolved different ejaculate depletion patterns, consistent with the predictions of sperm competition theory. Males evolving under female-biased sex ratios were able to maintain higher fertility for longer and showed less depletion of their accessory glands than males that had evolved under a male-biased sex ratio. The importance of the accessory glands in contributing to male and female fitness and as a potential target of selection is also demonstrated in the experiments in Chapter 4. Accessory gland size showed a strong and significant direct response to artificial selection. Males from the large accessory gland lines were found to be more successful in competition with rival males than were males from small or unselected accessory gland lines. No effect of accessory gland selection regime was detected in controlled tests of sperm displacement ability, but those same experiments did reveal significant positive associations between both accessory gland and testis size and sperm displacement ability. My results demonstrate the importance of accessory gland size for the outcome of sperm competition, and suggests that further attention should be paid to the effects of the quantity of seminal fluid proteins on male and female reproductive success. No consistent effects on female fitness of exposure to males with small or large accessory glands were found (Chapter 5). However, I could not fully separate out all the potential competing influences on female fitness; for example, differences in mating frequency and multiple mating between groups may have obscured potential variation in female fitness. This suggests that further investigation is required to establish the effects of exposure to males with different accessory gland sizes, and of seminal fluid quantity, on female fitness.

The accessory glands produce a large number of seminal fluid proteins that induce a range of physiological and behavioural post-mating changes in females (Gillott 2003). Among these changes is the inhibition of female sexual receptivity and an increase in egg laying caused by the action of SP (Chapman et al. 2003a; Liu and Kubli 2003). SP also makes a major contribution to the cost of mating suffered by females as a consequence of multiple mating (Wigby and Chapman 2005) and is thought to mediate sexual conflict. Chapter 6 describes experiments to determine whether another Acp, Acp62F, affects female survival, which might indicate that this protein is an additional potential agent of sexual conflict. Acp62F has previously been shown to be toxic to when ectopically expressed and has been proposed as a further candidate for contributing to the cost of mating in *D. melanogaster* (Lung et al. 2002). The results of my work here suggest in fact that Acp62F does not make a contribution to the cost of mating. This finding suggests that the results of ectopic expression studies need to be interpreted together with the results of fitness tests such as the ones I conducted, in order to gain a full and accurate picture of the effects of Acps.

In the following sections I describe the results of each of the experiments in Chapters 3 to 6, discuss their implications and consider additional questions arising from the findings and how these may provide further insights into the influence of sexual conflict and sexual selection on the mating system in *D. melanogaster*.

## **7.2 Ejaculate depletion patterns in male *Drosophila melanogaster* selected under increased and decreased levels of sperm competition (Chapter 3)**

Measures of ejaculate investment were made in males that had been maintained at male-biased (MB) and female-biased (FB) adult sex ratios, in which levels of sperm competition were high and low, respectively. During selection the frequency of mating and courtship bouts per female were highest in the male-biased lines whereas per male they were higher in the female-biased lines (Wigby and Chapman 2004). Therefore, MB males mate infrequently but face a high risk and intensity of sperm competition when they do mate. MB males should therefore be selected to increase their reproductive success by transferring large ejaculates, particularly in their first matings (Parker 1998). FB males on the other hand are likely to have many more opportunities to mate and when they do will face low sperm competition. Mates of

MB and FB males suffered increasingly reduced fertility with successive matings, but, as expected, the decline was significantly more pronounced for MB than for FB males. MB males showed weaker partitioning of ejaculates and hence suffered ejaculate exhaustion.

There was a corresponding and significant reduction in the size of the accessory glands and testes of males of MB and FB regimes after 5 successive matings. However, the accessory glands, but not testes, of MB males became depleted at a significantly faster rate than those of FB males. The results show that male reproductive traits have evolved in response to the risk of sperm competition and suggest that the ability to maintain fertility over successive matings is associated more strongly with the rate of accessory gland than testis depletion. A decrease in fertility and accessory gland depletion was not related to mating duration or the interval between mating, both of which did not differ between female and male-biased lines. It is not clear however whether males evolving under the different regimes differ in the rate in which Acps are transferred.

Previous studies have shown that males mated in succession have progressively more depleted accessory glands and become increasingly less able to elicit post-mating responses in females (Hihara 1981). The associated drop in fertility is caused by the depletion of the accessory gland and not the testis, which retain motile sperm (Hihara 1981). There was also no difference in the size of the testes between female and male-biased lines in Chapter 3 either before or after matings, although successive matings were found to reduce testis size, similar to the finding for accessory gland size. The results provide evidence of the importance of the quantity of accessory gland proteins transferred to females in determining male and female fitness.

