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The genetic basis of sexually selected interactive phenotypes

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I. **Pembury Smith, M. Q. R.** and Snook. R. The impact of indirect genetic effects on sperm ejection: how partner genotype influences shared sexually selected traits. *Manuscript.*
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Candidate contributions to thesis articles*

[#]Candidate contributions to thesis articles

	I	**II**
Conceived the study	**Minor**	**Minor**
Designed the study	**Significant**	**Significant**
Collected the data	**Substantial**	**Substantial**
Analysed the data	**Substantial**	**Substantial**
Manuscript preparation	**Substantial**	**Substantial**

[#]Contribution explanation

Minor: contributed in some way, but contribution was limited.

Significant: provided a significant contribution to the work.

Substantial: took the lead role and performed the majority of the work.

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Summary

In polyandrous mating systems, both pre- and post-copulatory traits determine the reproductive success of an individual. Many traits that arise from either episode of sexual selection cannot be defined outside the context of a reproductive interaction. Such traits are examples of interactive phenotypes, in which the total genetic variation that can respond to selection depends on partner genotype (indirect genetic effects; IGE). Additionally, when these traits describe the duration of a reproductive interaction, the phenotypic value expressed will be the same in each sex. As the phenotypic optima rarely aligns between the sexes, such interactive phenotypes are often antagonistic. Theoretical models have predicted that IGEs influence the outcome of sexual conflict as well as the evolutionary potential of a given trait. However, few studies have examined the impacts of IGEs on interactive antagonistic pre- and post-copulatory traits. In this thesis, I conducted a quantitative genetic study on three antagonistic sexually selected interactive phenotypes that represent sequential stages of the reproductive process. In all three traits, only a single shared phenotype is expressed in both sexes, despite the outcome likely mediated by sex-specific traits. ***Chapter I*** focuses on the post-copulatory sexually selected trait sperm ejection, which describes the amount of time during which sperm is retained in the female reproductive tract after copulation, influencing the outcome of competitive fertilisation. ***Chapter II*** examines the pre-copulatory trait mating latency (which acts as a proxy for sexual attractiveness) and copulation duration. Additionally, using data from ***Chapter I***, the phenotypic and genetic correlations between all three traits were examined. We found that sperm ejection and mating latency are heritable, and that direct and IGEs underly their phenotypic variation, suggesting that the evolutionary dynamics of these traits are likely influenced by partner genotype via sexually antagonistic coevolution. In comparison, we found limited evidence that copulation duration is influenced by IGEs despite showing that both male and female genotype individually influence phenotypic variation. We also observed significant phenotypic correlations between traits but weak evidence of additive genetic correlations, suggesting that episodes of selection may act independently allowing individual traits to evolve separately. Together, our findings demonstrate the quantitative genetic basis behind phenotypic variation in interactive traits subject to sexual conflict, and the potential relationship between pre- and post-copulatory episodes of selection.

Sammanfattning

I polyandriska parningssystem avgör både pre- och postkopulatoriska egenskaper individens reproduktiva framgång. Många egenskaper som uppstår som en konsekvens av pre- och postkopulatorisk sexuell selektion kan endast definieras i kontexten av en reproduktiv interaktion. Sådana egenskaper är exempel på interaktiva fenotyper, där den totala genetiska variationen som kan svara på selektion är beroende av partnergenotypen (indirekta genetiska effekter; IGE). Dessutom, när dessa egenskaper beskriver varaktigheten på en reproduktiv interaktion, kommer det fenotypiska värdet som uttrycks att vara detsamma i båda könen. Eftersom fenotypiska optimum sällan matchar båda könen, är sådana interaktiva fenotyper ofta antagonistiska. Enligt teoretiska modeller påverkar IGE både utfallet av sexuell konflikt såväl som den evolutionära potentialen för en given egenskap. Men få studier har undersökt effekterna av IGE på interaktiva antagonistiska pre- och postkopulatoriska egenskaper. I denna licentiatavhandling genomförde jag en kvantitativ genetisk studie på tre sexuellt selekterade antagonistiska interaktiva fenotyper som representerar steg i reproduktionsprocessen. I alla tre egenskaper uttrycks endast en gemensam fenotyp i båda könen, trots att utfallet troligtvis påverkas av könspecifika egenskaper. ***Kapitel I*** fokuserar på den sexuellt selekterade postkopulatoriska egenskapen spermie-ejektion, som beskriver den tid under vilken

spermier behålls i den honliga reproduktionskanalen efter kopulation, vilket i sin tur påverkar utfallet av befruktning under konkurrens. ***Kapitel II*** undersöker den prekopulatoriska egenskapen parningslatens (som fungerar som en proxy för sexuell attraktivitet) och kopulationsvaraktighet. Dessutom undersöktes fenotypiska och genetiska korrelationer mellan alla tre egenskaper med data från ***Kapitel I***. Vi fann att spermie-ejektion och parningslatens är ärftliga och att både direkta genetiska effekter och IGEs ligger bakom deras fenotypiska variation, vilket antyder att de evolutionära dynamikerna för dessa egenskaper troligtvis påverkas av partnergenotypen genom sexuellt antagonistisk samevolution. Jämförelsevis fann vi begränsade belägg för att kopulationsvaraktighet påverkas av IGE trots att vi visade att både hanars och honors individuella genotyper påverkar fenotypisk variation. Vi observerade också signifikanta fenotypiska korrelationer mellan egenskaper men svaga belägg för additiva genetiska korrelationer, vilket antyder att selektionsepisoder kan verka oberoende och möjliggöra att enskilda egenskaper utvecklas separat. Tillsammans visar våra resultat den kvantitativa genetiska grunden bakom fenotypisk variation i interaktiva egenskaper under sexuell konflikt samt den potentiella relationen mellan pre- och postkopulatoriska episoder av selektion.

Introduction

Interactive phenotypic framework

Understanding the evolution of behaviour is complex as its flexibility and environmental sensitivity makes it difficult to empirically determine the acts of selection (Bailey, Marie-Orleach and Moore, 2018). Specifically, traits that are only expressed during social interactions can be hard to define as explicit quantitative phenotypes, as they are determined, at least in part, by a conspecific individual (Moore, Brodie III and Wolf, 1997). Such traits, known as "interactive phenotypes", include behaviours that are influenced by the phenotype of a conspecific, such as learning (Agrawal, 2001), movement (Signor *et al.*, 2017a, 2017b), and egg laying rate (Brommer, Rattiste and Wilson, 2008). Other interactive phenotypes cannot be defined outside the context of an interaction, such as aggression (Camerlink *et al.*, 2013; Saltz, 2013; Anderson, Scott and Dukas, 2017), cooperation (Crespi, 2001; Edenbrow *et al.*, 2017) and predator-prey interactions (Bleakley and Brodie III, 2009). The latter also includes phenotypes that represent characteristics of an interaction where only a single shared phenotype can be measured in both conspecifics (Dingemanse and Araya-Ajoy, 2015), such as the latency to cannibalisation (Bleakley *et al.*, 2013) or mating (i.e. mounting latency in Bailey and Zuk, 2012).

In a standard quantitative genetics framework, phenotypes of a focal individual are partitioned into direct genetic and environmental effects (Falconer, 1996) (Figure 1 A). Here, the environmental effects describe non-genetic abiotic factors. However, interactive phenotypes are cases in which simple evolutionary models are inappropriate. During a social interaction, the genotype of the conspecific represents a component of the focal individual's environment (Moore, Brodie III and Wolf, 1997). If the conspecific genotype is variable and influences focal phenotype, the environmental component of focal trait expression can itself be heritable and evolve (Wolf, Brodie III and Moore, 1999; McGlothlin *et al.*, 2010). As a result, when determining the genetic architecture of an interactive phenotype, quantitative models must incorporate indirect genetic effects (IGEs) which describe the influence of interacting genotypes on a focal phenotype (Moore, Brodie III and Wolf, 1997; Wolf, Brodie III and Moore, 1999; Santostefano *et al.*, 2017). IGEs can alter evolutionary trajectories, resulting in phenotypes different to those predicted by traditional quantitative models (Moore, Brodie III and Wolf, 1997; McGlothlin *et al.*, 2010) as they can increase or decrease both trait evolution and trait variance (Bailey and Moore, 2012). As a result, identifying IGEs and studying their impact on evolutionary dynamics is required when describing the inheritance, evolution, and maintenance of adaptive variation in interactive phenotypes.

Recent quantitative genetic analyses have begun to model the effects of IGEs when examining the evolution of interactive phenotypes. Two main theoretical approaches have been used (McGlothlin and Brodie III, 2009; Baud *et al.*, 2022): Variance Partitioning and the Trait-Based Approach. The

former quantifies the magnitude with which IGEs influence a focal phenotype relative to direct genetic effects (reviewed in Cheverud, 1984). The latter approach uses a model to describe how the phenotype of a focal individual is influenced by an interaction with a conspecific (Moore, Brodie III and Wolf, 1997; Bijma, 2014). Using this approach, the focal individual's phenotype can be partitioned into:

$$z_i = a_i + e_i + \Psi z_j'$$

where

$$z_j' = a_j + e_j$$

Here z_i denotes the interactive phenotype of focal individual i, a_i the additive genetic effects, e_i the general environmental effects and z_j' the phenotype of the interacting individual which is, in turn, made up of the additive genetic effects a_j and the general environmental effects e_j of interacting individual j. The interaction coefficient Ψ describes the effect of the j partner's phenotype on the phenotype (z_i) of the focal individual i. It therefore outlines the strength and direction in which an interactive phenotype changes as a consequence of the genes expressed by a social partner (IGEs; Figure 1 B). For example, if Ψ is zero the interactive phenotype in the focal individual is unaffected by IGEs, whereas if $\Psi > 0$ it increases trait expression and *vice versa.* Interestingly, empirical work has shown that Ψ can vary by genotype (Kent *et al.*, 2008; Bleakley and Brodie III, 2009; Bailey and Zuk, 2012; Marie-Orleach *et al.*, 2017), sex (Edenbrow *et al.*, 2017) and environment (Signor *et al.*, 2017b). Ψ can therefore be used to make evolutionary predictions about the effects of IGEs across generations.

The importance of IGEs when investigating sexually selected traits

Sexually selected traits are interactive phenotypes as they arise from an interaction between two reproductive partners. Subsequently, when investigating the link between genotype and phenotype in order to examine trait evolutionary potential, IGEs must be identified, as this can change inferences on the causes of variation. For example, the lek paradox describes the phenomenon whereby substantial additive genetic variance is observed in many traits despite the expectation of strong directional sexual selection (Kirkpatrick and Barton, 1997; Qvarnström, Brommer and Gustafsson, 2006; Miller and Moore, 2007; Danielson-François, Zhou and Greenfield, 2009; Bailey and Moore, 2012). One explanation is that IGEs can result in selection based on genetic compatibility between copulating genotypes, producing indirect genetic benefits which drive non-directional trait selection. As a result, IGEs can contribute to the maintenance of genetic variation for a given sexually selected trait (Miller and Moore, 2007).

IGEs also play a critical role in sexually selected traits that mediate sexual conflict (Moore and Pizzari, 2005). Traditional models of sexual selection have predicted that the evolution of sexually selected traits occurs via mutual coevolution as it selects for genes that confer an overall reproductive advantage to both partners (Andersson, 1994). However, as the reproductive interests of the sexes rarely align, many sexually selected interactive phenotypes are antagonistic traits where phenotypic expression increases fitness in one sex whilst simultaneously reducing partner fitness (Arnqvist and Rowe, 2005). The "chase-away hypothesis" (Holland and Rice, 1998) predicts that antagonistic trait expression can stimulate cyclical evolution of adaptations and counteradaptations. Theoretical models of sexual conflict in interactive phenotypes using a quantitative genetic perspective have shown that IGEs can stimulate the rapid evolution of multiple antagonistic traits above standard predicted rates, even in the absence of additive genetic variation (Moore and Pizzari, 2005). Therefore, understanding the indirect genetic basis of an interactive antagonistic trait can provide additional insights into the role of selection on such traits, how this influences the evolutionary trajectories of the sexes, and how this may also contribute to the maintenance of trait variation in a population.

Applying an interactive framework to sexually selected behavioural traits

Recent work utilising an interactive phenotypic approach has examined IGEs on sexually selected trait expression by deriving Ψ. For example, the IGE of female genotype has been found to underly variance in sexually selected male cuticular hydrocarbon profile in *Drosophila* (Petfield *et al.*, 2005; Kent *et al.*, 2008), and male body mass and advertisement song in the lesser waxmoth *Achroia grisella* (Danielson-François, Zhou and Greenfield, 2009). Interestingly, population-level variation also appears to influence the strength of IGEs. For example, the direction and strength of IGEs on female choosiness varied between different geographically isolated populations of the field cricket *Teleogryllus oceanicus* (Bailey and Zuk, 2012).

Researchers have also quantified the IGE on antagonistic interactive traits. For example, in *D. melanogaster*, the genotype of male mating partners significantly influenced copulation duration (Edward *et al.*, 2014), a trait thought to mediate sexual conflict. Additionally, the hermaphroditic flatworm, *Macrostomum lignano*, performs a post-copulatory sucking behaviour to remove components of the ejaculate from storage after copulation (Schärer, Joss and Sandner, 2004; Vizoso, Rieger and Schärer, 2010). As the timing of this behaviour influences fertilisation success, selectively removing the ejaculate benefits the sperm recipient via cryptic "female" choice, at a cost to the sperm donor. The propensity of this sucking behaviour has been shown to be dependent on the genotype of the sperm donor and sperm recipient (Marie-Orleach *et al.*, 2017). As a result, genetic variance in both the ability resist sucking behaviour and propensity to suck suggests that phenotypic outcome is heavily influenced by IGEs and has likely evolved via sexually antagonistic coevolution (Marie-Orleach, Janicke and Schärer, 2013; Marie-Orleach *et al.*, 2017).

The challenges when using an interactive framework to examine sexually selected behavioural traits

While empirical work has begun to investigate the underlying quantitative genetic basis of interactive sexually selected phenotypes, a number of challenges remain. First, when examining behaviours that arise as a product of a sexual interaction, traditional interactive phenotypic models cannot be used to derive Ψ. This is because Ψ is calculated by regressing the phenotypic value in one individual onto the value of a separate measured phenotype expressed in the interacting partner. However, when the trait of interest is the duration of a reproductive interaction between two individuals (i.e. copulation duration), the phenotypic value measured is the same for each individual. When this occurs, Ψ must be derived in a different manner according to the relationship:

$$z_k^m = a_k^m + e_k^m + \Psi a'^f_j$$

Here, we examine to what extent interactive phenotypic expression (z_k^m) in a focal male genotype k is influenced by the genotype of its female partner j. z represents an interactive phenotype which is the product of an interaction between a focal male, k, and an interacting female, j. a_k^m represents the additive genetic effects and e_k^m the environmental effects of the focal male k, with a'^f_j defining the additive genetic effects of the interacting female partner j. Ψ is therefore the interaction coefficient, outlining the extent to which interacting partner genotype influences the phenotypic outcome in the focal individual (Figure 1 C; Figure 2 B). As this is a shared interactive trait, this can be examined reciprocally in each sex (Figure 1 D; Figure 2 B). As a result, quantifying the extent to which IGEs influence interactive phenotypes when only a shared phenotype is expressed provides valuable insights on trait evolution. However, to date this has not been examined.

A second challenge lies in the fact that, although Ψ can be calculated irrespective of specific partner traits through which IGEs are mediated, undetected latent variables may influence estimates of Ψ and therefore conclusions about the influence of IGEs (Bailey and Hoskins, 2014). Polyandry, in which a female copulates with multiple males within one reproductive cycle, generates intense pre-copulatory selection for traits that control mating success (Andersson, 1994). This mating system also drives post-copulatory selection for traits that influence successful fertilization (Parker, 1970; Eberhard, 1996; Birkhead and Pizzari, 2002) via sperm competition and/or cryptic female choice (Eberhard, 1996;

Snook and Hosken, 2004). Therefore, to ascertain the evolutionary potential of sexually selected interactive phenotypes, the total strength of selection on any given trait depends on the phenotypic and genetic relationship between pre- and post-copulatory traits. When investigating the phenotypic relationship, the phenotype-linked fertility hypothesis predicts a positive correlation between pre- and post-copulatory fitness if male secondary sexual characteristics reflect their fertility (Sheldon, 1994). Alternatively, a negative phenotypic correlation between pre- and post-copulatory fitness may be observed if there is a trade-off between investing in secondary sexual characteristics (in order to acquire matings) and ejaculate components (in order to increase fertilisation success) (Parker and Pizzari, 2010), known as sperm competition game theory (Simmons, Lüpold and Fitzpatrick, 2017). If there is positive genetic relationship between pre- and post-copulatory fitness (due to pleiotropy and/or linkage between traits), this results in correlated changes between phenotypes, enhancing trait evolutionary potential (Kvarnemo and Simmons, 2013), whereas negative genetic relationships slow the rate of evolutionary change (Nelson and Crone, 1999). Therefore, in order to accurately determine the consequences of sexual selection under polyandry, investigating both phenotypic and genetic relationship between pre- and post-copulatory traits is required as it has the potential to alter coevolutionary responses (Walsh and Lynch, 2018).

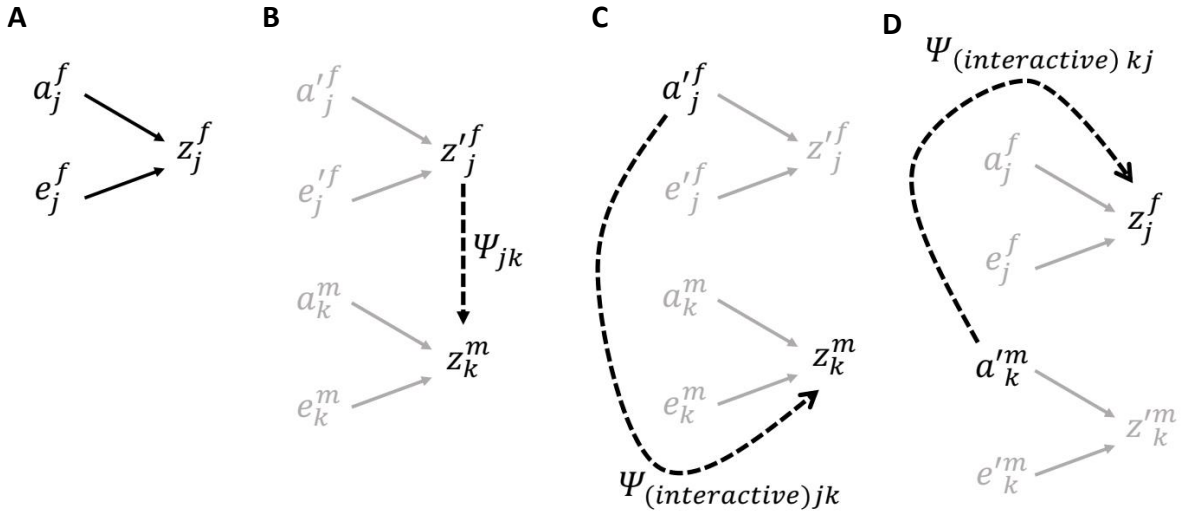

Figure 1. Path diagram depicting quantitative genitive analysis of noninteractive (A), interactive (B), and shared interactive (C & D) phenotypes. Variables associated with a male individual are shown with the m superscript, and variables associated with a female are shown with the f superscript. Variables associated with an interacting individual are denoted with an apostrophe. (A) Noninteractive phenotype, where the phenotype of female individual j (z_j^f) is determined by additive genetic effects (a_j^f) and the environment (e_j^f) (adapted from Moore et al. 1997). (B) Interactive phenotype, where the phenotype of a focal male k (z_k^m) is influenced by additive genetic and environmental effects but also by the (non-shared) phenotype of an interacting female (z'^f_j). The magnitude of this interaction is denoted by Ψ which is a partial regression coefficient obtained from regressing focal male phenotype (z_k^m) on the phenotype of his partner (z'^f_j). (C) Interactive phenotype, where the phenotype examined in the focal and interacting individual share the same value. As a result, you cannot regress the phenotype of one individual onto a separate phenotype in another individual. Instead, measurements of the interaction coefficient can be derived by examining effects arising from additive genetic effects of the interacting partner (in this case the female a'^f_j) on the shared phenotype in a focal sex (in this case the male z_k^m). The reciprocal of this analysis (D) can also be derived.

Aims

Using a quantitative genetics approach, we investigate how direct genetic effects and IGEs influence interactive phenotypic expression and variance in the promiscuous species *D. melanogaster.* In this species, the formation of structured ejaculates, described as a mating plug, is a critical component of reproduction as it acts as a mechanism to retain sperm in the uterus and therefore facilitate efficient sperm storage (Parker, 1970; Schneider, Atallah and Levine, 2017). However, only around 10-20% of sperm is stored, with a white sac comprising of both the sperm mass and the mating plug ejected by

the female after copulation (Lee *et al.*, 2015). The timing of this ejection (described as sperm ejection) is a mediator of post-copulatory sexual selection (Snook and Hosken, 2004; Manier *et al.*, 2010; Lüpold *et al.*, 2013; Firman *et al.*, 2017). Moreover, it is an interactive phenotype in which only a single shared phenotype can be measured in both sexes, despite the outcome likely mediated by sex-specific traits (Dingemanse and Araya-Ajoy, 2015). Longer plug retention provides more time in which sperm can be stored in the female's sperm storage organs, increasing paternity success (Manier *et al.*, 2010, 2013; Lüpold *et al.*, 2013, 2020). Therefore, ejection time is thought to be an intrinsic source of sexual conflict as phenotypic optima may differ between the sexes (Arnqvist and Rowe, 2005; McDonough-Goldstein, Pitnick and Dorus, 2022). Although recent investigations have shown a significant female-by-male genotypic interaction underlying variation in this phenotype (Lüpold *et al.*, 2020), the extent to which IGEs influence phenotypic variation has not yet been quantified.

Mating latency – the time from a pair being introduced to copulation beginning – is a pre-copulatory sexually selected trait which is inversely proportional to mating rate (Fulker, 1966; Jennions and Petrie, 1997). Increased mating rate increases male fitness, driving strong directional selection for traits that reduce latency (Arnold and Duvall, 1994). In contrast, females are expected to have an optimal mating rate (Arnqvist and Nilsson, 2000) that may not align with that of her partners, resulting in an evolutionary conflict between the sexes over mating latency (Holland and Rice, 1998; Arnqvist and Nilsson, 2000). Additionally, copulation duration – the length of time from the male copulatory organ entering the female until the male and female disengage – has been shown to influence paternity via mate guarding (Parker, 1970; Alcock, 1994), altering female post-mating behaviour (Chapman *et al.*, 1995), and facilitating the removal of rival sperm (Parker, 1970). As such, there may be selection on males to prolong copulation duration beyond what is optimal for females, resulting in antagonistic selection in both sexes to control duration. However, the extent with which direct and IGEs influence phenotypic variation in both traits is still debated.

In this thesis we investigate the extent to which direct and IGEs underly variation in pre- and post-copulatory sexually selected traits using a novel diallel quantitative genetics approach. To acquire these data, we crossed 11 isofemale lines from the *D. melanogaster* Genetic Reference Panel (DGRP; Mackay *et al.*, 2005; Huang *et al.*, 2014) in a full diallel mating design excluding reciprocal crosses (Figure 3). For each cross, mating latency, copulation duration (*Chapter II*) and ejection time (*Chapter I*) were measured (Figure 3).

In *Chapter I* we investigate the extent to which direct and IGEs underly variation in the post-copulatory sexually selected trait sperm ejection. Direct genetic effects are derived by identifying significant line-specific and sex-specific phenotypic responses (Figure 2 A). Line-specific (additive) effects ("l" in Figure 2 A) describes to what extent the overall mean phenotypic value of each genotype differs from the population average, independent of sex. It is therefore the additive effect of a genotype when male and when female on phenotypic outcome. Sex-specific effects ("s" in Figure 2 A) describe to what extent the mean phenotypic value of each genotype differs when it is male or female. To examine the potential for IGEs, we modify traditional models that calculate Ψ to derive values for an interactive trait in which both the male and female share the same phenotypic value (Figure 1 C & D). Ψ describes to what extent the overall mean phenotypic value of a given focal genotype of a given sex differs (positively or negatively) to the phenotype expressed when it is crossed with a specific interacting genotype of the opposite sex (Figure 1 C & D; Figure 2 B). In doing so we identify the capacity with which IGEs influence an antagonistic post-copulatory sexually selected trait.

To examine the total strength of selection on any given trait, the relationship between pre- and post-copulatory fitness must be taken into consideration. Prior work has emphasised the importance of examining the relationship between numerous interactive phenotypes when clarifying the evolutionary dynamics of a focal trait (Bailey and Hoskins, 2014; Bailey, Marie-Orleach and Moore, 2018). Therefore, in *Chapter II*, we apply the methods used in *Chapter I* to identify the extent to which direct genetic effects and IGEs underly variation in a pre-copulatory trait (mating latency) and copulation duration. By incorporating data collected for *Chapter I*, we examine three interactive

phenotypes that represent sequential stages of the reproductive process and investigate their phenotypic and genetic relationships. In doing so we identify whether these results support the phenotype-linked fertility hypothesis or the sperm competition game theory model, and examine how their relationships may influence the evolutionary potential of any given trait.

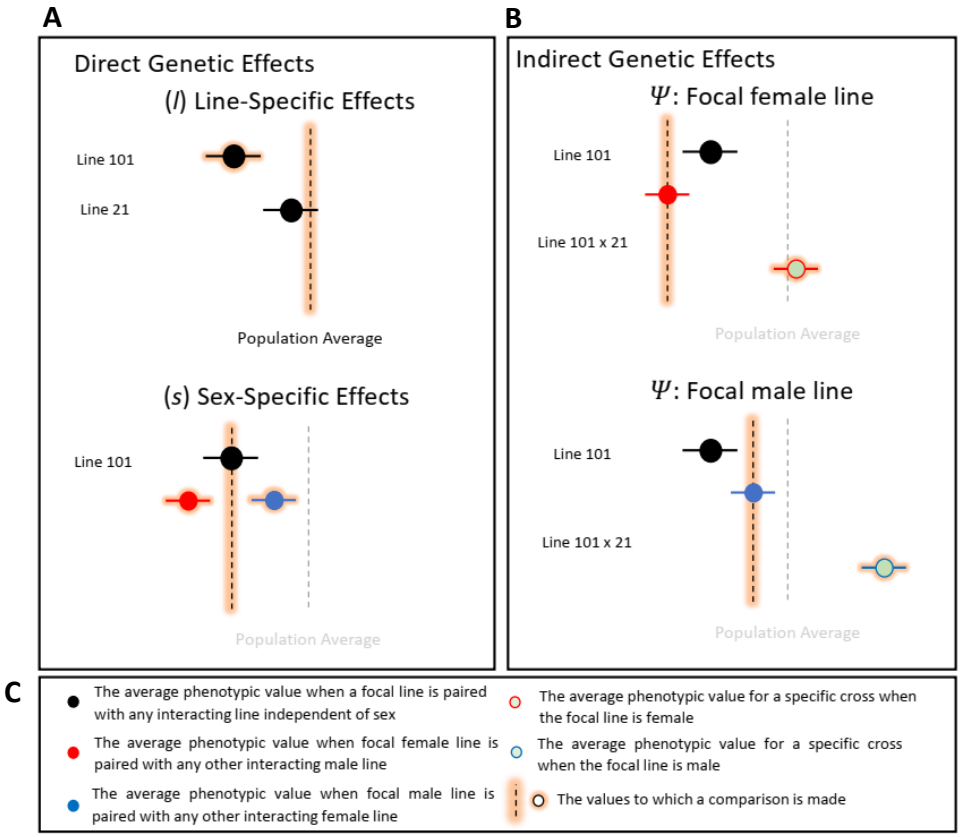

Figure 2. A schematic to describe how Direct Genetic Effects and Indirect Genetic Effects were quantified for a given phenotype. Direct genetic effects (A) are composed of Line-Specific Effects (*l*) and Sex-Specific Effects (*s*). Line-specific (additive) effects describe to what extent the overall mean phenotypic value of each genotype differs from the population average, independent of sex. Sex-Specific Effects (*s*) describe to what extent the mean phenotypic value of each genotype differs when it is male or female. Indirect Genetic Effects (B) describe how the genotype of an interacting individual influences the phenotype of a focal individual. Indirect genetic effects are examined for each sex. Ψ when the focal line is female quantifies the extent to which the phenotypic value expressed between a focal female and an interacting male differs from the focal female's phenotypic average. Ψ when the focal line is male quantifies the opposite. A legend (C) describes each component presented in figures (A) and (B). This figure is modified from Supplementary Figure S2 in both *Chapter I* and *II*.

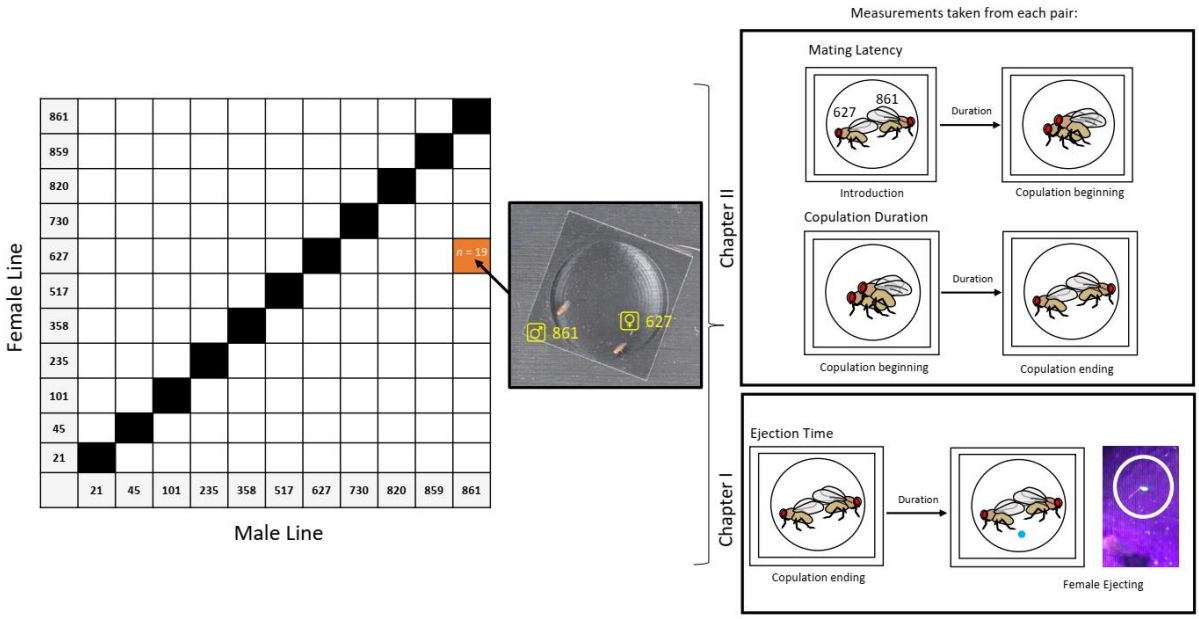

Figure 3. A schematic of the diallel mating design. Eleven isofemale lines were crossed in a full diallel mating design excluding reciprocal crosses (represented as a black square), representing 110 possible male-by-female genotypic crosses. For each genotypic cross, one male and one female from different lines were introduced into a chamber. Analysis of ejection time, the duration in minutes from copulation ending to the female ejecting, is presented in *Chapter I*. A photo of ejection is presented, with the ejection mass highlighted by a white circle. Analysis of mating latency, the time from a pair being introduced to copulation beginning, and copulation duration, the length of time from the male copulatory organ entering the female until the male and female disengage, is presented in *Chapter II*. Measurements of ejection from *Chapter I* were used when comparing the phenotypic and genetic relationship between all three traits in *Chapter II*. A pairing between male 861 and female 627 is illustrated in the figure, of which there were 19 replicates. This figure is modified from the Supplementary Figure S1 in *Chapter II*.

Future Directions

In *Chapter I*, the extent to which the genotype of both sexes influences the post-copulatory sexually selected trait sperm ejection is explored. Our results suggest that the timing of sperm ejection is a heritable trait and that direct genetic effects, IGEs, and sexual conflict play a clear role in the maintenance of trait variance. Knowledge of the genetic architecture of sperm ejection in both sexes is a prerequisite to understanding both its adaptive significance and phenotypic variation. In both chapters isofemale lines are used from the DGRP (Mackay *et al.*, 2005; Huang *et al.*, 2014). These are fully sequenced homozygous inbred *D. melanogaster* strains which enable association mapping between genomic regions and trait variance. A number of candidate genes that influence sperm storage and ejection have been identified (Lee *et al.*, 2015; Avila and Wolfner, 2017; Chen *et al.*, 2019; Wigby *et al.*, 2020). However, the underlying sequence variants that cause differences in sperm ejection remain largely unidentified. Identifying these causal variants would shed light on the genomic systems in which sexual selection acts, as well as provide a greater understanding on the role of sexual selection in phenotypic variance and diversification. Future work will provide this gap in knowledge.

In *Chapter II*, the extent to which IGEs influence pre- and post-copulatory traits was identified. We showed that the genotypes of both sexes and their interaction had a significant effect on mating latency but not copulation duration, highlighting that the influence of IGEs on phenotypic outcome is not consistent across all interactive phenotypes. Our data supports the phenotype-linked fertility hypothesis, showing a positive relationship between sperm ejection time (the length of time sperm is retained) and the speed at which an individual copulated (inverse of mating latency). However, our research did not directly identify specific traits that contribute to variation in mating latency and competitive fertilization. Therefore, further work is required to make predictions about their influence on trait diversification and the maintenance of phenotypic and genetic variation. Additionally, there was limited evidence to suggest that this phenotypic relationship was observed at the genetic level.

In our study only the correlations between additive genetic effects, which describes the average effect of each genotype independent of sex, were examined. As a result, future work examining how these relationships genetically covary with sex and cross would provide greater detail on whether the phenotypic correlations observed have a genetic basis.

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…..Oh….I should also thank *D. melanogaster* for behaving well enough to run this project….apart from line 517. You were a nightmare.

References

Agrawal, A.A. (2001) 'Phenotypic Plasticity in the Interactions and Evolution of Species', *Science*, 294(5541), pp. 321–326. Available at: https://doi.org/10.1126/science.1060701.

Alcock, J. (1994) 'Postinsemination Associations Between Males and Females in Insects: The Mate-Guarding Hypothesis', *Annual Review of Entomology*, 39(1), pp. 1–21. Available at: https://doi.org/10.1146/annurev.en.39.010194.000245.

Anderson, B.B., Scott, A. and Dukas, R. (2017) 'Indirect genetic effects on the sociability of several group members', *Animal Behaviour*, 123, pp. 101–106. Available at: https://doi.org/10.1016/j.anbehav.2016.10.028.

Andersson, M. (1994) *Sexual Selection*. Princeton University Press.

Arnold, S.J. and Duvall, D. (1994) 'Animal Mating Systems: A Synthesis Based on Selection Theory', *The American Naturalist*, 143(2), pp. 317–348. Available at: https://doi.org/10.1086/285606.

Arnqvist, G. and Nilsson, T. (2000) 'The evolution of polyandry: multiple mating and female fitness in insects', *Animal Behaviour*, 60(2), pp. 145–164. Available at: https://doi.org/10.1006/anbe.2000.1446.

Arnqvist, G. and Rowe, L. (2005) *Sexual Conflict*. Princeton University Press.

Avila, F.W. and Wolfner, M.F. (2017) 'Cleavage of the Drosophila seminal protein Acp36DE in mated females enhances its sperm storage activity', *Journal of Insect Physiology*, 101, pp. 66–72. Available at: https://doi.org/10.1016/j.jinsphys.2017.06.015.

Bailey, N.W. and Hoskins, J.L. (2014) 'DETECTING CRYPTIC INDIRECT GENETIC EFFECTS', *Evolution*, 68(7), pp. 1871–1882. Available at: https://doi.org/10.1111/evo.12401.

Bailey, N.W., Marie-Orleach, L. and Moore, A.J. (2018) 'Indirect genetic effects in behavioral ecology: does behavior play a special role in evolution?', *Behavioral Ecology*, 29(1), pp. 1–11. Available at: https://doi.org/10.1093/beheco/arx127.

Bailey, N.W. and Moore, A.J. (2012) 'Runaway Sexual Selection Without Genetic Correlations: Social Environments and Flexible Mate Choice Initiate and Enhance the Fisher Process', *Evolution*, 66(9), pp. 2674–2684. Available at: https://doi.org/10.1111/j.1558-5646.2012.01647.x.

Bailey, N.W. and Zuk, M. (2012) 'Socially flexible female choice differs among populations of the Pacific field cricket: geographical variation in the interaction coefficient psi (Ψ)', *Proceedings of the Royal Society B: Biological Sciences*, 279(1742), pp. 3589–3596. Available at: https://doi.org/10.1098/rspb.2012.0631.

Baud, A. *et al.* (2022) 'Indirect Genetic Effects: A Cross-disciplinary Perspective on Empirical Studies', *Journal of Heredity*, 113(1), pp. 1–15. Available at: https://doi.org/10.1093/jhered/esab059.

Bijma, P. (2014) 'The quantitative genetics of indirect genetic effects: a selective review of modelling issues', *Heredity*, 112(1), pp. 61–69. Available at: https://doi.org/10.1038/hdy.2013.15.

Birkhead, T.R. and Pizzari, T. (2002) 'Postcopulatory sexual selection', *Nature Reviews Genetics*, 3(4), pp. 262–273. Available at: https://doi.org/10.1038/nrg774.

Bleakley, B.H. *et al.* (2013) 'Cannibalism as an interacting phenotype: precannibalistic aggression is influenced by social partners in the endangered Socorro Isopod (Thermosphaeroma thermophilum)', *Journal of Evolutionary Biology*, 26(4), pp. 832–842. Available at: https://doi.org/10.1111/jeb.12098.

Bleakley, B.H. and Brodie III, E.D. (2009) 'Indirect Genetic Effects Influence Antipredator Behavior in Guppies: Estimates of the Coefficient of Interaction Psi and the Inheritance of Reciprocity', *Evolution*, 63(7), pp. 1796–1806. Available at: https://doi.org/10.1111/j.1558-5646.2009.00672.x.

Brommer, J.E., Rattiste, K. and Wilson, A.J. (2008) 'Exploring plasticity in the wild: laying date–temperature reaction norms in the common gull Larus canus', *Proceedings of the Royal Society B: Biological Sciences*, 275(1635), pp. 687–693. Available at: https://doi.org/10.1098/rspb.2007.0951.

Camerlink, I. *et al.* (2013) 'Indirect Genetic Effects and Housing Conditions in Relation to Aggressive Behaviour in Pigs', *PLOS ONE*, 8(6), p. e65136. Available at: https://doi.org/10.1371/journal.pone.0065136.

Chapman, T. *et al.* (1995) 'Cost of mating in Drosophila melanogaster females is mediated by male accessory gland products', *Nature*, 373(6511), pp. 241–244. Available at: https://doi.org/10.1038/373241a0.

Chen, D.S. *et al.* (2019) 'Female Genetic Contributions to Sperm Competition in Drosophila melanogaster', *Genetics*, 212(3), pp. 789–800. Available at: https://doi.org/10.1534/genetics.119.302284.

Cheverud, J.M. (1984) 'Evolution by Kin Selection: A Quantitative Genetic Model Illustrated by Maternal Performance in Mice', *Evolution*, 38(4), pp. 766–777. Available at: https://doi.org/10.2307/2408388.

Crespi, B.J. (2001) 'The evolution of social behavior in microorganisms', *Trends in Ecology & Evolution*, 16(4), pp. 178–183. Available at: https://doi.org/10.1016/S0169-5347(01)02115-2.

Danielson-François, A.M., Zhou, Y. and Greenfield, M.D. (2009) 'Indirect genetic effects and the lek paradox: inter-genotypic competition may strengthen genotype × environment interactions and conserve genetic variance', *Genetica*, 136(1), pp. 27–36. Available at: https://doi.org/10.1007/s10709-008-9297-z.

Dingemanse, N.J. and Araya-Ajoy, Y.G. (2015) 'Interacting personalities: behavioural ecology meets quantitative genetics', *Trends in Ecology & Evolution*, 30(2), pp. 88–97. Available at: https://doi.org/10.1016/j.tree.2014.12.002.

Eberhard, W. (1996) *Female Control: Sexual Selection by Cryptic Female Choice*. Princeton University Press.

Edenbrow, M. *et al.* (2017) 'The Evolution of Cooperation: Interacting Phenotypes among Social Partners', *The American Naturalist*, 189(6), pp. 630–643. Available at: https://doi.org/10.1086/691386.

Edward, D.A. *et al.* (2014) 'Sexual Conflict and Interacting Phenotypes: A Quantitative Genetic Analysis of Fecundity and Copula Duration in Drosophila Melanogaster', *Evolution*, 68(6), pp. 1651–1660. Available at: https://doi.org/10.1111/evo.12376.

Falconer, D.S. (1996) *Introduction To Quantitative Genetics 4th Edition*. Available at: http://archive.org/details/IntroductionToQuantitativeGenetics (Accessed: 17 April 2023).

Firman, R.C. *et al.* (2017) 'Postmating Female Control: 20 Years of Cryptic Female Choice', *Trends in Ecology & Evolution*, 32(5), pp. 368–382. Available at: https://doi.org/10.1016/j.tree.2017.02.010.

Fulker, D.W. (1966) 'Mating Speed in Male Drosophila melanogaster: A Psychogenetic Analysis', *Science*, 153(3732), pp. 203–205. Available at: https://doi.org/10.1126/science.153.3732.203.

Holland, B. and Rice, W.R. (1998) 'Perspective: Chase-Away Sexual Selection: Antagonistic Seduction Versus Resistance', *Evolution*, 52(1), pp. 1–7. Available at: https://doi.org/10.1111/j.1558-5646.1998.tb05132.x.

Huang, W. *et al.* (2014) 'Natural variation in genome architecture among 205 Drosophila melanogaster Genetic Reference Panel lines', *Genome Research*, 24(7), pp. 1193–1208. Available at: https://doi.org/10.1101/gr.171546.113.

Jennions, M.D. and Petrie, M. (1997) 'VARIATION IN MATE CHOICE AND MATING PREFERENCES: A REVIEW OF CAUSES AND CONSEQUENCES', *Biological Reviews*, 72(2), pp. 283–327. Available at: https://doi.org/10.1017/S0006323196005014.