Future work could examine whether male-biased males are also selected to have higher competitive ability. Male-biased males have to compete with many rival males to fertilise females both before and after mating. MB males might be expected to be more aggressive, both towards other males and in their courtship of females, and to court females more frequently. Therefore, placing male-biased and female-biased males in a competitive environment and assaying male aggressiveness, mating and courtship attempts, or placing them in sperm precedence studies, would

further elucidate the mechanisms of sperm allocation. As well as evolving in their patterns of ejaculate depletion patterns, males may also vary in their plastic response to sperm competition. It would be interesting for example to place males from the male and female-biased lines in the presence or absence of other males and test their post-mating success. Such a simple set up could establish whether differences exist in the ejaculate volume (both sperm and Acp) delivered under varying sperm competition risk and intensity, and whether differences exist between the lines. Additional measures of male mating traits could also elucidate the mechanisms underlying the responses of males to sperm competition. For examples, do male-biased males transfer more ejaculate more quickly than female-biased males? This question could be addressed by using Enzyme Linked ImmunoSorbent Assays (ELISA; see also section 7.3) to quantify and assess the rate of Acp transfer. This approach can also be related to post-mating responses in females, for example, to test whether male-biased males induce a greater response in egg laying following single matings or whether they induce longer periods of female refractoriness to mating.

It might also be expected that different Acp variants that are more or less effective under competition will have increased or decreased in frequency in the male or female-biased lines respectively. This could be tested by asking whether associations exist between relative success in sperm competition and the sequences of candidate Acps. In addition, microarray analysis across all Acps, or qPCR on candidate Acps, could be employed to test whether the expression of Acps differs between the lines to determine in what respects male Acp expression patterns evolve in response to sexual conflict and sperm competition.

It would also be interesting to examine the evolutionary responses of females in these lines. Sexual conflict theory predicts that females should counter-adapt to minimise male-induced harm by reducing mating frequencies or evolving physiological resistance to harmful traits (Holland and Rice 1999; Wigby and Chapman 2004). In contrast to the role of sexual conflict in *Sepsis cynipsea* (Martin and Hosken 2003), Wigby and Chapman (2004) found no evidence in these lines that females evolve resistance to male-induced harm by reducing their willingness to mate. Although females that had been maintained at high conflict (male-biased lines) were not less likely to remate than females from low conflict lines (female-

biased lines), females may evolve resistance in response to the harmful effects of SP or other Acps. These selection lines could be used further to test whether females evolving under high conflict are more resistant to males with increased accessory gland size (that may transfer more Acps) and/or males that lack specific proteins, such as SP, than females evolving under low conflict.

### **7.3 Accessory gland size responds to selection and is associated with male reproductive success (Chapter 4)**

Chapter 4 examined the contribution of male reproductive morphology to male reproductive success. I documented the response to replicated bi-directional artificial selection on male accessory gland size. Accessory gland size showed a clear and significant response to selection with males in 'large' lines having significantly larger accessory glands than males in 'small' lines. These changes were independent of testis and body size, which did not differ significantly between the regimes.

The nature of the response is particularly interesting in light of the potential importance of the role of the accessory gland in determining male post-copulatory success that I discussed in Chapter 3. In mating systems in which females mate multiply and store sperm, selection is expected to act upon morphological and behavioural traits in males that maximize success in post-copulatory competition. Among the traits contributing to male success in sperm competition are the Acps that are passed from the male accessory gland during mating. While the effect of a number of individual Acps are known, for example, in reducing female receptivity and increasing egg production and sperm storage, less is known of the functional significance of the size of the accessory glands themselves. The response to selection on accessory gland size, and the consequences for male competitive ability, suggests that the quantity of Acps is an important determinant of male reproductive success and also that accessory gland size may be an important target of selection in mating systems where males must compete in sperm competition.

When in competition with other males, males with larger accessory glands sired significantly more offspring than did males from the small or unselected lines, in the absence of any significant differences in mating frequency. One likely explanation for this is the transfer of greater quantities of Acps by males with larger accessory

glands. If all or some Acps act in a dose-dependent way, males with larger accessory glands would therefore realize greater reproductive success. One way to assess whether Acps are being transferred in greater quantity by large accessory gland males, and hence to understand more directly the potential increased reproductive success of such males, is to utilize ELISAs to detect and quantify the transfer of Acps. This technique is currently being used in a collaboration with Laura Sirot and Mariana Wolfner (Cornell University) to test whether large and small accessory gland males from my selection lines differ in the amount of SP and ovulin (Acp26Aa) they transfer during mating to females. This approach could also reveal whether males with larger accessory glands transfer greater quantities of Acps because they have more of them available or because they are transferred at a faster rate. Using this technique with a larger range of Acp antibodies, it should be possible to establish whether for example, males that transfer greater amounts of specific sperm storage proteins such as Acp36DE (Neubaum and Wolfner 1999) have more sperm stored by their partners and have greater success in sperm competition.