Kent, C. *et al.* (2008) 'Social Context Influences Chemical Communication in D. melanogaster Males', *Current Biology*, 18(18), pp. 1384–1389. Available at: https://doi.org/10.1016/j.cub.2008.07.088.

Kirkpatrick, M. and Barton, N.H. (1997) 'The strength of indirect selection on female mating preferences', *Proceedings of the National Academy of Sciences of the United States of America*, 94(4), pp. 1282–1286. Available at: https://doi.org/10.1073/pnas.94.4.1282.

Kvarnemo, C. and Simmons, L.W. (2013) 'Polyandry as a mediator of sexual selection before and after mating', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 368(1613), p. 20120042. Available at: https://doi.org/10.1098/rstb.2012.0042.

Lee, K.-M. *et al.* (2015) 'A Neuronal Pathway that Controls Sperm Ejection and Storage in Female Drosophila', *Current Biology*, 25(6), pp. 790–797. Available at: https://doi.org/10.1016/j.cub.2015.01.050.

Lüpold, S. *et al.* (2013) 'Female mediation of competitive fertilization success in *Drosophila melanogaster*', *Proceedings of the National Academy of Sciences*, 110(26), pp. 10693–10698. Available at: https://doi.org/10.1073/pnas.1300954110.

Lüpold, S. *et al.* (2020) 'How female × male and male × male interactions influence competitive fertilization in Drosophila melanogaster', *Evolution Letters*, 4(5), pp. 416–429. Available at: https://doi.org/10.1002/evl3.193.

Mackay, T.F.C. *et al.* (2005) 'Genetics and genomics of Drosophila mating behavior', *Proceedings of the National Academy of Sciences*, 102(suppl_1), pp. 6622–6629. Available at: https://doi.org/10.1073/pnas.0501986102.

Manier, M.K. *et al.* (2010) 'Resolving Mechanisms of Competitive Fertilization Success in Drosophila melanogaster', *Science*, 328(5976), pp. 354–357. Available at: https://doi.org/10.1126/science.1187096.

Manier, M.K. *et al.* (2013) 'Postcopulatory Sexual Selection Generates Speciation Phenotypes in Drosophila', *Current Biology*, 23(19), pp. 1853–1862. Available at: https://doi.org/10.1016/j.cub.2013.07.086.

Marie-Orleach, L. *et al.* (2017) 'Indirect genetic effects and sexual conflicts: Partner genotype influences multiple morphological and behavioral reproductive traits in a flatworm', *Evolution*, 71(5), pp. 1232–1245. Available at: https://doi.org/10.1111/evo.13218.

Marie-Orleach, L., Janicke, T. and Schärer, L. (2013) 'Effects of mating status on copulatory and postcopulatory behaviour in a simultaneous hermaphrodite', *Animal Behaviour*, 85(2), pp. 453–461. Available at: https://doi.org/10.1016/j.anbehav.2012.12.007.

McDonough-Goldstein, C.E., Pitnick, S. and Dorus, S. (2022) 'Drosophila female reproductive glands contribute to mating plug composition and the timing of sperm ejection', *Proceedings of the Royal Society B: Biological Sciences*, 289(1968), p. 20212213. Available at: https://doi.org/10.1098/rspb.2021.2213.

McGlothlin, J.W. *et al.* (2010) 'Interacting Phenotypes and the Evolutionary Process. Iii. Social Evolution', *Evolution*, 64(9), pp. 2558–2574. Available at: https://doi.org/10.1111/j.1558-5646.2010.01012.x.

McGlothlin, J.W. and Brodie III, E.D. (2009) 'HOW TO MEASURE INDIRECT GENETIC EFFECTS: THE CONGRUENCE OF TRAIT-BASED AND VARIANCE-PARTITIONING APPROACHES', *Evolution*, 63(7), pp. 1785–1795. Available at: https://doi.org/10.1111/j.1558-5646.2009.00676.x.

Miller, C.W. and Moore, A.J. (2007) 'A potential resolution to the lek paradox through indirect genetic effects', *Proceedings of the Royal Society B: Biological Sciences*, 274(1615), pp. 1279–1286. Available at: https://doi.org/10.1098/rspb.2006.0413.

Moore, A.J., Brodie III, E.D. and Wolf, J.B. (1997) 'Interacting Phenotypes and the Evolutionary Process: I. Direct and Indirect Genetic Effects of Social Interactions', *Evolution*, 51(5), pp. 1352–1362. Available at: https://doi.org/10.1111/j.1558-5646.1997.tb01458.x.

Moore, A.J. and Pizzari, T. (2005) 'Quantitative Genetic Models of Sexual Conflict Based on Interacting Phenotypes.', *The American Naturalist*, 165(S5), pp. S88–S97. Available at: https://doi.org/10.1086/429354.

Nelson, W.A. and Crone, E.E. (1999) '*Genetics and Analysis of Quantitative Traits* . Michael Lynch , Bruce Walsh', *The Quarterly Review of Biology*, 74(2), pp. 225–225. Available at: https://doi.org/10.1086/393101.

Parker, G.A. (1970) 'Sperm Competition and Its Evolutionary Consequences in the Insects', *Biological Reviews*, 45(4), pp. 525–567. Available at: https://doi.org/10.1111/j.1469-185X.1970.tb01176.x.

Parker, G.A. and Pizzari, T. (2010) 'Sperm competition and ejaculate economics', *Biological Reviews*, 85(4), pp. 897–934. Available at: https://doi.org/10.1111/j.1469-185X.2010.00140.x.

Petfield, D. *et al.* (2005) 'Genetic variance in female condition predicts indirect genetic variance in male sexual display traits', *Proceedings of the National Academy of Sciences*, 102(17), pp. 6045–6050. Available at: https://doi.org/10.1073/pnas.0409378102.

Pitnick, S. (1996) 'Investment in Testes and the Cost of Making Long Sperm in Drosophila', *The American Naturalist*, 148(1), pp. 57–80. Available at: https://doi.org/10.1086/285911.

Pizzari, T. and Snook, R.R. (2003) 'Perspective: Sexual Conflict and Sexual Selection: Chasing Away Paradigm Shifts', *Evolution*, 57(6), pp. 1223–1236. Available at: https://doi.org/10.1111/j.0014-3820.2003.tb00331.x.

Qvarnström, A., Brommer, J.E. and Gustafsson, L. (2006) 'Testing the genetics underlying the co-evolution of mate choice and ornament in the wild', *Nature*, 441(7089), pp. 84–86. Available at: https://doi.org/10.1038/nature04564.

Saltz, J.B. (2013) 'Genetic composition of social groups influences male aggressive behaviour and fitness in natural genotypes of Drosophila melanogaster', *Proceedings of the Royal Society B: Biological Sciences*, 280(1771), p. 20131926. Available at: https://doi.org/10.1098/rspb.2013.1926.

Santostefano, F. *et al.* (2017) 'Indirect genetic effects: a key component of the genetic architecture of behaviour', *Scientific Reports*, 7(1), p. 10235. Available at: https://doi.org/10.1038/s41598-017-08258-6.

Schärer, L., Joss, G. and Sandner, P. (2004) 'Mating behaviour of the marine turbellarian Macrostomum sp.: these worms suck', *Marine Biology*, 145(2), pp. 373–380. Available at: https://doi.org/10.1007/s00227-004-1314-x.

Schneider, J., Atallah, J. and Levine, J.D. (2017) 'Social structure and indirect genetic effects: genetics of social behaviour', *Biological Reviews*, 92(2), pp. 1027–1038. Available at: https://doi.org/10.1111/brv.12267.

Sheldon, Ben.C. (1994) 'Male phenotype, fertility, and the pursuit of extra-pair copulations by female birds', *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 257(1348), pp. 25–30. Available at: https://doi.org/10.1098/rspb.1994.0089.

Signor, S.A. *et al.* (2017a) 'Conservation of social effects (Ψ) between two species of Drosophila despite reversal of sexual dimorphism', *Ecology and Evolution*, 7(23), pp. 10031–10041. Available at: https://doi.org/10.1002/ece3.3523.

Signor, S.A. *et al.* (2017b) 'Social effects for locomotion vary between environments in Drosophila melanogaster females', *Evolution*, 71(7), pp. 1765–1775. Available at: https://doi.org/10.1111/evo.13266.

Simmons, L.W., Lüpold, S. and Fitzpatrick, J.L. (2017) 'Evolutionary Trade-Off between Secondary Sexual Traits and Ejaculates', *Trends in Ecology & Evolution*, 32(12), pp. 964–976. Available at: https://doi.org/10.1016/j.tree.2017.09.011.

Singh, B.N. and Singh, S.R. (1999) 'Female remating inDrosophila ananassae: shorter duration of copulation during second mating as compared to first mating', *Journal of Biosciences*, 24(4), pp. 427–431. Available at: https://doi.org/10.1007/BF02942652.

Snook, R.R. and Hosken, D.J. (2004) 'Sperm death and dumping in Drosophila', *Nature*, 428(6986), pp. 939–941. Available at: https://doi.org/10.1038/nature02455.

VIZOSO, D.B., RIEGER, G. and SCHÄRER, L. (2010) 'Goings-on inside a worm: functional hypotheses derived from sexual conflict thinking', *Biological Journal of the Linnean Society*, 99(2), pp. 370–383. Available at: https://doi.org/10.1111/j.1095-8312.2009.01363.x.

Walsh, B. and Lynch, M. (2018) *Evolution and Selection of Quantitative Traits*. Oxford University Press.

Wigby, S. *et al.* (2020) 'The Drosophila seminal proteome and its role in postcopulatory sexual selection', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 375(1813), p. 20200072. Available at: https://doi.org/10.1098/rstb.2020.0072.

Wolf, J.B., Brodie III, E.D. and Moore, A.J. (1999) 'Interacting Phenotypes and the Evolutionary Process. II. Selection Resulting from Social Interactions.', *The American Naturalist*, 153(3), pp. 254–266. Available at: https://doi.org/10.1086/303168.

Chapter 1

The impact of indirect genetic effects on sperm ejection: how partner genotype influences shared sexually selected traits

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Abstract

Sexual reproduction requires an interaction between the sexes. When females mate multiply, sexual selection and sexually antagonistic coevolution (SAC) can occur, resulting in the widespread evolution of sex-specific traits that influence male and female fitness. One such trait is sperm ejection, which is when females eject sperm from the reproductive tract after copulation, the timing of which is sexually antagonistic. Like many sexually selected traits, it is also an interactive phenotype, in which expression is influenced or defined by conspecific interactions. In such cases, the genotype of the interacting partner becomes a heritable component of the focal individual's environment, generating indirect genetic effects (IGEs). Both SAC and IGEs are predicted to influences the evolutionary dynamics of a trait, and thus a standard quantitative genetic approach that assumes environmental variation is not heritable is inappropriate to understanding the evolution of these ubiquitous sexually selected interactive phenotypes. Here we use a novel diallel quantitative genetics approach which partitions direct and indirect genetic architecture, using multiple *Drosophila melanogaster* isofemale lines, to understand the underlying genetics and evolution of sperm ejection time. We show that sperm ejection is heritable, and that both an individual's own genotype (direct effect) and the genotype of their partner (IGEs) influence the timing of sperm ejection. By using this unique approach, we are also able to show that both direct effects and the magnitude of IGEs are sex-specific, providing evidence that IGEs influence antagonistic coevolution in this trait. Together, these data demonstrate the underlying quantitative genetic basis behind phenotypic variation on a key fitness-related trait subject to sexual conflict.

1. Introduction

Polyandry, in which females mate with multiple males within one reproductive cycle, promotes the overlap of male ejaculates which compete to fertilize the female ova (Birkhead and Møller, 1993). Females can benefit from extra copulations (Arnqvist and Nilsson, 2000; Jennions and Petrie, 2000), whereas, for each polyandrous mating, male paternity assurance and reproductive success decreases (Chapman *et al.*, 2003). This fitness difference between the sexes creates conflict over optimal remating rate, generating post-copulatory sexual selection (Parker, 1970; Birkhead and Pizzari, 2002) via intra-sexual selection (sperm competition; Parker, 1970), and inter-sexual selection (cryptic female choice; Eberhard, 1996; Birkhead, 1998). Both processes can stimulate sexually antagonistic coevolution between males and females which selects for shared antagonistic traits (i.e. intra-locus conflict; Bonduriansky and Chenoweth, 2009; Van Doorn, 2009; Schenkel *et al.*, 2018) and sex-specific manipulation and/or resistance traits (i.e. inter-locus conflict; Rice and Holland, 1997; Pennell and Morrow, 2013; Dapper and Wade, 2016; Firman *et al.*, 2017) in order to control paternity.

One mechanism in which males attempt to control female remating and subsequent paternity share is via mating plugs. Such plugs have independently evolved in a diverse range of taxa as a post-copulatory mate guarding strategy (Reviewed in Parker, 1970). These structures influence male

fertilization success as they reduce sperm loss, facilitate sperm storage, and control female post-mating responses by reducing receptivity to remating (Schneider, Mangels and Dean, 2016). In *Drosophila melanogaster*, the mating plug is formed within the female reproductive tract (FRT) and is largely composed of proteins (i.e. seminal fluid proteins; SFPs) (Lung and Wolfner, 2001) as well as pheromones, such as cis-vaccenyl acetate, which decrease female attractiveness in future copulations (Laturney and Billeter, 2016). The timing of plug ejection influences the amount of time sperm has to move into storage, biasing sperm use and subsequently influencing the outcome of competitive fertilization (Snook and Hosken, 2004; Manier *et al.*, 2010; Lüpold *et al.*, 2013; Firman *et al.*, 2017). As paternity in this species is largely determined by the proportion of each male's sperm in storage (Manier *et al.*, 2010; Lüpold *et al.*, 2012), males benefit from long ejection times (Lüpold *et al.*, 2013). Whether longer ejection times maximise female fitness is unclear (Lüpold *et al.*, 2013). Thus, the timing of ejection is thought to be a source of sexual conflict (Arnqvist and Rowe, 2005; McDonough-Goldstein, Pitnick and Dorus, 2022).

Multiple sex-specific effects have been linked to the timing of its ejection in *D. melanogaster*, such as SFP composition and FRT secretions (McDonough-Goldstein, Pitnick and Dorus, 2022). The role of both sexes in mediating sperm ejection timing is unsurprising as this trait is an interactive phenotype. Interactive phenotypes describe traits which require or are influenced by conspecific interactions, generating indirect genetic effects (IGEs) as the interacting conspecific genotype becomes a heritable environmental component of the focal individual (Moore, Brodie III and Wolf, 1997). IGEs can influence among-individual variation and the evolution of the trait by either accentuating or diminishing the rate of selection (Moore, Brodie III and Wolf, 1997; Wolf, Brodie III and Moore, 1999; Bleakley and Brodie III, 2009; McGlothlin *et al.*, 2010; Bailey and Moore, 2012; Dingemanse and Araya-Ajoy, 2015). For example, IGEs are expected to influence antagonistic coevolution and the outcome of sexual conflict, stimulating strong selection for adaptations and counter-adaptations above standard predicted rates (Moore and Pizzari, 2005). In doing so, IGEs can facilitate the maintenance of genetic variation for a given trait, potentially resolving the lek paradox (Kirkpatrick and Barton, 1997; Qvarnström, Brommer and Gustafsson, 2006; Miller and Moore, 2007; Danielson-François, Zhou and Greenfield, 2009; Bailey and Moore, 2012). Subsequently, interactive trait evolution may be different when using an interactive phenotypic framework from those predicted using a standard genetic framework, in which the environmental component is considered not heritable, as the indirect effect of one sex may have evolutionary relevance on the direct effect of the opposite sex.

For many interactive phenotypes that exist exclusively as a product of a reproductive interaction, such as sperm ejection, only a single shared phenotype can be measured for both sexes, despite the outcome likely mediated by sex-specific traits (Dingemanse and Araya-Ajoy, 2015). Examining how IGEs influence the outcome of interactive traits where there is one shared phenotype should improve understanding of phenotypic variance in sexually selected traits and subsequent evolutionary patterns (Moore, Brodie III and Wolf, 1997; Moore and Pizzari, 2005; McGlothlin *et al.*, 2010; Bailey, Marie-Orleach and Moore, 2018). Despite this relevance, relatively few studies have examined the role of IGEs on sexually selected interactive phenotypes (but see Danielson-François, Zhou and Greenfield, 2009; Bailey and Zuk, 2012; Marie-Orleach, Janicke and Schärer, 2013; Bailey and Hoskins, 2014; Marie-Orleach *et al.*, 2017), and even fewer have examined this in traits where phenotypic value is shared by both sexes, with those that have not quantifying the direction and magnitude of IGEs (Edward *et al.*, 2014).

We use an interactive phenotypic framework to identify the extent to which sperm ejection is directly influenced by the genotype of each focal sex and indirectly by the interacting sex. We partition these effects by taking a quantitative genetic diallel approach (Lenarcic *et al.*, 2012) using eleven isofemale lines from the *D. melanogaster* Genetic Reference Panel (DGRP; Mackay *et al.*, 2012). This allows us to directly and reciprocally manipulate the genetic component of the social environment to identify direct genetic effects and quantify the influence of IGEs using the parameter Ψ (Moore, Brodie III and Wolf, 1997). In this framework, the focal individual represents the direct genetic component and the opposite interacting sex represents the indirect environmental component. We find that sperm ejection is a heritable trait, and that phenotypic variance is attributed to both direct and IGEs, that are both genotype and sex-specific. Our results outline the role of IGEs on a key fitness-related trait subject to sexual conflict and sexual selection, and expose the underlying quantitative genetic basis behind phenotypic variation in this trait.

2. Materials and Methods

2.1 Fly Stocks

Eleven randomly selected isogenic lines from the *D. melanogaster* Genetic Reference Panel were used for this study (DGRP-21, -45, -101, -235, -358, -517, -627, -730, -820, -859, -861; Mackay *et al.*, 2012; Huang *et al.*, 2014). DGRP lines originate from a single wild population collected in Raleigh, North Carolina, in 2003, where 20 generations of full-sibling matings were conducted for each line, resulting in a panel of 205 inbred lines that have been sequenced. In our lab, all lines were housed in standard culture vials containing 5ml of a standard food medium (1L water: 80g medium cornmeal, 18g dried yeast, 10g soya flour, 80g malt extract, 40g molasses, 8g agar, 25 mL of 10% Nipagin, 4 mL of propionic acid) at 12-h light:12-h dark cycle. No ethical approval was required for the work. These stocks were used to generate experimental animals. Flies and all experiments were kept at 25°C.

2.2 Production of focal individuals

To generate focal individuals, each line was placed in food vials. Each vial had a ca. 1:1 sex ratio and 20 individuals per vial. Parent flies were removed after three days and, ca. eight days later, virgin focal offspring were collected within 2h after eclosion under light CO_2 anaesthesia. Sexes were housed separately with 10-15 individuals per vial prior to experiments. Focal individuals were collected across five consecutive days, followed by five consecutive days of experiments, making up a single ten-day block. 14 blocks were performed. Thus, focal individuals for subsequent experiments were six days old.

2.3 Quantifying sperm ejection

We measured ejection time as the time from when the male's copulatory organ disengages from the female until the time at which the female ejects. To acquire these data, all isofemale lines were crossed in a full diallel mating design excluding reciprocal crosses, producing 110 crosses in total (Figure S1). Between 14 and 26 matings were conducted for each cross (Figure 1).

One male and one female from different randomly selected DGRP lines were introduced into a 3D-printed black plastic chamber, consisting of a cuboid of 34 mm x 33 mm x 9 mm with a hemispherical cavity of diameter 20 mm and depth 7 mm (Hopkins *et al.*, 2019) (Figure S1). A glass coverslip was used to cover the cavity as each sex was introduced. Each chamber contained a drop of an agar-sugar solution to avoid desiccation stress. The male was always introduced into the chamber first.

Approximately 90 pairs were mated each day and all chambers were filmed with a camcorder (Panasonic HC-V180 or Sony HDR-CX405). All chambers were observed every 3-5 minutes for 1 hour after the pair was introduced to identify the end of copulation (note that most copulations in the lines we used occur within the first hour). Following the end of copulation, each chamber was scanned using a fluorescent light at ca. ten-minute intervals to identify the time of ejection, with exact timings verified using video playback. If the pair had not ejected after nine hours following copulation, then the chamber was filmed overnight. If ejection was clearly visible on the video recording, then this data point was kept, otherwise the pair was excluded from the analysis.

2.4 Statistical Analysis

2.4.1 Analytical Approach

We first examine direct genetic effects: how an individual's genotype influences phenotypic outcome (Figure S2 A). This is divided into two components: line-specific (additive) effects and sex-specific effects. Line-specific (additive) effects ("l" in Figure S2 A) describes to what extent the mean phenotypic value of each genotype in turn differs from the population average, independent of sex. For example, genotype 45 may have an overall mean ejection time of 60 minutes which is significantly shorter than the population average which is 120 minutes. Sex-specific effects ("s" in Figure S2 A) describe to what extent the mean phenotypic value of each genotype differs when it is male or female. For example, genotype 45 may have an overall mean ejection time of 60 minutes, however, there may be a strong contrast between the sexes (i.e. 30 minutes when male and 90 minutes when female), or the sex-specific mean ejection times could be very similar (i.e. 58 minutes when male and 62 minutes when female). The former case would indicate a strong sex-specific effect, and the latter a weak or insignificant effect.