No significant differences between males from the large and small accessory gland regimes in sperm displacement ability in a non-competitive situation were found, but there were overall significant positive associations between accessory gland and testis size and sperm displacement ability. The reason that a significant difference was not found when comparing between the different selection regimes may reflect variability within the lines or replicates. Given also the highly variable nature of sperm precedence, more accurate correlations between traits such as sperm displacement and Acp transfer will be possible by quantifying the Acps using the ELISA technique. Experiments are also currently being performed to test whether males from the large and small accessory gland selected lines differ significantly in the amount, or rate of transfer, of Acps to females during single matings in competitive and non-competitive situations. These experiments will reveal whether males from the different lines transfer more Acps when they are in the presence of rival males and whether the ratio of different Acps transferred to females can change.

As well as probing further mechanisms through which males with larger accessory glands realize greater post-mating success, premating differences between males

from the different lines may also exist. Presumably there is some trade-off/cost to having larger accessory glands since we might otherwise expect all males to have large glands. Development time or time to sexual maturity are potential characters that could trade off against accessory gland size, as occurs in stalk-eyed flies (Baker et al. 2002). It will also be interesting to see how females from these lines have responded to selection. Males selected for large accessory glands may have greater productivity with females from their own lines with which they have coevolved than they have with females from the other lines.

#### **7.4 The effect of accessory gland size on the survival and fitness of female *Drosophila melanogaster* (Chapter 5)**

Females suffer a cost of mating through mating multiply (Fowler and Partridge 1989) which has been shown to be mediated by the accessory gland proteins transferred by males (Chapman et al. 1995) and in large part by the action of SP (Wigby and Chapman 2005). In addition, SP causes an increase in egg laying and induces females to be unreceptive to mating. Other Acps are involved in the efficient transfer and storage of sperm and later in the utilization of that sperm in fertilizing eggs and in ensuring that it is not displaced by the ejaculates of rival males. Where the functions of Acps have been determined, this has mostly been achieved by looking at the phenotypic responses in the presence or absence or addition of the Acp under investigation. There is also evidence that the quantity of Acps is important in influencing the post-mating responses in females and in determining male and female fitness (Hihara 1981; Chapter 4). If the quantity of Acps is important in determining male and female fitness, we should expect that females mated to males with larger accessory glands would perhaps suffer reduced survival but have greater egg laying and fertility. The experiments in Chapter 5 utilised males from the lines selected for large and small accessory gland size (described in Chapter 4) and examined the effect of exposure to these males on female survival and fitness.

The results show that although females housed with large accessory gland males had significantly shorter lifespans than those housed with small males in both replicates, the survival of females housed with unselected males was variable, being significantly shorter lived than either of the other groups in replicate 1, but not

significantly different from females housed with small males in replicate 2. One important contributing cause for this inconsistency may have been differences in mating frequency between groups. Males in the unselected group in replicate 1 mated more frequently than males in the other groups. If the effect of SP, or other cost causing agents, is dose dependent, it would be expected that in this group females would suffer relatively reduced survival. This would not be predicted to be as strong an effect in females mated to males with smaller accessory glands since such males may transfer only small amounts of Acps at each mating. They would therefore have a smaller effect on female survival for any given mating rate than males with large accessory glands. To clarify whether the quantity of seminal fluid proteins has an effect on female survival the males selected for large and small accessory gland size could be used in assays that controlled the number of matings, for example mating females at regular intervals during female lifespan. Correspondingly, quantitative Western blot or ELISA analysis could also be used, to confirm whether males with larger accessory glands transfer larger quantities of specific Acps (i.e. SP) and whether Acps are passed more rapidly than by males with small accessory glands. These experiments, with additional complementary fitness assays, could further reveal the importance of accessory gland size as a trait influencing male and female fitness, and the mechanisms by which Acps influence female reproductive physiology and behaviour.

Although males with larger accessory glands are able to mate more frequently (Bangham et al. 2002), they are also expected to induce longer refractory periods in females. This may explain why females mated to males with larger accessory glands mated less often than females in the other groups. Females housed with males with large glands were courted more frequently than females housed with males with small or unselected accessory glands suggesting that these males with larger glands were willing to mate more frequently. Mating assays that provide males with sequential mating opportunities as employed elsewhere (Bangham et al 2002; Rogers et al 2005) will be required to confirm that males with larger accessory glands are able to mate more frequently.