Next, we examine indirect genetic effects: how the genotype of an interacting individual influences the phenotype of a focal individual (Figure S2 B). In these cases, for each copulating pair, one sex will represent the "focal genotype" and the partner will be the "interacting genotype". IGEs are measured for each sex in turn and describe to what extent the mean phenotypic value of the focal genotype differs when it is paired with an interacting genotype. When we are examining the IGE on females, the focal genotype will be female and the interacting genotype will be male ("Ψ: Focal female line" in Figure S2 B), and *vice versa* ("Ψ: Focal male line" in Figure S2 B).

For each sex and genotype, we measure *(i)* the strength and direction of each IGE for each interacting genotype, and *(ii)* the overall magnitude of the IGE on the focal genotype. The strength and direction of IGEs describes to what extent the mean phenotypic value of a given focal genotype differs (positively or negatively) when it is crossed with a specific interacting genotype. For example, female genotype 45 may have an overall mean ejection time of 90 minutes. However, when it is paired with male genotype 21 it has a mean ejection time of 120 minutes: this implies a strong positive IGE. The overall magnitude of the IGE can be quantified by observing how the mean phenotypic value of a focal genotype (of a given sex) varies when it is crossed with all other interacting genotypes. For example, in female genotype 45 we could observe that *(i)* the ejection time of female genotype 45 takes a large range of values when paired with different male genotypes, that differ from the mean ejection time of female genotype 45 – the overall magnitude of IGE is large; *(ii)* only a few interacting male genotypes drive an ejection time with a large deviation from the phenotypic average of female genotype 45 – the overall magnitude of IGE is small; or *(iii)* the ejection time of line 45 females does

not deviate from their overall average for any interacting male genotype – the overall magnitude of IGE is close to or equal to 0.

2.4.2 Direct Genetic Effects

To assess line-specific (additive) genetic effects (the phenotype without regard to focal sex) and sex-specific effects (the phenotypic value when considering the sex of the focal individual), analyses were performed using the package BayesDiallel (Lenarcic *et al.*, 2012) in R v 3.4.4 (R Core Team, 2016). Bayesian Diallel models are described by a quote string of characters, with the full model containing seven heritable components ($BSabmvw$; Lenarcic *et al.*, 2012). Our model included four components from the full model and the random covariate batch (labelled 1 to 14) to predict how much of the total interactive phenotypic variance is explained by each component in the model, which is given below:

$$y_i = \mu + \underbrace{\sum_{r=1}^{R} u_i^{(r)}}_{\widetilde{Random}} + \underbrace{(l_{j[i]} + l_{k[i]})}_{\widetilde{line}\ (l)} + \underbrace{(s_{j[i]} - s_{k[i]})}_{\widetilde{sex}\ (s)} + \underbrace{(I_{\{j[i]\neq k[i]\}} v_{(jk)[i]})}_{\widetilde{cross-specific}\ (v)} + \underbrace{(I_{\{j[i]\neq k[i]\}} w_{(jk)[i]})}_{\widetilde{cross-specific\ sex}\ (w)}$$

Raw data for ejection time (y_i) is measured for all individual pairings where $j_{[i]}$, $k_{[i]}$, and $(jk)_{[i]}$, respectively describe the female, male and female-male combination relevant to the specific pairing i where $i \in \{1, \ldots, n\}$. The $\sum_{r=1}^{R} u_i^{(r)}$term represents the contribution of the random effect which for single phenotypic outcome always includes an effect of experimental batch as $u_i^{(r)} \sim N(0, \tau_r^2)$ for each $r \in \{1, \ldots, R\}$. Genotypic line-specific effects l are modelled as random effects and provide estimates of the average ejection time of a genotype for female j in combination with male k and is equivalent to the proportion of additive genetic variability. Sex-specific effects s are modelled as symmetric (random effect) deviations from the l model and describes an additional increase or decrease in the mean ejection time induced by a line being female, with male as a reference (Cockerham and Weir, 1977). The components l and s are equivalent to a and m in Equation 16 of Lenarcic *et al.* (2012), and outline the direct genetic effects that influence ejection time (Figure S2 A). BayesDiallel analysis also outlines IGEs which describe interactions between specific copulatory pairs. These are modelled as two types of random effect departures from the ls model: cross-specific effects v (model differences specific to a given pair independent of reciprocal effects, i.e. crosses jk and kj have the same effect), and cross-specific sex effects w (model deviations from cross-specific effects due to differences between reciprocal crosses, i.e. crosses jk and kj have different effects). Overall both describe the extent to which ejection time from a specific cross varies from what would be expected based on the average performance of the genotypes involved (Murphy *et al.*, 2008); and, in the case of w, if this is sex-specific (Figure S2 B). However, as cross-specific effects represent fewer observations, these results are strongly subject to Bayesian adaptive shrinkage which pulls extreme but sparsely supported means towards the middle (Lenarcic *et al.,* 2012). As a result, IGEs using this method are often vague meaning that other, more direct approaches are more appropriate when calculating IGEs. Here our direct approach is to calculate Ψ (see section 2.4.3 below).

Ejection time for all estimates were log-transformed and calculated from multiple posterior draws, leading to a complete posterior distribution of each model component. These are summarized as highest posterior density intervals (HPD) such that credibility intervals excluding zero indicate strong evidence that an effect is different from the average. The variance of each group, e.g. τ_a^2, was modeled with a weak inverse gamma prior τ_a^{-2} (*df* = 0.02, *mean* = 0.2), and the prior for fixed effect μ is set to

a vague normal distribution $\mu \sim N(0, 10^3)$ as described in Lenarcic *et al.* (2012). Posterior distributions were estimated for all parameters using an efficient MCMC Gibbs sampler with 5 chains, 10, 000 iterations and a burn-in of 100.

In order to report the overall relative contribution of each model component, diallel variance projections (VarP) were calculated (Crowley *et al.*, 2014). This approach is a heritability-like measure which uses the posterior predictive distribution of effects from the model to simulate future, complete, perfectly balanced diallels of the same genotypic lines. Unlike traditional heritability, it is calculated based on heritable components of the diallel rather than variance components, which increases interpretability, stability and accuracy (Crowley *et al.*, 2014). In each simulated dataset, the contribution of each component in the model (i.e. l and s) is calculated as its sum of squares divided by the total phenotype sum of squares. The resulting proportion, the VarP, provides a prospective summary describing how much each component in the model influences phenotypic variation. Subsequently, the total VarP[$l + s + v + w$] is equivalent to broad-sense heritability and VarP[l] is related to narrow-sense heritability (Lenarcic *et al.*, 2012; Maurizio *et al.*, 2017). Estimates for VarPs are calculated in the same way as the HPD summaries with credibility intervals excluding zero providing strong evidence that an effect explains a significant proportion of the phenotypic variance.

2.4.3 Indirect Genetic Effects

IGEs were derived by calculating Ψ for each male-by-female interaction using R v 4.2.0 (R Core Team, 2016). Up until now the interaction coefficient Ψ has be calculated for traits in which the phenotypes of interest can be measured in both focal and interacting individuals. In these cases, Ψ is calculated by regressing focal phenotype onto a separate interacting phenotype. However, sperm ejection requires a different approach. We provide a framework in which Ψ can be calculated for phenotypes when separate measurements cannot be taken for each sex. Separate models were derived for each sex-specific focal line (Figure S2 B). The below formula describes how Ψ is derived for a single focal female genotype j, but is equally applicable to a focal male with appropriate change of notation. We define

$$z_{k[i]} = \beta_0 + \beta_1 \bar{z} + \boldsymbol{\Psi X_K} + \boldsymbol{\omega}_b \boldsymbol{Y}_B + \varepsilon_{[i]}$$

where

$$\boldsymbol{X_K} = \begin{cases} 1 \; if \; K = k \\ 0 \; if \; K \neq k \end{cases}$$

Here, $z_{k[i]}$ denotes the measured ejection time for the i^{th} trial within the k^{th} interacting male genotype. $\bar{z}$ is the mean phenotype of the focal female line. β_0 is the intercept and β_1 the slope of $\bar{z}$. $\boldsymbol{X}_K$ is a vector representing each individual kth interacting male genotype. This means that K always takes the value of one of our interacting eleven lines. For example, when examining sperm ejection in a specific cross ($z_{k[i]}$) between focal female line 101 and interacting male line 21, k = genotype 21 ("Ψ: Focal female line" in Figure S2 B). The vector $\boldsymbol{X_K}$ = 1 when $K = k$, otherwise $\boldsymbol{X_K}$ will = 0 (i.e. if you are deriving Ψ for focal female line 101 when crossed with male line 21, you will only derive a value of Ψ when k is 21). Strictly speaking, Ψ is an intercept term from the random effect's model. However, as $\boldsymbol{X}$ is a binary variable, it can also be interpreted as the gradient describing to what extent each interacting male genotype influences focal female genotype. ω_B denotes an effect of batch, fitted as a random effect. $\boldsymbol{Y}_B$ has the same properties as $\boldsymbol{X_K}$ but describes each batch. ε is the residual error

term. Ejection time was standardized within line to have a mean of 0 and a standard deviation of 1, meaning that the average phenotype ($\bar{z}$) and intercept (β_0) for a given line for each sex is 0. By doing so, the formula simplifies to:

$$z_{k[i]} = \boldsymbol{\Psi X_K} + \boldsymbol{\omega}_b \boldsymbol{Y}_B + \varepsilon_{[i]}$$

and:

$$z_{j[i]} = \boldsymbol{\Psi X_J} + \boldsymbol{\omega}_b \boldsymbol{Y}_B + \varepsilon_{[i]}$$

when describing a single focal male genotype where, $z_{j[i]}$ denotes the measured ejection time for the i^{th} trial within the j^{th} interacting female genotype ("Ψ: Focal male line" in Figure S2 B).

Restricted Maximum Likelihood Model was used to fit the model parameters which were fitted for each sex separately within each line, with ejection time log transformed. The model was fitted using the *lme4* function. When Ψ is measured on standardized traits it takes values between -1 and 1. When values of Ψ were outside this range due to large variation around model estimates they were reported as -1 and 1 respectively. For focal genotypes unaffected by the interacting genotype, Ψ = 0. Ψ is negative for phenotypes where the interacting genotype reduces trait expression from the phenotypic average of the focal line, and positive when it increases trait expression. This analysis depicts the strength and direction of IGEs for each male-by-female cross. To analyse the overall magnitude of IGEs for each focal genotype, we quantified the overall variance in Ψ when male and female respectively. For a given focal genotype, if the variance in Ψ is large for a given sex, then the magnitude of IGEs is strong with interacting genotypes having an overall strong effect on phenotypic outcome. For a given focal genotype, if the variance in Ψ is small for a given sex, the opposite conclusion can be drawn. An F-test was used to determine if variance in Ψ was significantly different between the sexes.

3. Results

3.1 Direct Genetic Effects

The timing of sperm ejection displayed substantial phenotypic variation (Figure 1). This variation was heritable with narrow-sense heritability (additive line-specific genetic effects), and sex-specific effects explaining a significant proportion of the phenotypic variance (Figure S3 A), and results are robust to the small variation in sample size between cells (Figure S4). The significant line-specific effect was largely driven by two genotypes (101 and 45), both displaying ejection times significantly shorter than the population average (Figure 2 A). Significant sex-specific effects were observed in three genotypes (517, 627 and 820; Figure 2 B). Genotypes 517 and 627 displayed significantly longer ejection times when the focal individual of that line was female mated to males from different lines, compared to when the focal individual of those lines were male mated to females from different lines (Figure 2 B). Genotype 820 displayed a significant sex-specific effect in the opposite direction (Figure 2 B).

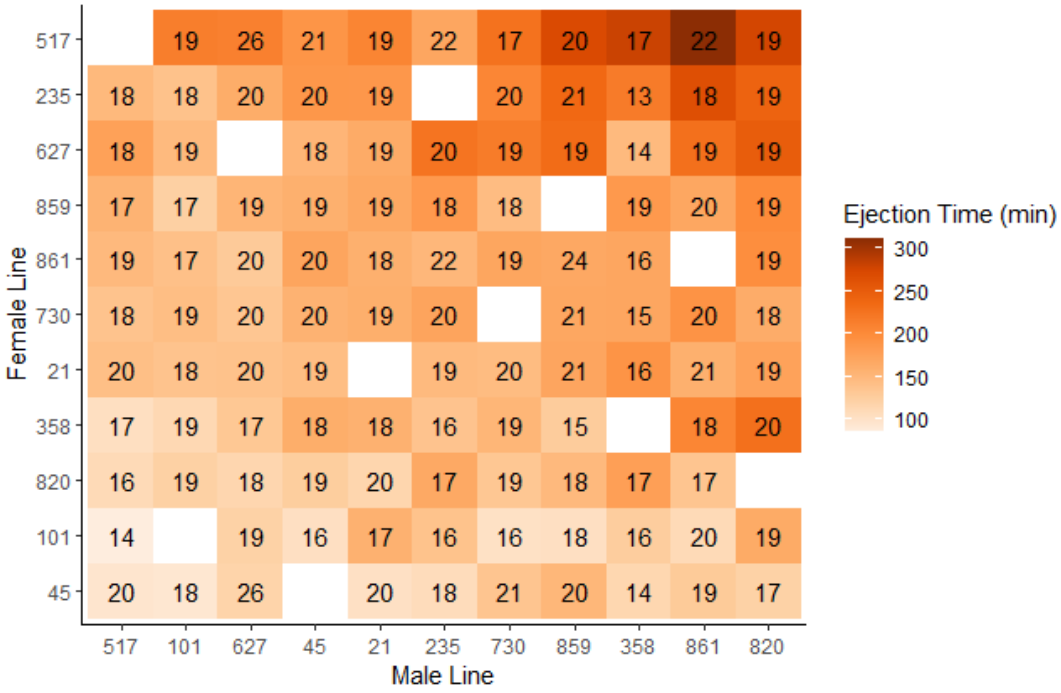


Figure 1. Variation in the timing of sperm ejection. The colour of each cell represents the shared mean sperm ejection time expressed by a male and a female from two different DGRP lines. The lines are ordered left to right from the line displaying the shortest duration to the longest duration, when male and female respectively. Cell colour represents mean ejection time for each cross, the darker the colour the longer the ejection time. Within line crosses were not conducted and are denoted in white. The number in each cell is the sample size for each pairing.

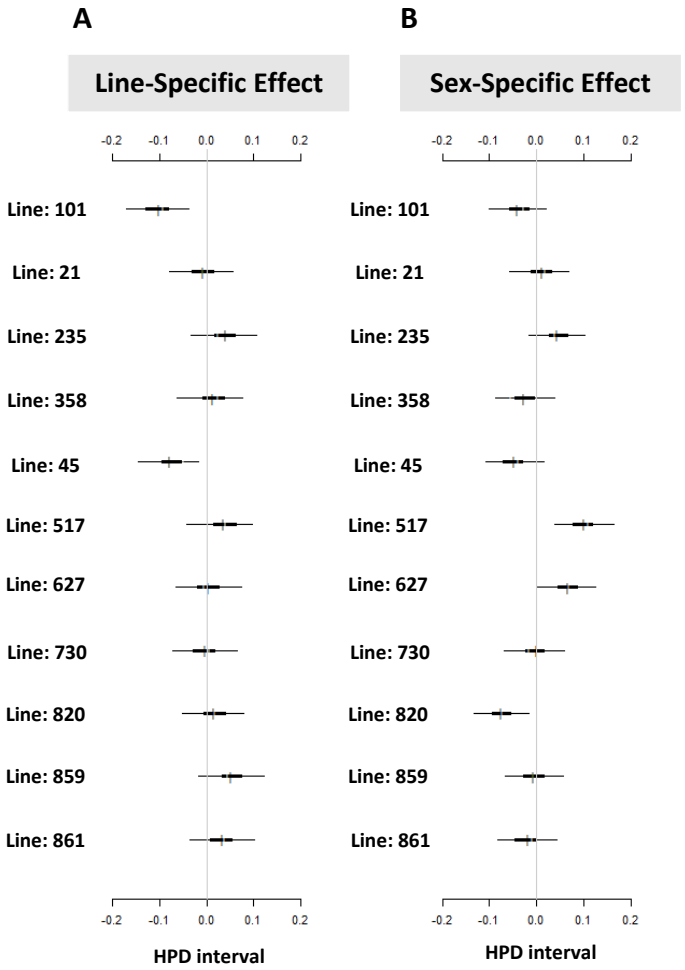


Figure 2. Highest posterior density (HPD) intervals identifying genotypic lines that display significant direct genetic effects for ejection time based on the $lsvw$ model. Line-Specific Effects (A) denote how mean ejection time for a given genotype is related to the population average (vertical grey line), independent of sex. Any bar to the left of the vertical line suggests that the mean ejection time for this genotype, independent of sex, is shorter than the population average. Sex-Specific Effects (B) denote the average deviation in ejection time when a genotype is female compared to the overall average ejection time of that genotype (vertical grey line), with male as a baseline. Any bar that does not overlap zero indicates that, for that genotype, mean ejection time between the sexes is significantly different from each other. Any bar to the left of the vertical line suggests that the mean ejection time for this genotype, is significantly longer when male than female, and *vice versa* when to right of the vertical line. For each effect, thin and thick horizontal lines show the 95 and 50% HPD intervals, respectively, with short vertical bars indicating the posterior mean. HPD intervals that do not include zero we consider to be statistically significant at 95% credibility. Details on how Line-Specific and Sex-Specific effects were calculated can be found in Supplementary Figure S2. Note that line order in this figure contrasts from the other figures and is not in increasing numerical order.

3.2 Indirect Genetic Effects

IGEs will be seen when trait expression of the focal individual is strongly influenced by the genotype of the interacting partner. In this analysis, IGEs will be observed when specific crosses between a focal genotype and an interacting partner genotype deviate from the focal genotype's sperm ejection average. Within each genotypic line, mean ejection time displayed considerable diallel cross-specific variation in comparison to the population average for both sexes (Figure 3). That is, the focal sex sperm ejection time could be either greater or lesser than the population average when paired with a specific interacting genotype (Figure 3). This pattern indicates that, for a given focal genotype, phenotypic outcome will vary depending on the interacting genotype.

To quantify IGEs we estimate Ψ which describes to what extent an interacting genotype (the heritable environmental component) influences focal individual phenotype for each genotypic line. For almost all genotypic lines, the direction and strength of Ψ, depicted by the sign and size respectively, was cross-specific (Figure 4). That is, the strength and direction with which an interacting genotype alters the phenotypic outcome of a focal genotype is dependent on both the focal and interacting genotype. Additionally, the magnitude of Ψ for each focal genotype was sex-specific, with males displaying significantly larger variation compared to females (Figure 4; $F = 0.33$, $df = 109$, $p < 0.001$). This result indicates that males have a weaker IGE on focal female phenotype compared to the IGE of females on focal male phenotype. In addition, two genotypes (21 and 730) showed Ψ values close to or equal to zero when examining focal female trait expression. This result indicates that the interacting male genotype did not shift trait expression in these focal females from their average phenotype. In comparison, no focal male genotype displayed Ψ values that were close to or equal to zero across all female interacting genotypes. IGEs (cross-specific and cross-specific sex effects) were also calculated using a BayesDiallel approach (Figure S3), however, due to Bayesian shrinkage as outlined in the methods, calculations of Ψ are more robust when analysing IGEs.

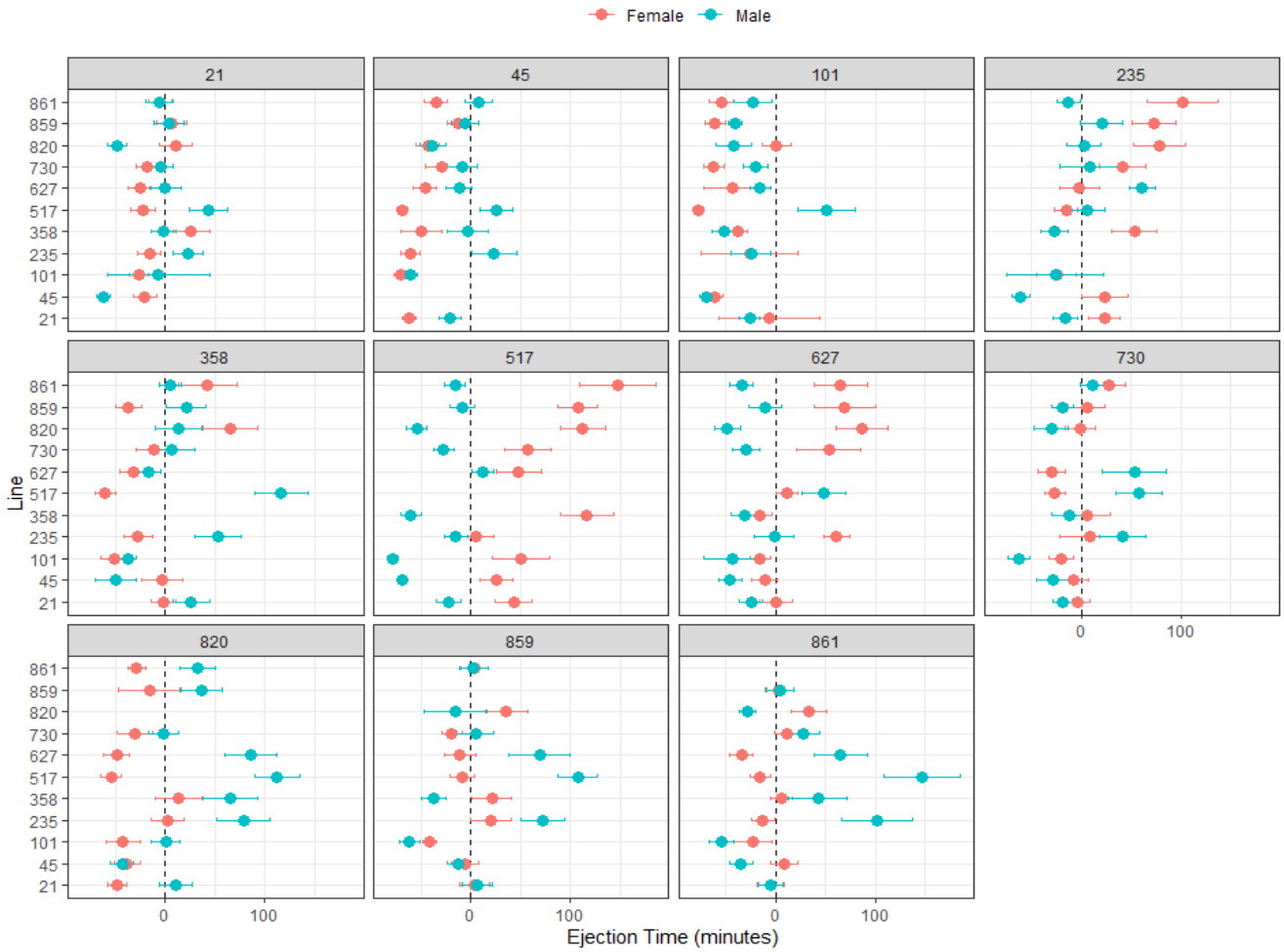


Figure 3. Variation in sex-specific mean ejection time for each focal genotype when crossed with an interacting genotype compared to the population average. Each box represents a focal genotype, denoted by the grey label above each graph. Within each box, each point represents the mean ejection time and standard deviation when each focal male (blue) or female (red) genotype is crossed with a specific interacting genotype, denoted on the y axis. The vertical dashed line represents the average ejection time of the population. A point left of the dashed line suggests that the mean ejection time for the cross involving those lines is shorter than the population average.

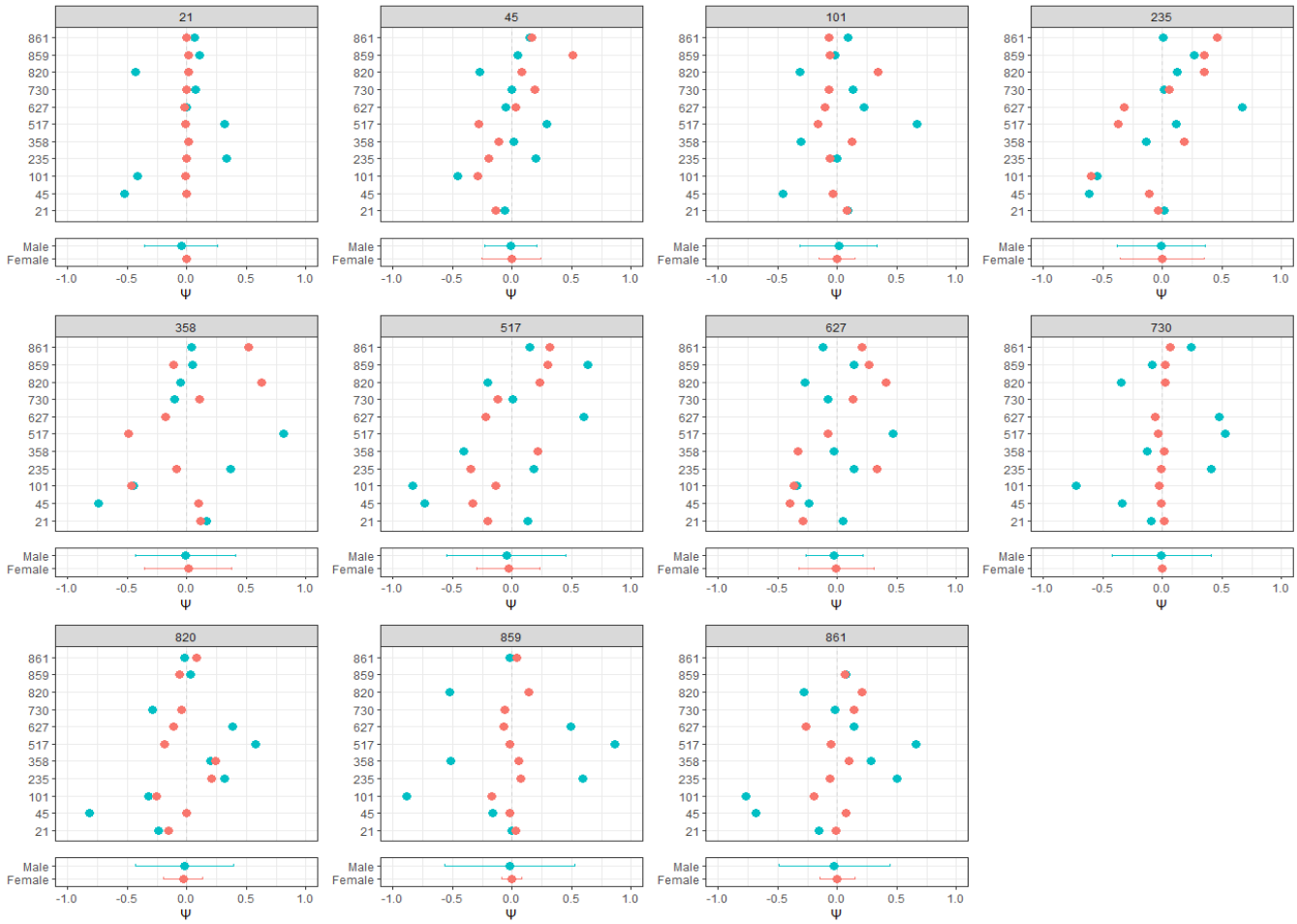


Figure 4. The sex-specific estimates of Ψ for sperm ejection time when each focal genotypic line is paired with each interacting genotype. Each box above represents a focal genotype, denoted by the grey label above each graph. Within each box, each point represents the Ψ value when each focal male (blue) or female (red) genotype is crossed with a specific interacting genotype, denoted on the y axis. The vertical dashed line represents the average sperm ejection time of the focal genotype, when male and female respectively. The further away a point is from the dashed line, the greater the phenotype deviates from the focal genotypes' phenotypic average when crossed with that specific interacting genotype: representing the strength of the IGE. A point left of the dashed line suggests that an interacting genotype drives an ejection time shorter than the focal genotypes' phenotypic average: representing the direction of the IGE. Each box below summarises the overall mean ± SD of Ψ when the focal male (blue) or female (red) genotype is crossed with all interacting genotypes of the opposite sex: representing the magnitude of IGEs. Details on how Ψ is calculated when the focal genotype is male and female can be found in Supplementary Figure S2.

4. Discussion

We aimed to reveal the underlying quantitative genetic basis of sperm ejection, a sexually selected trait, by considering that this is an interactive phenotype, subject to both genetic influences of the focal individual and the heritable environment component of the interacting sex. Using this modified quantitative genetic framework, we show that sperm ejection timing is heritable and that both direct effects and IGEs play a key role in trait expression. By using a diallel approach we found significant line- and sex-specific effects influence sperm ejection timing, and identify the specific genotypes that drive these significant effects. We also show that the magnitude with which IGEs influence phenotypic variation was genotype- and sex-specific, with focal female ejection time less affected by interactive male genotypes than in the opposite direction. To date, work examining interactive phenotypes has largely been dominated by experiments focusing on traits in which separate phenotypic values can be measured in each sex. Our work fills a research gap by quantifying the role of direct and IGEs on an antagonistic trait in which the phenotypic value is shared between the sexes and whose fitness consequences are well-described (Lüpold *et al.*, 2012, 2013). In doing so we identify the capacity with which direct and IGEs maintain post-copulatory trait variation, and examine how sexual conflict plays a role in the evolutionary trajectories of the sexes.

A traditional quantitative genetics framework derives heritability by examining the additive influence of parent genes on offspring phenotype. Here, additive line-specific genotypic effects represent a heritability-like measure, indicating whether intrinsic effects of genotype, independent of sex, significantly contribute to ejection time. Our diallel study found that the timing of sperm ejection is heritable and that line-specific genotypic effects and sex-specific effects significantly contribute to phenotypic variation. Taken together, these patterns suggest that there is substantial phenotypic variation in the population that is maintained by direct genotypic effects, providing significant genetic variation for evolution to act on. Additionally, significant sex-specific effects support the idea that sperm ejection is a sexual conflict trait and that antagonistic interactions contribute to the phenotypic variation observed.

Identifying the direct genetic contribution is insufficient to understanding putative evolutionary responses to selection when considering the evolution of interactive phenotypes. As predicted, we show that IGEs influence phenotypic variance in sperm ejection. Within each focal genotypic line, both the strength and the direction of Ψ varied depending on the interacting genotype, showing that the phenotypic outcome clearly depends on the reproductive partner. IGEs likely contribute to the persistence of sperm ejection time variance within a population, providing support for the idea that IGEs provide a resolution to the lek paradox (Miller and Moore, 2007). Such variation may be maintained through genotype-dependent trait preferences in each sex, and/or sexual conflict. The latter would mediate the evolution of multiple antagonistic male persistence and/or female resistance traits among the tested lines, meaning that the ability to disrupt a partner's influence on phenotypic outcome is cross dependent (Moore and Pizarri, 2005).

We also show that the magnitude of this IGE is large, and displays significant sex-specific variation, with male interacting phenotype having less influence on focal female phenotype than the reverse. Although it seems reasonable to assume that females would benefit from flexibly adjusting ejection time according to partner genotype (Lüpold *et al.*, 2013), it has been suggested that, under sexual conflict, limited variation in Ψ represents a reduced effect of manipulation by an interacting genotype (Moore and Pizarri, 2005). Subsequently, our results indicate strong selection for traits that counteract

male manipulation across all lines via sexually antagonistic coevolution, with certain genotypes better able to resist male manipulation than others. These results corroborate previous work suggesting that the timing of ejection is explained by the genotypes of both sexes (Lüpold *et al.*, 2020) using a different *D. melanogaster* genetic background. As this previous study used limited numbers of isofemale lines and did not use a quantitative genetic framework that considers the effect of the interacting phenotypes, our analysis expands on what was previously known about this trait, quantifying the extent to which genotype- and sex-specific IGEs influence phenotypic outcome.

Additionally, our results show that Ψ shows genotype-specific variation within a population, meaning that Ψ itself, the extent with which interacting genotypes influence focal phenotype, can itself respond to selection, the prerequisite conditions for Ψ to evolve (Chenoweth, Rundle and Blows, 2010; Kazancıoğlu, Klug and Alonzo, 2012). Our experimental design ensures that variation in Ψ is not an experimental artifact as we eliminated sources of within-line and abiotic variation. Consequently, within population variation in Ψ observed provides evidence that the strength and direction of IGEs on sperm ejection may evolve over time and provides an additional mechanism by which IGEs can shape the evolution of this sexually selected trait.

The present study cannot address the genetic basis underlying variance in sperm ejection time, but there are several genetic mechanisms that have been proposed. Allelic variation in candidate genes associated with sperm ejection likely contribute to cross-specific phenotypic variance (Wigby *et al.*, 2020). Numerous ejaculatory bulb seminal fluid protein genes have been identified as candidates influencing sperm ejection time. For example, *PEBme* is required for efficient coagulation of the mating plug and the maintenance of sperm in the female reproductive tract (Avila, Cohen, *et al.*, 2015), and *PEBII* influences plug size and female post mating responses (Avila, Wong, *et al.*, 2015). Specifically in females, the receptor *Dh44R1*, as part of the *Dh44* neuronal pathway, has been shown to affect sperm retention and storage (Lee *et al.*, 2015). Similarly, the thermosensitive cation channel *TRPA1* (*dTrpA1*) in *doublesex*-expressing cells influences sperm ejection, with higher activation resulting in the suppression of mating plug ejection (Laturney and Billeter, 2016). After transfer, many SFPs are processed (e.g., via proteolytic cleavage) (Avila and Wolfner, 2017), or bind to receptors within the FRT in order to function (Chapman, 2001). As a result, any variation in the FRT, even under a constant male genotype, may change SFP function depending on female genotype, influencing phenotypic outcome. Other candidates that are known to influence sperm storage and female remating rate, traits that are linked to the timing of ejection, may also represent useful candidates. For example, *Acp36DE* (Neubaum and Wolfner, 1999) and *Acp29AB* (Wong *et al.*, 2008) have been shown to influence sperm storage, and genomic variation in sex peptide (*SP*), a seminal fluid protein gene that affects female post mating response, influences paternity success depending on allelic variation in the female *SP* receptor *SPR* (Chow, Wolfner and Clark, 2010). However, confirmation that allelic variation has a direct influence on sperm ejection in any of the genes outlined above has not yet been tested and warrants further investigation in order to pinpoint the exact genetic variants underlying this trait.

In conclusion, by treating ejection time as an interactive phenotype, we provide a more comprehensive understanding of its underlying genetic mechanisms. Our results support the idea that IGEs have a strong influence on sexually selected phenotypes likely evolving via sexually antagonistic coevolution. We also show that IGEs contribute to the maintenance of phenotypic and genetic variation, which may be an underappreciated mechanism to explain the lek paradox. We also provide evidence that Ψ itself can evolve, for which empirical support has been observed only a handful of times. By incorporating greater genetic variation than previously utilized, and capitalizing on

sequenced isofemale lines, this work will enable future research to pinpoint new candidate genes, and identify allelic variation in existing candidate genes, that underlie the phenotypic variation in sperm ejection time.

5. References

Arnqvist, G. and Nilsson, T. (2000) 'The evolution of polyandry: multiple mating and female fitness in insects', *Animal Behaviour*, 60(2), pp. 145–164. Available at: https://doi.org/10.1006/anbe.2000.1446.

Arnqvist, G. and Rowe, L. (2005) *Sexual Conflict*. Princeton University Press.

Avila, F.W., Wong, A., *et al.* (2015) 'Don't pull the plug! the Drosophila mating plug preserves fertility', *Fly*, 9(2), pp. 62–67. Available at: https://doi.org/10.1080/19336934.2015.1120931.

Avila, F.W., Cohen, A.B., *et al.* (2015) 'Retention of Ejaculate by Drosophila melanogaster Females Requires the Male-Derived Mating Plug Protein PEBme', *Genetics*, 200(4), pp. 1171–1179. Available at: https://doi.org/10.1534/genetics.115.176669.

Avila, F.W. and Wolfner, M.F. (2017) 'Cleavage of the Drosophila seminal protein Acp36DE in mated females enhances its sperm storage activity', *Journal of Insect Physiology*, 101, pp. 66–72. Available at: https://doi.org/10.1016/j.jinsphys.2017.06.015.

Bailey, N.W. and Hoskins, J.L. (2014) 'DETECTING CRYPTIC INDIRECT GENETIC EFFECTS', *Evolution*, 68(7), pp. 1871–1882. Available at: https://doi.org/10.1111/evo.12401.

Bailey, N.W., Marie-Orleach, L. and Moore, A.J. (2018) 'Indirect genetic effects in behavioral ecology: does behavior play a special role in evolution?', *Behavioral Ecology*, 29(1), pp. 1–11. Available at: https://doi.org/10.1093/beheco/arx127.

Bailey, N.W. and Moore, A.J. (2012) 'Runaway Sexual Selection Without Genetic Correlations: Social Environments and Flexible Mate Choice Initiate and Enhance the Fisher Process', *Evolution*, 66(9), pp. 2674–2684. Available at: https://doi.org/10.1111/j.1558-5646.2012.01647.x.

Bailey, N.W. and Zuk, M. (2012) 'Socially flexible female choice differs among populations of the Pacific field cricket: geographical variation in the interaction coefficient psi (Ψ)', *Proceedings of the Royal Society B: Biological Sciences*, 279(1742), pp. 3589–3596. Available at: https://doi.org/10.1098/rspb.2012.0631.

Birkhead, T. and Møller, A. (1993) 'Female control of paternity', *Trends in Ecology & Evolution*, 8(3), pp. 100–104. Available at: https://doi.org/10.1016/0169-5347(93)90060-3.

Birkhead, T.R. (1998) 'CRYPTIC FEMALE CHOICE: CRITERIA FOR ESTABLISHING FEMALE SPERM CHOICE', *Evolution*, 52(4), pp. 1212–1218. Available at: https://doi.org/10.1111/j.1558-5646.1998.tb01848.x.

Birkhead, T.R. and Pizzari, T. (2002) 'Postcopulatory sexual selection', *Nature Reviews Genetics*, 3(4), pp. 262–273. Available at: https://doi.org/10.1038/nrg774.

Bonduriansky, R. and Chenoweth, S.F. (2009) 'Intralocus sexual conflict', *Trends in Ecology & Evolution*, 24(5), pp. 280–288. Available at: https://doi.org/10.1016/j.tree.2008.12.005.

Chapman, T. (2001) 'Seminal fluid-mediated fitness traits in Drosophila', *Heredity*, 87(5), pp. 511–521. Available at: https://doi.org/10.1046/j.1365-2540.2001.00961.x.

Chapman, T. *et al.* (2003) 'The sex peptide of Drosophila melanogaster: Female post-mating responses analyzed by using RNA interference', *Proceedings of the National Academy of Sciences*, 100(17), pp. 9923–9928. Available at: https://doi.org/10.1073/pnas.1631635100.

Chenoweth, S.F., Rundle, H.D. and Blows, M.W. (2010) 'EXPERIMENTAL EVIDENCE FOR THE EVOLUTION OF INDIRECT GENETIC EFFECTS: CHANGES IN THE INTERACTION EFFECT COEFFICIENT, PSI (ψ), DUE TO SEXUAL SELECTION', *Evolution*, 64(6), pp. 1849–1856.

Chow, C.Y., Wolfner, M.F. and Clark, A.G. (2010) 'The Genetic Basis for Male × Female Interactions Underlying Variation in Reproductive Phenotypes of Drosophila', *Genetics*, 186(4), pp. 1355–1365. Available at: https://doi.org/10.1534/genetics.110.123174.

Cockerham, C.C. and Weir, B.S. (1977) 'Quadratic Analyses of Reciprocal Crosses', *Biometrics*, 33(1), pp. 187–203. Available at: https://doi.org/10.2307/2529312.

Collet, J.M. and Sztepanacz, J.L. (2022) 'Mating traits are phenotypically but not genetically correlated to fitness', *Behavioral Ecology*, 33(4), pp. 833–843. Available at: https://doi.org/10.1093/beheco/arac047.

Crowley, J.J. *et al.* (2014) 'Genetics of Adverse Reactions to Haloperidol in a Mouse Diallel: A Drug–Placebo Experiment and Bayesian Causal Analysis', *Genetics*, 196(1), pp. 321–347. Available at: https://doi.org/10.1534/genetics.113.156901.

Danielson-François, A.M., Zhou, Y. and Greenfield, M.D. (2009) 'Indirect genetic effects and the lek paradox: inter-genotypic competition may strengthen genotype × environment interactions and conserve genetic variance', *Genetica*, 136(1), pp. 27–36. Available at: https://doi.org/10.1007/s10709-008-9297-z.

Dapper, A.L. and Wade, M.J. (2016) 'The evolution of sperm competition genes: The effect of mating system on levels of genetic variation within and between species', *Evolution*, 70(2), pp. 502–511. Available at: https://doi.org/10.1111/evo.12848.

Dingemanse, N.J. and Araya-Ajoy, Y.G. (2015) 'Interacting personalities: behavioural ecology meets quantitative genetics', *Trends in Ecology & Evolution*, 30(2), pp. 88–97. Available at: https://doi.org/10.1016/j.tree.2014.12.002.

Eberhard, W. (1996) *Female Control: Sexual Selection by Cryptic Female Choice*. Princeton University Press.

Edward, D.A. *et al.* (2014) 'Sexual Conflict and Interacting Phenotypes: A Quantitative Genetic Analysis of Fecundity and Copula Duration in Drosophila Melanogaster', *Evolution*, 68(6), pp. 1651–1660. Available at: https://doi.org/10.1111/evo.12376.

Firman, R.C. *et al.* (2017) 'Postmating Female Control: 20 Years of Cryptic Female Choice', *Trends in Ecology & Evolution*, 32(5), pp. 368–382. Available at: https://doi.org/10.1016/j.tree.2017.02.010.

Hopkins, B.R. *et al.* (2019) 'BMP signaling inhibition in Drosophila secondary cells remodels the seminal proteome and self and rival ejaculate functions', *Proceedings of the National Academy of Sciences*, 116(49), pp. 24719–24728. Available at: https://doi.org/10.1073/pnas.1914491116.

Huang, W. *et al.* (2014) 'Natural variation in genome architecture among 205 Drosophila melanogaster Genetic Reference Panel lines', *Genome Research*, 24(7), pp. 1193–1208. Available at: https://doi.org/10.1101/gr.171546.113.