There was no consistent pattern of differences in egg laying or egg to adult viability of females mated to males from the different lines. The transfer of greater quantities of Acps by males with larger accessory glands might have been expected to elicit



larger post-mating responses, including increased egg laying or increased egg to adult viability. However, since large mating differences existed between groups, this may have obscured any dose-dependent effects of the Acps. The patterns of egg laying and egg to adult viability may have been further complicated by the interaction of competing male ejaculates. For example, although males transferring larger ejaculates may transfer greater quantities of Acps that assist in sperm storage, rival males may transfer larger quantities of Acps involved in sperm displacement. It is also possible that sufficient sperm and Acps were transferred during multiple mating making it impossible to detect differences in egg laying and egg to adult viability. Performing controlled single or multiple matings, and looking for post-mating responses in individual females, could help to resolve the effects of the transfer of greater quantities of Acps. The response of selection line females to individual Acps could be assessed by using RNAi/knockdown males to establish evolved responses in females, such as whether females from large lines differ in their response to the presence or absence of SP. Females from large lines may, for example, suffer less from the transfer of SP. Selection line females could also be used to investigate whether females evolving where they receive larger quantities are able to detoxify harmful Acps.

## **7.5 The effect of Acp62F delivery on female lifespan and reproductive success (Chapter 6)**

Female mating costs are mediated by male Acps (Chapman et al. 1995). Recent work has identified SP as being a major contributor to these costs. Acp62F has also been proposed as a candidate for the cost of mating after the finding that it is toxic when ectopically expressed. Chapter 6 uses transgenic males expressing undetectable levels of Acp62F through RNAi and males with a null mutation for Acp62F to test the effects of Acp62F on female longevity and fecundity. The results suggest that Acp62F is not responsible for the cost of mating.

Although no Acp62F protein was detectable in males where RNAi reduced expression of the protein, it is possible that there are small residual levels of Acp62F that could be transferred to females. The possibility that Acp62F may contribute to physiological changes in females, including having effects on female survival could not then be excluded. However, this scenario seems unlikely in light of the results of

the experiment using males that are null for Acp62F. Just as in the RNAi experiments, no differences in female survival were found that could be attributed to the presence or absence of Acp62F. A further possibility is that mating frequency may not have been high enough to detect an effect on survival. However, this would suggest that the contribution of Acp62F to the cost of mating is either very small, or at least makes a small contribution compared to SP (Wigby and Chapman 2005) and could be avoided by females simply not mating at very high frequencies. The results of both experiments provide no evidence that Acp62F mediates the cost of mating in females.

The demonstration of direct mating costs that result from the receipt of SP suggests that the *SP* gene is under the influence of sexual selection and sexual conflict. The SP therefore represents a proximate mechanism underlying conflict between the sexes in *D. melanogaster*. It will also be interesting to establish whether the other Acp loci (CG10433 and CG8137) that have proven to be toxic to adults upon ectopic expression are also harmful to females as a result of normal mating, and therefore establish whether SP alone causes mating costs. Having identified the genes involved in this conflict the challenge remains to whether such female-harm genes have caused correlated responses in female resistance genes, thus demonstrating evidence of sexually antagonistic coevolution. In addition the mechanisms by which SP (and any other factors) causes harm are now being investigated. For example, SP elevates oogenesis in females by stimulating the release of juvenile hormone (Moshitzky et al. 1996). JH has been implicated in lowering immunity and therefore represents a potential mechanism by which female costs may be induced.

The function of Acp62F remains unclear. Although it has been hypothesised that Acp62F could regulate processing of other seminal fluids, such as Acp26Aa and Acp70A, upon transfer to the female (Mueller 2006), no effects of Acp62F have been found in reproductive traits related to these Acps. Future exploration of the function of Acp62F might investigate potential functional redundancy between Acp62F and other Acps transferred, particularly other serine protease inhibitors. This might be achieved if double deletion mutations could be generated. Sperm from males that do not transfer Acp62F have been found to be significantly better at resisting displacement from subsequent males to mate than males that did transfer

Acp62F (Mueller 2006). Further investigation will be required to clarify and probe this mechanism and other possible functions of Acp62F. Furthermore, other reproductive traits associated with the predicted function of Acp62F, such as sperm longevity and male fertilisation success could be compared in females mated to either males transferring Acp62F or males lacking Acp62F. For example, the effect of Acp62F on sperm longevity could be assessed by performing single matings between wild-type females and males null or control for Acp62F. Female storage organs could then be dissected and motile sperm counted at regular intervals following mating to assess sperm longevity. In parallel, undissected females could be kept to test the effect of Acp62F on egg fertility.

The results presented in this thesis offer insights into the mechanisms underlying the mating system of *D. melanogaster*. I investigated how reproductive traits, strategies and morphologies have been shaped by sexual selection and sexual conflict and present results that add to an extensive base of sperm competition theory. Female promiscuity has potent consequences for males. I have demonstrated the strength of this selection pressure in driving evolutionary change and the importance of male reproductive structures particularly in determining male success in sperm competition.

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**Appendix I. Ejaculate depletion patterns evolve in response to experimental manipulation of sex ratio in *Drosophila melanogaster*. Evolution.**

**Appendix II. Mating and Immunity in Invertebrates. Trends in Ecology and Evolution 22:48-55.**