Jennions, M.D. and Petrie, M. (2000) 'Why do females mate multiply? A review of the genetic benefits', *Biological Reviews of the Cambridge Philosophical Society*, 75(1), pp. 21–64. Available at: https://doi.org/10.1017/s0006323199005423.

Kazancıoğlu, E., Klug, H. and Alonzo, S.H. (2012) 'The Evolution of Social Interactions Changes Predictions About Interacting Phenotypes', *Evolution*, 66(7), pp. 2056–2064. Available at: https://doi.org/10.1111/j.1558-5646.2012.01585.x.

Kirkpatrick, M. and Barton, N.H. (1997) 'The strength of indirect selection on female mating preferences', *Proceedings of the National Academy of Sciences of the United States of America*, 94(4), pp. 1282–1286. Available at: https://doi.org/10.1073/pnas.94.4.1282.

Laturney, M. and Billeter, J.-C. (2016) 'Drosophila melanogaster females restore their attractiveness after mating by removing male anti-aphrodisiac pheromones', *Nature Communications*, 7(1), p. 12322. Available at: https://doi.org/10.1038/ncomms12322.

Lee, K.-M. *et al.* (2015) 'A Neuronal Pathway that Controls Sperm Ejection and Storage in Female Drosophila', *Current Biology*, 25(6), pp. 790–797. Available at: https://doi.org/10.1016/j.cub.2015.01.050.

Lenarcic, A.B. *et al.* (2012) 'A General Bayesian Approach to Analyzing Diallel Crosses of Inbred Strains', *Genetics*, 190(2), pp. 413–435. Available at: https://doi.org/10.1534/genetics.111.132563.

Lung, O. and Wolfner, M.F. (2001) 'Identification and characterization of the major Drosophila melanogaster mating plug protein', *Insect Biochemistry and Molecular Biology*, 31(6), pp. 543–551. Available at: https://doi.org/10.1016/S0965-1748(00)00154-5.

Lüpold, S. *et al.* (2012) 'How Multivariate Ejaculate Traits Determine Competitive Fertilization Success in Drosophila melanogaster', *Current Biology*, 22(18), pp. 1667–1672. Available at: https://doi.org/10.1016/j.cub.2012.06.059.

Lüpold, S. *et al.* (2013) 'Female mediation of competitive fertilization success in *Drosophila melanogaster*', *Proceedings of the National Academy of Sciences*, 110(26), pp. 10693–10698. Available at: https://doi.org/10.1073/pnas.1300954110.

Lüpold, S. *et al.* (2020) 'How female × male and male × male interactions influence competitive fertilization in Drosophila melanogaster', *Evolution Letters*, 4(5), pp. 416–429. Available at: https://doi.org/10.1002/evl3.193.

Mackay, T.F.C. *et al.* (2012) 'The Drosophila melanogaster Genetic Reference Panel', *Nature*, 482(7384), pp. 173–178. Available at: https://doi.org/10.1038/nature10811.

Manier, M.K. *et al.* (2010) 'Resolving Mechanisms of Competitive Fertilization Success in Drosophila melanogaster', *Science*, 328(5976), pp. 354–357. Available at: https://doi.org/10.1126/science.1187096.

Marie-Orleach, L. *et al.* (2017) 'Indirect genetic effects and sexual conflicts: Partner genotype influences multiple morphological and behavioral reproductive traits in a flatworm', *Evolution*, 71(5), pp. 1232–1245. Available at: https://doi.org/10.1111/evo.13218.

Marie-Orleach, L., Janicke, T. and Schärer, L. (2013) 'Effects of mating status on copulatory and postcopulatory behaviour in a simultaneous hermaphrodite', *Animal Behaviour*, 85(2), pp. 453–461. Available at: https://doi.org/10.1016/j.anbehav.2012.12.007.

Maurizio, P.L. *et al.* (2017) 'Bayesian Diallel Analysis Reveals Mx1-Dependent and Mx1-Independent Effects on Response to Influenza A Virus in Mice', *G3: Genes|Genomes|Genetics*, 8(2), pp. 427–445. Available at: https://doi.org/10.1534/g3.117.300438.

McDonough-Goldstein, C.E., Pitnick, S. and Dorus, S. (2022) 'Drosophila female reproductive glands contribute to mating plug composition and the timing of sperm ejection', *Proceedings of the Royal Society B: Biological Sciences*, 289(1968), p. 20212213. Available at: https://doi.org/10.1098/rspb.2021.2213.

McGlothlin, J.W. *et al.* (2010) 'Interacting Phenotypes and the Evolutionary Process. Iii. Social Evolution', *Evolution*, 64(9), pp. 2558–2574. Available at: https://doi.org/10.1111/j.1558-5646.2010.01012.x.

Miller, C.W. and Moore, A.J. (2007) 'A potential resolution to the lek paradox through indirect genetic effects', *Proceedings of the Royal Society B: Biological Sciences*, 274(1615), pp. 1279–1286. Available at: https://doi.org/10.1098/rspb.2006.0413.

Moore, A.J., Brodie III, E.D. and Wolf, J.B. (1997) 'Interacting Phenotypes and the Evolutionary Process: I. Direct and Indirect Genetic Effects of Social Interactions', *Evolution*, 51(5), pp. 1352–1362. Available at: https://doi.org/10.1111/j.1558-5646.1997.tb01458.x.

Moore, A.J. and Pizzari, T. (2005) 'Quantitative Genetic Models of Sexual Conflict Based on Interacting Phenotypes.', *The American Naturalist*, 165(S5), pp. S88–S97. Available at: https://doi.org/10.1086/429354.

Murphy, K. *et al.* (2008) 'Response to selection, combining ability and heritability of coleoptile length in winter wheat', *Euphytica*, 164(3), pp. 709–718. Available at: https://doi.org/10.1007/s10681-008-9692-7.

Neubaum, D.M. and Wolfner, M.F. (1999) 'Mated Drosophila melanogaster Females Require a Seminal Fluid Protein, Acp36DE, to Store Sperm Efficiently', *Genetics*, 153(2), pp. 845–857. Available at: https://doi.org/10.1093/genetics/153.2.845.

Parker, G.A. (1970) 'Sperm Competition and Its Evolutionary Consequences in the Insects', *Biological Reviews*, 45(4), pp. 525–567. Available at: https://doi.org/10.1111/j.1469-185X.1970.tb01176.x.

Pennell, T.M. and Morrow, E.H. (2013) 'Two sexes, one genome: the evolutionary dynamics of intralocus sexual conflict', *Ecology and Evolution*, 3(6), pp. 1819–1834. Available at: https://doi.org/10.1002/ece3.540.

Qvarnström, A., Brommer, J.E. and Gustafsson, L. (2006) 'Testing the genetics underlying the co-evolution of mate choice and ornament in the wild', *Nature*, 441(7089), pp. 84–86. Available at: https://doi.org/10.1038/nature04564.

R Core Team (2016) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Rice, W. and Holland, B. (1997) 'The enemies within: Intergenomic conflict, interlocus contest evolution (ICE), and the intraspecific Red Queen', *Behavioral Ecology and Sociobiology*, 41, pp. 1–10. Available at: https://doi.org/10.1007/s002650050357.

Schenkel, M.A. *et al.* (2018) 'Making sense of intralocus and interlocus sexual conflict', *Ecology and Evolution*, 8(24), pp. 13035–13050. Available at: https://doi.org/10.1002/ece3.4629.

Schneider, M.R., Mangels, R. and Dean, M.D. (2016) 'The molecular basis and reproductive function(s) of copulatory plugs', *Molecular Reproduction and Development*, 83(9), pp. 755–767. Available at: https://doi.org/10.1002/mrd.22689.

Snook, R.R. and Hosken, D.J. (2004) 'Sperm death and dumping in Drosophila', *Nature*, 428(6986), pp. 939–941. Available at: https://doi.org/10.1038/nature02455.

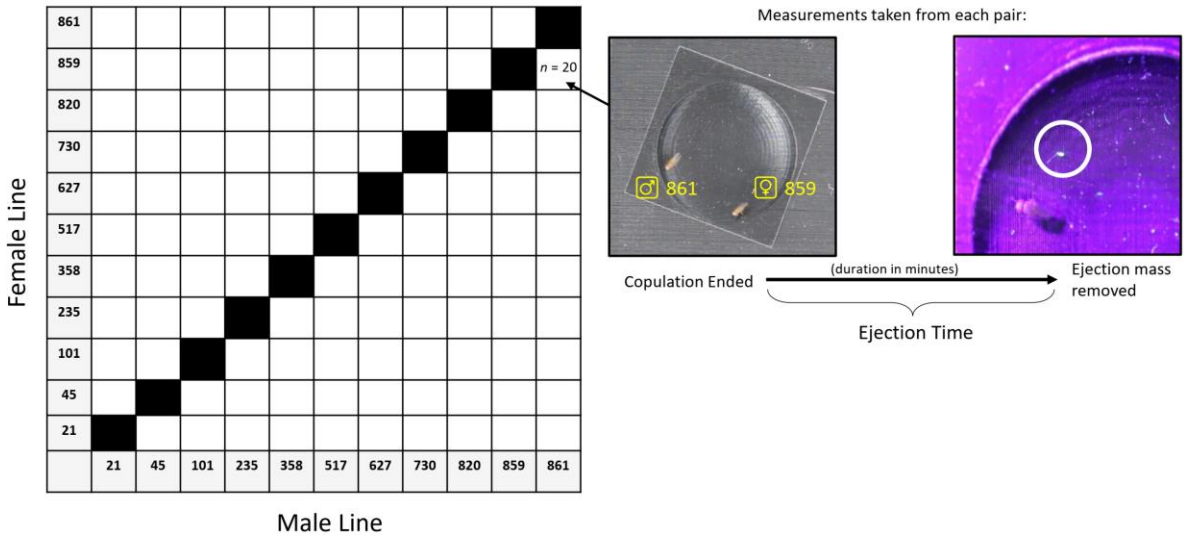
Taylor, M.L., Evans, J.P. and Garcia-Gonzalez, F. (2013) 'No Evidence for Heritability of Male Mating Latency or Copulation Duration across Social Environments in Drosophila melanogaster', *PLOS ONE*, 8(10), p. e77347. Available at: https://doi.org/10.1371/journal.pone.0077347.

Van Doorn, G.S. (2009) 'Intralocus Sexual Conflict', *Annals of the New York Academy of Sciences*, 1168(1), pp. 52–71. Available at: https://doi.org/10.1111/j.1749-6632.2009.04573.x.

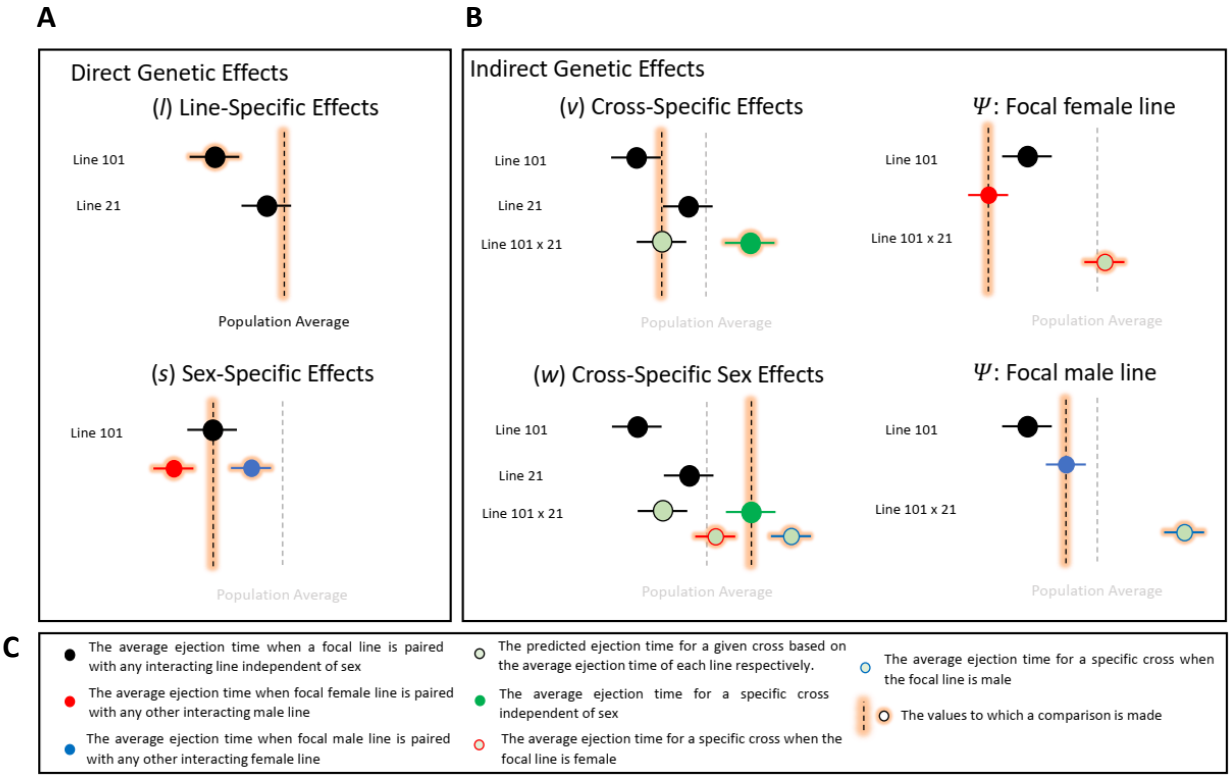
Wigby, S. *et al.* (2020) 'The Drosophila seminal proteome and its role in postcopulatory sexual selection', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 375(1813), p. 20200072. Available at: https://doi.org/10.1098/rstb.2020.0072.

Wong, A. *et al.* (2008) 'A role for Acp29AB, a predicted seminal fluid lectin, in female sperm storage in Drosophila melanogaster', *Genetics*, 180(2), pp. 921–931. Available at: https://doi.org/10.1534/genetics.108.092106.

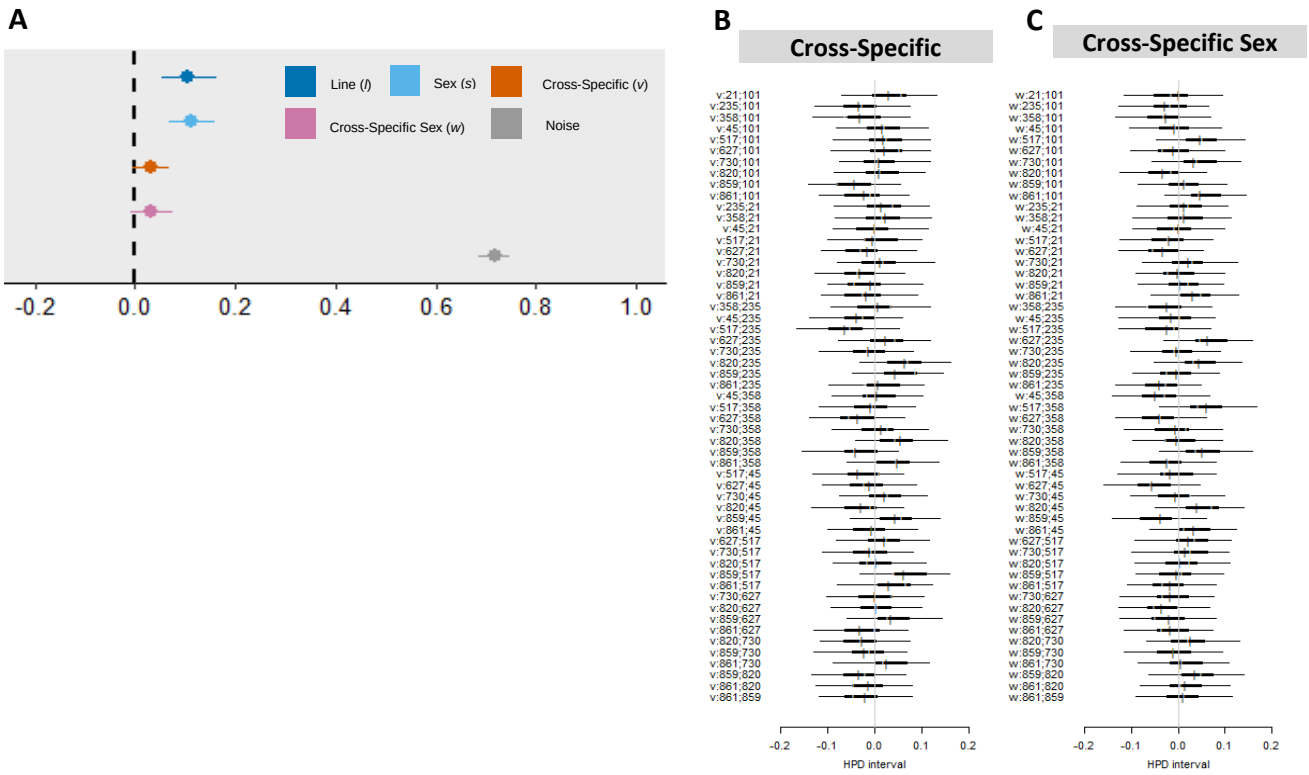
6. Chapter 1 – Supplementary Material



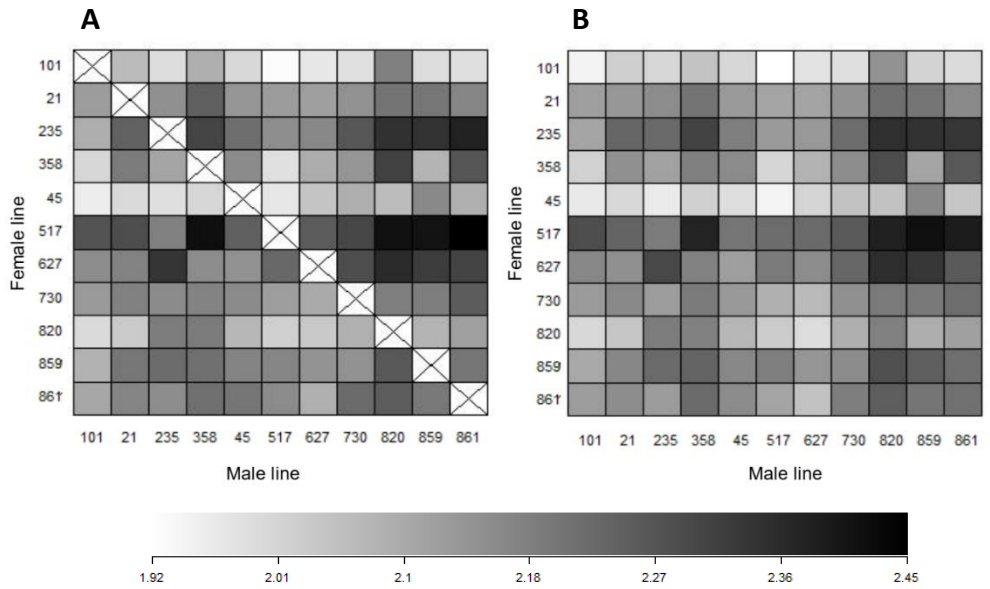
Supplementary Figure S1. A schematic of the diallel mating design. Eleven isofemale lines were crossed in a full diallel mating design excluding reciprocal crosses (represented as a black square), representing 110 possible male-by-female genotypic crosses. For each genotypic cross, one male and one female from different lines were introduced into a chamber. The duration in minutes from copulation ending to the female ejecting was recorded as the ejection time. A photo of the latter is shown, with the ejection mass highlighted by a white circle. Between 14 and 26 matings were recorded for each cross. A pairing between male 861 and female 859 is illustrated in the figure. A pairing between these two lines was conducted 20 times.



Supplementary Figure S2. A schematic to describe how Direct Genetic Effects and Indirect Genetic Effects were quantified for ejection time. Direct genetic effects (A) are composed of Line-Specific Effects (*l*) and Sex-Specific Effects (*s*). Line-Specific Effects are derived by comparing the average ejection time when a focal line is paired with all other interacting genotypic lines, independent of sex, to the population average. Sex-Specific Effects (*s*) are derived by comparing the average ejection time deviation when the focal line is female, with male as a baseline, compared to the overall average ejection time of that line. Indirect Genetic Effects (B) are composed of Cross-Specific Effects (*v*), Cross-Specific Sex Effects (*w*), Ψ when the focal line is female and Ψ when the focal line is male. Cross-Specific Effects (*v*) are derived by comparing the predicted ejection time for a given cross (between a focal genotype and an interacting genotype) based on the average ejection time of each line respectively to the actual average ejection time of that specific cross, independent of sex. Cross-Specific Sex Effects (*w*) are derived by comparing the average ejection time deviation when the focal line is female for a given cross to the average ejection time of that specific cross (with the focal line when male as a baseline; i.e. 101 x 21 vs 21 x 101). Ψ: Focal female line is quantified by identifying the extent to which the average ejection time for a given focal line when female (101) differs from the average ejection time when that focal female line is paired to a specific interacting male genotype (21). Ψ: Focal male line is quantified by identifying the extent to which the average ejection time for a given focal line when male (101) differs from the average ejection time when that focal male line is paired to a specific interacting female genotype (21). A legend (C) describes each component presented in figures (A) and (B).



Supplementary Figure S3. BayesDiallel analysis of sperm ejection time. (A) VarP Plot describing the variance projection of each diallel class. It predicts how much of the total phenotypic sum of squares is explained by each model component. The percentage of the variance in ejection time explained by diallel effects, a broad-sense heritability like measure, is 28%. Additive genotypic effects (l), a narrow-sense heritability like measure, explain 11% ± 3%. Sex-specific effects (s) account for 11% ± 2%. Cross-specific interactions (v and w) both account for 6% ± 2%. The black dotted line represents the significance threshold, so any model component that overlaps the dotted line does not explain a significant amount of the phenotypic variance. (B) Highest posterior density (HPD) intervals of sperm ejection based on the cross-specific effects (v) from the $lsvw$ model. (C) Highest posterior density (HPD) intervals of sperm ejection based on the cross-specific sex effects (w) from the $lsvw$ model. For each effect in (B) and (C), thin and thick horizontal lines show the 95 and 50% HPD intervals, respectively, with short vertical bars indicating the posterior mean. HPD intervals that do not include zero we consider to be statistically significant at 95% credibility. Overall there was no significant cross-specific or cross-specific sex effects. Details on how model effects (l , s, v, w) are calculated can be found in Supplementary Figure S2.



Supplementary Figure S4. Observed (A) and Predicted (B) means from the BayesDiallel model. Shading indicates log ejection time on a scale from 1.92 log-mins (lighter) to 2.45 log-mins (darker) from a total of 2056 pairings between eleven male and female DGRP lines. (A) The average phenotypic value for each pairing represented as shaded cell, where darker shading represents a longer ejection time. Crossed boxes indicate the absence of pairings. (B) The average phenotypic value for each pairing that would be expected on the basis of the model and the observed data, incorporating all uncertainty due to finite sampling and prior uncertainty about the parameters. The observed and predicted mean ejection times were largely similar, suggesting that the distributions predicted by the model largely represent our raw data. Note that genotype order is not in numerical order and differs from the other figures.

Chapter 2

Quantitative genetics of interactive pre- and post-copulatory traits

Matilda Q.R. Pembury-Smith and Rhonda R. Snook

Abstract

In polyandrous mating systems, reproductive success is dependent on both pre- and post-copulatory traits. These traits may either trade-off between each other or positively correlate when the pre-copulatory trait is indicative of overall fitness. Simultaneously, many pre- and post-copulatory traits are interactive phenotypes, where the total genetic variation that can respond to selection depends on heritable indirect genetic effects (IGEs), in this case the genotype of the social partner. Models predict that IGEs play an important role in the evolutionary potential of a given trait. However, the impacts of IGEs on interactive pre- and post-copulatory traits, and how this may influence the relationship between pre- and post-copulatory episodes of sexual selection have rarely been examined. Here we take a quantitative genetic approach to identify the direct and indirect genetic architecture of three traits that span pre- and post-copulatory sexual selection in multiple isofemale lines of *D. melanogaster*. We found strong evidence that both direct and IGEs maintain phenotypic variation in mating latency, which measures mate attractiveness, and sperm ejection, which measures post-mating fertilisation success. We found limited evidence that copulation duration is influenced by IGEs despite both male and female genotype individually influencing phenotypic variation. While we observed significant phenotypic correlations between traits there was only weak evidence of additive genetic correlations, suggesting that for these traits, episodes of selection may act independently. Together, these data outline the underlying quantitative genetic basis behind phenotypic variation in interactive phenotypes that represent sequential stages of the reproductive process, to provide a better understanding of trait evolutionary dynamics across episodes of selection.

1. Introduction

Pre-copulatory sexual selection drives the evolution of traits that influence mating success (Andersson, 1994). Likewise, in polyandrous mating systems, spatial and temporal overlap of competing sperm from rival males drives post-copulatory sexual selection. Post-copulatory sexual selection favours the evolution of traits that influence fertilisation success via increasing sperm competitive ability (sperm competition; Parker, 1970) and traits that enable females to bias sperm use and storage of competing ejaculates (cryptic female choice; Thornhill, 1983; Eberhard, 1996; Birkhead, 1998). Consequently, in polyandrous species, paternity success is affected by secondary sexual characteristics and traits that influence fertilisation success. Relative pre- and post-copulatory trait investment may depend on their energetic costs and the importance of both selection episodes to fitness (Lüpold *et al.*, 2014). The phenotype-linked fertility hypothesis (Sheldon, 1994) predicts a positive correlation between pre- and post-copulatory fitness if the former is indicative of genetic quality (reviewed by Sheldon, 1994; Johnstone, 1995; Rowe and Houle, 1997). In contrast, as trait investment is metabolically expensive (Remick, 1992; Rowe and Houle, 1997), sperm competition game theory predicts a trade-off between pre- and post-copulatory trait investment, resulting in a negative correlation between pre- and post-copulatory fitness (Pitnick, 1996; Simmons, Lüpold and Fitzpatrick, 2017).

To understand the evolution of traits subject to sexual selection, the genetic basis of these correlations must also be examined (Simmons, Lüpold and Fitzpatrick, 2017). Positive genetic covariance between

pre- and post-copulatory fitness (due to pleiotropy and/or linkage between traits) suggests correlated changes between phenotypes, enhancing trait evolutionary potential (Kvarnemo and Simmons, 2013). In comparison, negative covariance slows the rate of evolutionary change (Nelson and Crone, 1999). Existing work has demonstrated both positive (Simmons and Kotiaho, 2002; Hosken *et al.*, 2008) and negative (Evans, 2010; Simmons, Tinghitella and Zuk, 2010) genetic correlations between traits involved in pre- and post-copulatory episodes of selection. However, other work has found that pre- and post-copulatory traits are genetically uncorrelated, suggesting that each episode of selection acts independently (Travers, Garcia-Gonzalez and Simmons, 2016; Collet and Sztepanacz, 2022). Therefore, investigating both phenotypic and genetic correlations between pre- and post-copulatory traits is required to fully understand their relationship, and the capacity for coevolutionary responses to selection.

Understanding how selection causes evolutionary change in a given trait requires discerning its underlying genetic variance. This is particularly interesting when examining the genetic basis of interactive phenotypes, traits which require or are influenced by conspecific interactions. Here, the conspecific genotype becomes a heritable environmental component of the focal individual, generating indirect genetic effects (IGEs). IGEs are the influence of conspecific genotypes on phenotypic outcome (Moore, Brodie III and Wolf, 1997; Wolf, Brodie III and Moore, 1999; Bleakley and Brodie III, 2009; McGlothlin *et al.*, 2010), and can therefore facilitate the maintenance of phenotypic variance, and influence the rate of selection and evolution of a given trait (Moore, Brodie III and Wolf, 1997; Wolf, Brodie III and Moore, 1999; Bleakley and Brodie III, 2009; McGlothlin *et al.*, 2010; Bailey and Moore, 2012; Dingemanse and Araya-Ajoy, 2015).

Many sexually selected interactive phenotypes represent characteristics of an interaction between two copulating individuals, such as the latency to mate. In these traits, only a single shared phenotype can be measured for both sexes (Dingemanse and Araya-Ajoy, 2015). For such phenotypes, IGEs are expected to generate inter-locus sexual conflict (Rice and Holland, 1997; Pennell and Morrow, 2013; Dapper and Wade, 2016; Firman *et al.*, 2017). This is because phenotypic outcome affects the fitness of both sexes, yet the phenotypic optima rarely align, resulting in antagonistic selection on sex-specific traits. As a result, studies that only examine genetic variance in a single sex and ignore IGEs limit their ability to detect underlying genetic variation in antagonistic traits. Despite this, to date no study has quantified the impact of direct and IGEs on single shared pre- and post-copulatory traits, and how this may influence the relationship between episodes of selection. To address this research gap, we take a quantitative genetics approach using the polyandrous species *Drosophila melanogaster*. We identify the extent to which phenotypic variance is directly influenced by the genotype of each focal sex and indirectly by the interacting sex, and their phenotypic and genetic correlation, in three traits that span pre- and post-copulatory sexual selection episodes: the latency to mate, copulation duration and the timing of sperm ejection.

Mating latency, the time from a pair being introduced to copulation beginning, is an interactive pre-copulatory trait and a standard measure of female preference and male attractiveness (Fulker, 1966; Jennions and Petrie, 1997). As mating latency influences individual copulation frequency, there will be selection in males to reduce duration. Whether longer mating latencies maximise female fitness is unclear; however, existing work has suggested that females benefit from mating rates lower than the male optima (Holland and Rice, 1998). As a result, antagonistic selection pressures likely act on both sexes to control phenotypic outcome. Despite this, in *D. melanogaster*, the quantitative genetic

mechanisms underlying this trait are ambiguous, with conflicting results reported in the literature. For example, existing work has suggested that female genotype alone (Mackay *et al.*, 2005), the genotypes of both sexes individually (Tennant, Sonser and Long, 2014), or their joint interaction (Ratterman *et al.*, 2014) contribute to phenotypic variance. In addition, work examining heritability specifically has found that mating latency is heritable (Hoffmann, 1999), whereas others have found no such evidence of heritability, with neither genotype significantly contributing to phenotypic variance (Taylor, Evans and Garcia-Gonzalez, 2013). However, in all mentioned studies different populations or genetic lines were used which may contribute to the inconsistencies, and in the latter study mating latency was measured using a random sample of females which may inflate estimates of phenotypic variance and thus underestimate heritability. Therefore, replication using additional genotypes would provide further insight on trait heritability, the extent with which each sex contributes to phenotypic variation, and subsequently the degree to which this trait is under conflict in this species.

Copulation duration is defined as the length of time from the male copulatory organ entering the female until the male and female disengage. In some species there is a positive correlation between copulation duration and the amount of sperm that enters the female. This means that longer copulations facilitate sperm transfer, increasing paternity success (Edvardsson and Canal, 2006). In other species, such as *D. melanogaster*, sperm transfer is unrelated to the duration of copulation. However, copulation duration can also have functions additional to sperm transfer that influence paternity — these include mate guarding (Parker, 1970; Alcock, 1994), altering female post-mating behaviour (Chapman *et al.*, 1995; Singh and Singh, 1999), and facilitating the removal of rival sperm (Parker, 1970). As such, there may be selection in males to prolong copulation beyond what is optimal for females, resulting in antagonistic selection in both sexes to control duration. However, the extent to which the genotype of each sex contributes to the timing of copulation and whether this trait is under conflict in *D. melanogaster* is still ambiguous. Some studies have suggested that copulation duration is a heritable trait and that the genotype of both sexes contribute to phenotypic outcome (Edward *et al.*, 2014). In contrast, others have found no evidence of heritability (Taylor, Evans and Garcia-Gonzalez, 2013; Travers, Garcia-Gonzalez and Simmons, 2016). However, as mentioned previously, the latter work may have under estimated heritability due to random female sampling, necessitating further experiments.

Finally, the timing of sperm ejection is a known mechanism of post-copulatory sexual selection. In *D. melanogaster*, mating plugs (a post-copulatory sex-specific trait) form within the female reproductive tract (Parker, 1970; Lung and Wolfner, 2001; Schneider, Mangels and Dean, 2016). Plug formation alters male paternity as it increases sperm storage potential and reduces female attractiveness in future copulations (Schneider, Mangels and Dean, 2016). Therefore, the timing of plug ejection influences the outcome of competitive fertilisation (Snook and Hosken, 2004; Manier *et al.*, 2010; Lüpold *et al.*, 2013; Firman *et al.*, 2017). Specifically, longer ejection times increase male paternity (Lüpold *et al.*, 2013) as this is largely determined by the proportion of each male's sperm in storage (Manier *et al.*, 2010; Lüpold *et al.*, 2012). The timing of ejection is therefore thought to be an intrinsic source of sexual conflict as females may optimise fitness based on an ejection time different from her partner's optima (Arnqvist and Rowe, 2005; McDonough-Goldstein, Pitnick and Dorus, 2022). Previous work has shown that sperm ejection in *D. melanogaster* is determined by genetic variance in both sexes, and their interaction (Lüpold *et al.*, 2020), with recent work quantifying the magnitude and direction with which conspecific genotypes influence a focal individual's phenotype (Pembury Smith & Snook, unpublished; *Chapter I*). However, the extent to which the timing of sperm ejection

correlates phenotypically and genetically with the other two fitness-related traits in this species remains unknown.

We use a quantitative genetic framework to examine to what extent phenotypic variance is influenced by the genotype of each focal sex and indirectly by the interacting sex in pre- and post-copulatory traits, and to what extent their relationship phenotypically and genetically correlate. We employ a full diallel cross design using eleven isofemale lines from the *D. melanogaster* Genetic Reference Panel (DGRP; Mackay *et al.*, 2005; Huang *et al.*, 2014). This framework allows us to directly and reciprocally manipulate the genetic component of the social environment (the interacting sex) to identify direct genetic effects, as well as to what extent male and female genotype, and their interaction, contribute to phenotypic variation. This design improves upon existing work by increasing the power to detect underlying genetic variation. In cases where phenotypic variation is influenced by a significant male-by-female genotypic interaction, we quantify the influence of IGEs using the parameter Ψ (Moore, Brodie III and Wolf, 1997). After experimentally determining direct and IGEs for each trait, we then ascertain the relationship between all pair-wise comparisons of the three traits. In doing so, we test whether phenotypic and genetic correlations are consistent with the mutually exclusive prediction of the phenotype-link fertility hypothesis or the sperm competition game theory model. As both hypotheses have strong implications for trait evolutionary potential, examining this relationship provides insight into the capacity with which an interaction between both episodes of selection influences trait evolution.

2. Materials and Methods

2.1 Fly Stocks

Eleven randomly selected isogenic lines from the DGRP were used for this study (DGRP-21, -45, -101, -235, -358, -517, -627, -730, -820, -859, -861; Mackay *et al.*, 2005; Huang *et al.*, 2014). DGRP lines originate from a single wild population collected in Raleigh, North Carolina, in 2003, where 20 generations of full-sibling matings were conducted for each line, resulting in a panel of 205 inbred lines that have been sequenced. In our lab, all lines were housed in standard culture vials containing 5ml of a standard food medium (1L water: 80g medium cornmeal, 18g dried yeast, 10g soya flour, 80g malt extract, 40g molasses, 8g agar, 25 mL of 10% Nipagin, 4 mL of propionic acid) at 12h light:12h dark cycle. No ethical approval was required for the work. These stocks were used to generate experimental animals. Flies and all experiments were kept at 25°C.

2.2 Production of focal individuals

To generate focal individuals, each line was placed in food vials. Each vial had a ca. 1:1 sex ratio and 20 individuals per vial. Parent flies were removed after three days and, ca. eight days later, virgin focal offspring were collected within 2h after eclosion under light CO_2 anaesthesia. Sexes were housed separately with 10-15 individuals per vial prior to experiments. Focal individuals were collected across five consecutive days, followed by five consecutive days of experiments, making up a single ten-day block. 14 blocks were performed. Thus, focal individuals for subsequent experiments were six days old.

2.3 Quantifying phenotypic measurements

We measured mating latency (the delay between a pair being introduced into a vial and the time at which copulation started), copulation duration (the length of time from the male copulatory organ

entering the female until the male and female disengage), and ejection time (the delay between the end of copulation and the time at which the female ejected). To acquire these data, all isofemale lines were crossed in a full diallel mating design excluding reciprocal crosses, producing 110 crosses in total (Figure S1).

One male and one female from different randomly selected DGRP lines were introduced into a 3D-printed black plastic chamber, consisting of a cuboid of 34 mm x 33 mm x 9 mm with a hemispherical cavity of diameter 20 mm and depth 7 mm (Hopkins *et al.*, 2019) (Figure S1). A glass coverslip was used to cover the cavity as each sex was introduced. Each chamber contained a drop of an agar-sugar solution to avoid desiccation stress. The male was always introduced to the chamber first.

Approximately 90 pairs were mated each day and all chambers were filmed with a camcorder (Panasonic HC-V180 or Sony HDR-CX405). The exact time at which a pair was introduced into a chamber was recorded. All chambers were observed every 3-5 minutes for 1 hour after the pair was introduced to identify the end of copulation (note that most copulations in the lines we used occur within the first hour). Following the end of copulation, each chamber was scanned using a fluorescent light at ca. ten-minute intervals to identify the time of ejection. Exact timing of mating latency, copulation duration and ejection was verified using video playback. If the pair had not ejected after nine hours following copulation, then the chamber was filmed overnight. If ejection was clearly visible on the video recording, then this data point was kept, otherwise the pair was excluded from the analysis.

Between 17 and 31 matings were observed for each cross producing a total of 2247 copulations for which we have measurements of mating latency and copulation duration (Figure 1). Analysis on Direct Genetic Effects (Section 2.4.2) and Indirect Genetic Effects (Section 2.4.3) for mating latency and copulation duration used this data set. Measurements for ejection time were excluded for 191 matings due to a technical fault with the camera. These datapoints were excluded from all analysis including sperm ejection.

2.4 Statistical Analysis

2.4.1 Analytical Approach

First, we identify whether there is a significant effect of male genotype, female genotype and their interaction on phenotypic outcome.

Next, we examine direct genetic effects: how an individual's genotype influences phenotypic outcome (Figure S2 A). This is divided into two components: line-specific (additive) effects and sex-specific effects. Line-specific (additive) effects ("l" in Figure S2 A) describes to what extent the mean phenotypic value of each genotype in turn differs from the population average, independent of sex. For example, genotype 45 may have an overall mean mating latency of 60 minutes which is significantly shorter than the population average which is 120 minutes. Sex-specific effects ("s" in Figure S2 A) describe to what extent the mean phenotypic value of each genotype differs when it is male or female. For example, genotype 45 may have an overall mean mating latency of 60 minutes, however, there may be a strong contrast between the sexes (i.e. 30 minutes when male and 90 minutes when female), or the sex-specific mean mating latency could be very similar (i.e. 58 minutes when male and 62 minutes when female). The former case would indicate a strong sex-specific effect, and the latter a weak or insignificant effect.

Finally, if there is a significant interactive effect between male and female genotype of phenotypic outcome we examine indirect genetic effects: how the genotype of an interacting individual influences the phenotype of a focal individual (Figure S2 B). In these cases, for each copulating pair, one sex will represent the "focal genotype" and the partner will be the "interacting genotype". IGEs are measured for each sex in turn and describe to what extent the mean phenotypic value of the focal genotype differs when it is paired with an interacting genotype. When we are examining the IGE on females, the focal genotype will be female and the interacting genotype will be male ("Ψ: Focal female line" in Figure S2 B), and *vice versa* ("Ψ: Focal male line" in Figure S2 B).

For each sex and genotype, we measure *(i)* the strength and direction of each IGE for each interacting genotype, and *(ii)* the overall magnitude of the IGE on the focal genotype. The strength and direction of IGEs describes to what extent the mean phenotypic value of a given focal genotype differs (positively or negatively) when it is crossed with a specific interacting genotype. For example, female genotype 45 may have an overall mean mating latency of 90 minutes. However, when it is paired with male genotype 21 it has a mean mating latency of 120 minutes: this implies a strong positive IGE. The overall magnitude of the IGE can be quantified by observing how the mean phenotypic value of a focal genotype (of a given sex) varies when it is crossed with all other interacting genotypes. For example, in female genotype 45 we could observe that *(i)* the mating latency of female genotype 45 takes a large range of values when paired with different male genotypes, that differ from the mean mating latency of female genotype 45 – the overall magnitude of IGE is large; *(ii)* only a few interacting male genotypes drive a mating latency with a large deviation from the phenotypic average of female genotype 45 – the overall magnitude of IGE is small; or *(iii)* the mating latency of line 45 females does not deviate from their overall average for any interacting male genotype – the overall magnitude of IGE is close to or equal to 0.

2.4.2 Direct Genetic Effects

Sources of variation were analysed using mixed model nested ANOVA with type III sum of squares in R v 4.2.0 (R Core Team, 2016). Variance component estimation were conducted using Restricted Maximum Likelihood in a mixed model fitted with the *lmer* function (package lme4; Bates *et al.*, 2014). All phenotypic variables were log-transformed to ensure normality. For each response variable (mating latency, copulation duration and ejection time) we built a model with male genotype, female genotype and their interaction as independent variables. Batch (labelled 1 to 14) was included as a random effect.

To assess line-specific (additive) genetic effects (the phenotype without regard to focal sex) and sex-specific effects (the phenotypic value when considering the sex of the focal individual), analyses were performed using the package BayesDiallel (Lenarcic *et al.*, 2012) in R v 3.4.4 (R Core Team, 2016). Bayesian Diallel models are described by a quote string of characters, with the full model containing seven heritable components ($BSabmvw$; Lenarcic *et al.*, 2012). Our model included four components from the full model and the random covariate batch to predict how much of the total interactive phenotypic variance is explained by each component in the model, which is given below:

$$y_i = \mu + \underbrace{\sum_{r=1}^{R} u_i^{(r)}}_{Random} + \underbrace{(l_{j[i]} + l_{k[i]})}_{line\ (l)} + \underbrace{(s_{j[i]} - s_{k[i]})}_{sex\ (s)} + \underbrace{(I_{\{j[i]\neq k[i]\}} v_{(jk)[i]})}_{cross-specific\ (v)} + \underbrace{(I_{\{j[i]\neq k[i]\}} w_{(jk)[i]})}_{cross-specific\ sex\ (w)}$$

Raw data for a given phenotype (y_i) is measured for all individual pairings where $j_{[i]}$, $k_{[i]}$, and $(jk)_{[i]}$, respectively describe the female, male and female-male combination relevant to the specific pairing i, where $i \in \{1, \ldots, n\}$. The $\sum_{r=1}^{R} u_i^{(r)}$ term represents the contribution of the random effect which for single phenotypic outcome always includes an effect of experimental batch as $u_i^{(r)} \sim N(0, \tau_r^2)$ for each $r \in \{1, \ldots, R\}$. Genotypic line-specific effects l are modelled as random effects and provide estimates of the average phenotypic value of a genotype for female j in combination with male k, and is equivalent to the proportion of additive genetic variability. Sex-specific effects s are modelled as symmetric (random effect) deviations from the l model, and describe an additional increase or decrease in the mean phenotype induced by a line being female, with male as a reference (Cockerham and Weir, 1977). The components l and s are equivalent to a and m in Equation 16 of Lenarcic *et al.* (2012), and outline the direct genetic effects that influence a given phenotype (Figure S2 A). BayesDiallel analysis also outlines IGEs which describe interactions between specific copulatory pairs. These are modelled as two types of random effect departures from the ls model: cross-specific effects v (model differences specific to a given pair regardless independent of reciprocal effects, i.e. crosses jk and kj have the same effect), and cross-specific sex effects w (model deviations from cross-specific effects due to differences between reciprocal crosses. i.e. crosses jk and kj have different effects). Overall both outline the IGEs, describing the extent to which a phenotypic value from a specific cross varies from what would be expected based on the average performance of the genotypes involved (Murphy *et al.*, 2008); and, in the case of w, if this is sex-specific (Figure S2 B). However, as cross-specific effects represent fewer observations, these results are strongly subject to Bayesian adaptive shrinkage which pulls extreme but sparsely supported means towards the middle (Lenarcic *et al.*, 2012). As a result, cross-specific effects using this method are often vague, meaning that other more direct approaches are more appropriate when calculating IGEs. Here our direct approach is to calculate Ψ (see section 2.4.3 below).

Mating latency, copulation duration, and ejection time for all estimates were log-transformed and calculated from multiple posterior draws, leading to a complete posterior distribution of each model component. These are summarised as highest posterior density intervals (HPD), such that credibility intervals excluding zero indicate strong evidence that an effect is different from the average. The variance of each group, e.g. τ_a^2, was modelled with a weak inverse gamma prior τ_a^{-2} (*df* = 0.02, *mean* = 0.2), and the prior for fixed effect μ is set to a vague normal distribution $\mu \sim N(0, 10^3)$ as described in Lenarcic *et al.* (2012). Posterior distributions were estimated for all parameters using an efficient MCMC Gibbs sampler with 5 chains, 10,000 iterations and a burn-in of 100. Direct genetic effects for ejection time were derived prior to this experiment (Pembury Smith and Snook, unpublished; *Chapter I*).

In order to report the overall relative contribution of each model component, diallel variance projections (VarP) were calculated (Crowley *et al.*, 2014). This approach is a heritability-like measure which uses the posterior predictive distribution of effects from the model to simulate future, complete, perfectly balanced diallels of the same genotypic lines. Unlike traditional heritability, it is calculated based on heritable components of the diallel rather than variance components, which increases interpretability, stability and accuracy (Crowley *et al.*, 2014). In each simulated dataset, the contribution of each component in the model (i.e. l and s) is calculated as its sum of squares divided by the total phenotype sum of squares. The resulting proportion, VarP, provides a prospective summary describing how much each component in the model influences phenotypic variation.

Subsequently, the total VarP[$l + s + v + w$] is equivalent to broad-sense heritability and VarP[l] is related to narrow-sense heritability (Lenarcic *et al.*, 2012; Maurizio *et al.*, 2017). Estimates for each VarP are calculated in the same way as the HPD summaries, where credibility intervals excluding zero provide strong evidence that an effect explains a significant proportion of the phenotypic variance.

2.4.3 Indirect Genetic Effects

IGEs were derived by calculating Ψ for each male-by-female interaction using R v 4.2.0 (R Core Team, 2016). Up until now the interaction coefficient Ψ had be calculated for traits in which the phenotypes of interest can be measured in both focal and interacting individuals. We provide a framework in which Ψ can be calculated for phenotypes when separate measurements cannot be taken for each sex. Separate models were derived for each sex-specific focal line (Figure S2 B). The below formula describes how Ψ is derived for a single focal female genotype j, but is equally applicable to a focal male with appropriate change of notation. We define

$$z_{k[i]} = \beta_0 + \beta_1 \bar{z} + \boldsymbol{\Psi X_K} + \boldsymbol{\omega}_b \boldsymbol{Y}_B + \varepsilon_{[i]}$$

where

$$\boldsymbol{X_K} = \begin{cases} 1 \; if \; K = k \\ 0 \; if \; K \neq k \end{cases}$$

Here, $z_{k[i]}$ denotes the measured phenotype for the i^{th} trial within the k^{th} interacting male genotype. $\bar{z}$ is the mean phenotype of the focal female line. β_0 is the intercept and β_1 the slope of $\bar{z}$. $\boldsymbol{X}_K$ is a vector representing each individual kth interacting male genotype. This means that K always takes the value of one of our interacting eleven lines. For example, when examining a given phenotype $z_{k[i]}$ in a specific cross between focal female line 101 and interacting male line 21, k = genotype 21 ("Ψ: Focal female line" in Figure S2 B). The vector $\boldsymbol{X_K}$ = 1 when $K = k$, otherwise $\boldsymbol{X_K}$ will = 0 (i.e. if you are deriving Ψ for focal female line 101 when crossed with male line 21, you will only derive a value of Ψ when k is 21). Strictly speaking, Ψ as calculated here is an intercept term from the random effect model. However, as $\boldsymbol{X}$ is a vector of binary variables, it can also be interpreted as the gradient describing to what extent each interacting male genotype influences focal female genotype. ω_B denotes an effect of batch, fitted as a random effect. $\boldsymbol{Y}_B$ has the same properties as $\boldsymbol{X_K}$ but describes each batch. $\varepsilon_{[i]}$is the residual error term. All phenotypes were standardised within line to have a mean of 0 and a standard deviation of 1, meaning that the average phenotype ($\bar{z}$) and intercept (β_0) for a given line for each sex is 0. By doing so, the formula simplifies to:

$$z_{k[i]} = \boldsymbol{\Psi X_K} + \boldsymbol{\omega}_b \boldsymbol{Y}_B + \varepsilon_{[i]}$$

and:

$$z_{j[i]} = \boldsymbol{\Psi X_J} + \boldsymbol{\omega}_b \boldsymbol{Y}_B + \varepsilon_{[i]}$$

when describing a single focal male genotype where, $z_{j[i]}$ denotes the measured phenotype for the i^{th} trial within the j^{th} interacting female genotype ("Ψ: Focal male line" in Figure S2 B).

Restricted Maximum Likelihood Model was used to fit the model parameters which were fitted for each sex separately within each line, with all phenotypic variables log transformed. The model was fitted using the *lmer* function. When Ψ is measured on standardised traits it takes values between -1 and 1. When values of Ψ were outside this range due to large variation around model estimates they were reported as -1 and 1 respectively. For genotypes unaffected by the interacting genotype, Ψ = 0.

Ψ is negative for phenotypes where the interacting genotype reduced trait expression from the phenotypic average of the focal line, and positive when it increases trait expression. This analysis depicts the strength and direction of IGEs for each male-by-female cross. To analyse the overall magnitude of IGEs for each focal genotype, we quantified the overall variance in Ψ when male and female respectively. For a given focal genotype, if the variance in Ψ is large for a given sex, then the magnitude of IGEs is strong with interacting genotypes having an overall strong effect on phenotypic outcome. If the variance in Ψ is small for a given sex, the opposite conclusion can be drawn. An F-test was used to determine if variance in Ψ was significantly different between the sexes. IGEs for ejection time were derived prior to this experiment (Pembury Smith and Snook, unpublished; *Chapter I*).

2.4.3 Phenotypic and Genetic Correlation between Phenotypic Traits

Phenotypic correlations were analysed using linear mixed models in R v 4.2.0 (R Core Team, 2016). Variance component estimations were conducted using Restricted Maximum Likelihood in a mixed model fitted with the *lmer* function (Bates *et al.*, 2014). Batch, male genotype and female genotype were included as random effects. Two models were produced, the first included copulation duration as the dependent variable and mating latency as the independent variable. Here the dependent variable copulation duration was log transformed to assume normality. The second included ejection time as the dependent variable, and mating latency, copulation duration and their interaction as independent variables. Ejection time was log transformed to assume normality and mating latency and copulation duration were scaled to reduce the effect of multicollinearity. Collinearity was checked using the *vif* function.

The pairwise genetic relationship between the three traits were examined using Pearson's correlation coefficients between line-specific (additive) effect estimates from the BayesDiallel analysis (See section 2.4.2 above) (Turner *et al.*, 2018) using R v 3.4.4 (R Core Team, 2016). This identifies whether the derived intrinsic effect of each genotype has a significant genetic effect on the relationship between each trait.

3. Results

3.1 Direct Genetic Effects

Mating latency displayed substantial phenotypic variation (Figure 1 A). This phenotypic variation was heritable, with (additive) line-specific effects (narrow-sense heritability) and sex-specific effects explaining a significant proportion of the phenotypic variance (Figure S3 A). These results are robust to the small variation in sample size between cells (Figure S5 A & B). We also found that both male genotype and female genotype independently had a significant effect on phenotypic outcome (Table 1). Examining each genotype individually, the significant line-specific effect observed was largely driven by four genotypes (45, 517, 861 and 235; Figure 2 A). The first two displayed significantly longer mating latencies than the population average whereas the latter two displayed the opposite. The significant sex-specific effect was largely driven by two genotypes, 517 and 21, which displayed opposite sex-specific patterns (Figure 2 B). The former showed a significantly longer mating latency when the focal individual of that line was male mated to females from different lines compared to when the focal individual of that line was female mated to males from different lines (Figure 2 B). The latter showed the opposite sex-specific pattern.

In comparison to mating latency, copulation duration showed limited phenotypic variance, with mean duration only ranging from 13 minutes to 23 minutes (Figure 1 B). Although both male genotype and

female genotype contributed significantly to phenotypic variance (Table 1), with VarP analysis showing significant narrow-sense heritability (Figure S4 A), HPD plots revealed no significant line-specific or sex-specific deviations from the population average (Figure 2 C & D). This result is robust, despite small variation in sample size between cells (Figure S5 C & D).

Similar to mating latency, male genotype, female genotype (Table 1), narrow-sense heritability (line-specific effects) and sex-specific effects (Pembury Smith and Snook, unpublished; *Chapter I*) significantly influenced variation in sperm ejection time. Taken together, these results suggest that phenotypic variation in the timing of ejection is significantly influenced by direct genetic effects.

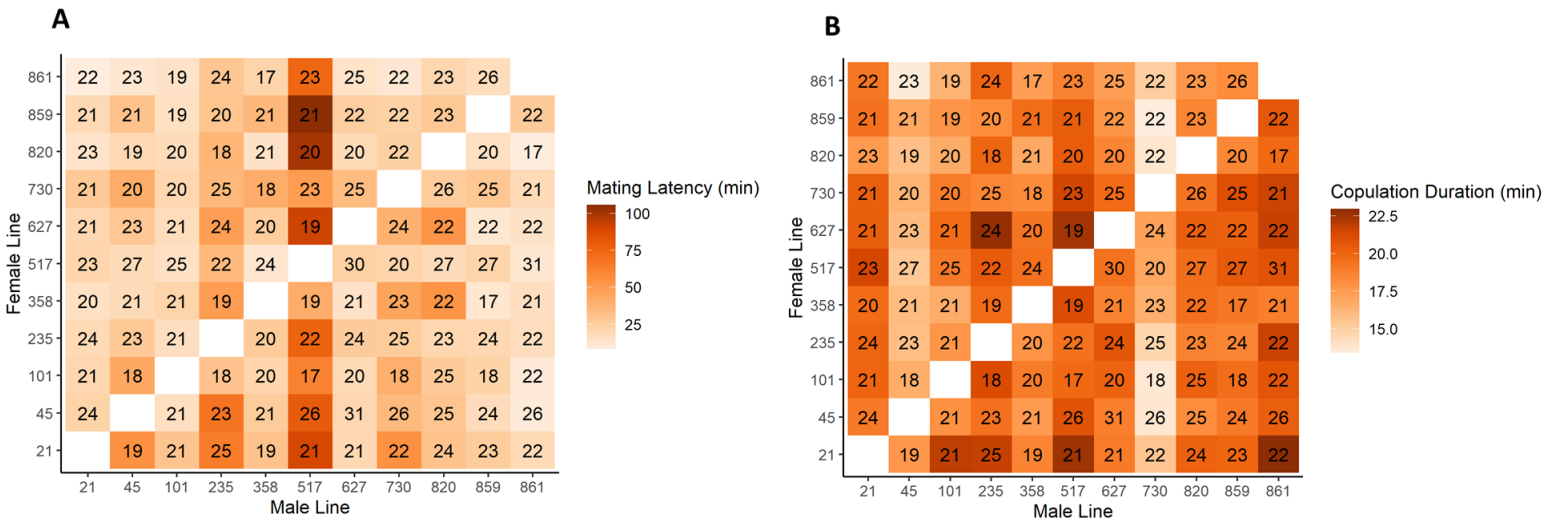
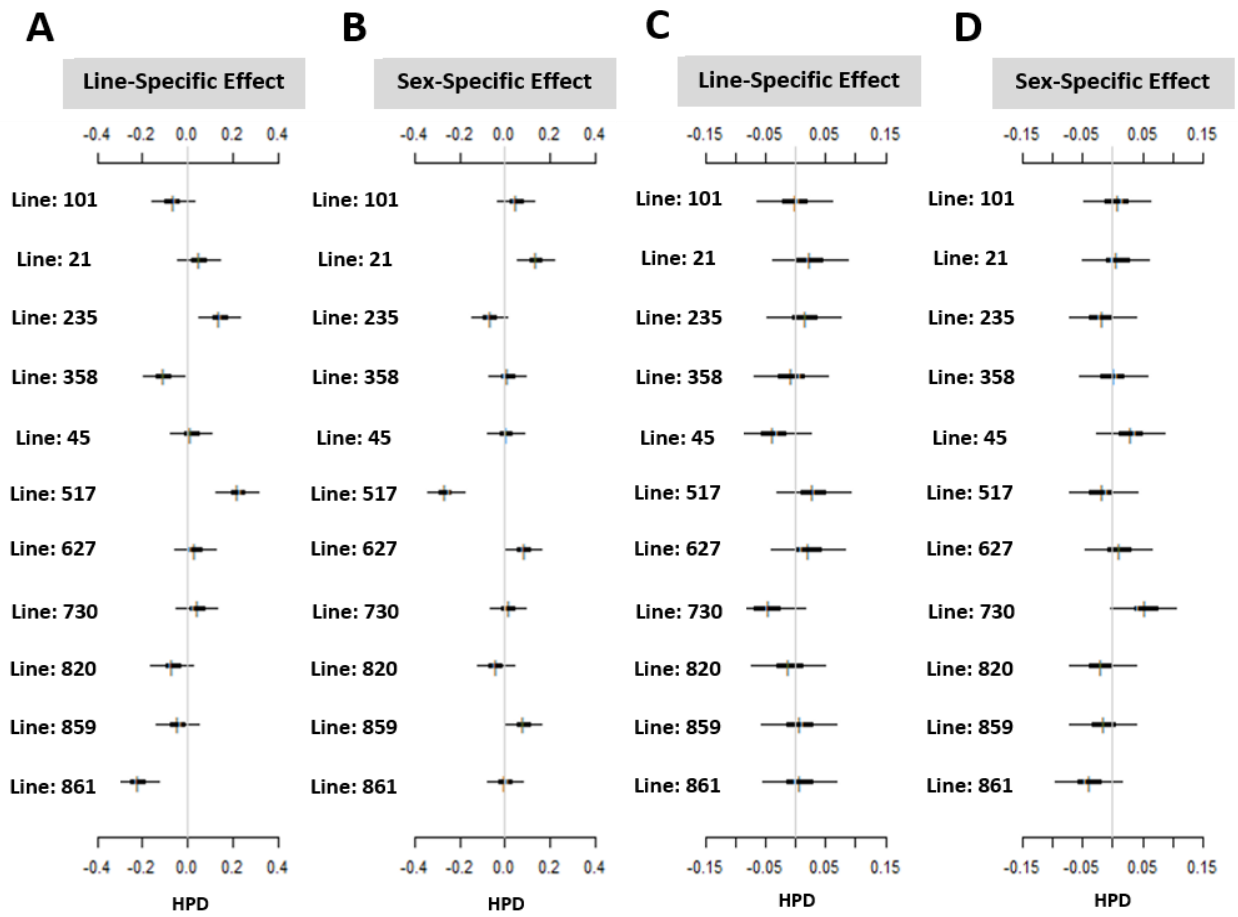


Figure 1. Variation in interactive phenotypic traits. The colour of each cell represents the shared mean mating latency (A) and copulation duration (B) expressed by a male and a female from two different DGRP lines. The darker the colour the longer the duration. The specific genotypic crosses than generate comparatively longer or shorter durations differ between traits. Although, both interactive traits display some phenotypic variation, the scale of phenotypic variation in copulation duration (B) is five times smaller than mating latency (A). Within line crosses were not conducted and are denoted in white. The number in each cell is the sample size for each pairing.

Table 1. Mixed model nested ANOVAs for interactive phenotypes.

Interactive Phenotype	Source of Variance	DF	MS	DenDF	F	P
Mating Latency	Male Genotype	10	45.9	1783.0	45.5	**< 0.001**
	Female Genotype	10	15.9	2005.4	15.8	**< 0.001**
	Male Genotype x Female Genotype	89	1.7	2250.4	1.7	**0.0001**
Copulation Duration	Male Genotype	10	2.3	2146.9	47.0	**< 0.001**
	Female Genotype	10	0.5	2224.7	9.6	**< 0.001**
	Male Genotype x Female Genotype	89	0.1	2296.5	1.2	0.09
Ejection Time	Male Genotype	10	2.9	1889.7	16.2	**< 0.001**
	Female Genotype	10	7.2	1921.9	40.0	**< 0.001**
	Male Genotype x Female Genotype	89	0.2	1935.3	1.3	**0.03**



388 **Figure 2. Highest posterior density (HPD) intervals for mating latency (A-B) and copulation duration (C-D) based on the**
389 ***lsvw* model.** For each effect, thin and thick horizontal lines show the 95 and 50% HPD intervals, respectively, with short
390 vertical bars indicating the posterior mean. HPD intervals that do not include zero we consider to be statistically significant
391 at 95% credibility. Line-Specific Effects (A & C) denote how mean phenotype for a given genotype is related to the population
392 average (vertical grey line), independent of sex. Any bar to the left of the grey vertical line suggests that the mean phenotypic
393 value for this genotype, independent of sex, is shorter than the population average. Sex-Specific Effects (B & D) denote the
394 average phenotypic deviation when a genotype is female compared to the overall average phenotypic value of that genotype
395 (vertical grey line), with male as a baseline. Any bar to the left of the grey vertical line suggests that the mean phenotypic
396 value for that genotype is significantly longer when male than female. Details on how Line-Specific and Sex-Specific effects
397 were calculated can be found in Supplementary Figure S2. Note that the line order in this figure contrasts from the other
398 figures and is not increasing in numerical order.

3.2 Indirect Genetic Effects

IGEs will be seen when trait expression of the focal individual is strongly influenced by the genotype of the interacting partner. In this analysis, IGEs will be observed when specific crosses between a focal genotype and an interacting partner genotype deviate from the focal genotype's phenotypic average. Variation in mating latency was significantly influenced by an interaction between male and female genotype (Table 1). Within each genotypic line, mean mating latency displayed considerable cross-specific variation in comparison to the population average for both sexes (Figure 3). That is, mating latency in the focal sex could either be greater or lesser than the population average when paired with a specific interacting genotype. Taken together, these results suggest that for a given focal genotype, mating latency will vary depending on the interaction between a specific male and female genotype.

To calculate the effect of IGEs on mating latency we derive Ψ which describes to what extent an interacting genotype influences the phenotype of the focal individual for each genotypic line. The direction and strength of Ψ , depicted by the sign and size respectively, was cross-specific (Figure 4). This result suggests the strength and direction with which an interacting genotype alters the phenotypic outcome of a focal genotype is dependent on both the focal and interacting genotype. This was observed in both sexes across all focal genotypes. Additionally, the magnitude of Ψ was sex-specific (Figure 4). We found that, for a given focal genotype, overall variation in Ψ was significantly larger when female than when male (Figure 4; F = 2.69, df = 109, p > 0.001). This suggests that interacting males have a comparatively stronger effect on the focal female phenotype compared to the indirect genetic effect of interacting females on focal male phenotype. This effect was largely driven by interacting male genotype 517 which had a strong positive influence on phenotypic outcome for almost all focal female lines, displaying Ψ values consistently close to 1. IGEs (cross-specific and cross-specific sex effects) were also calculated using a BayesDiallel approach (Figure S3), however, due to Bayesian shrinkage as outlined in the methods, calculations of Ψ are more robust when analysing IGEs.

When investigating the effect of IGEs on copulation duration, we observed no significant interactive effect (Table 1), and no cross-specific effects (Figure S4). Taken together, this suggests that IGEs have limited influence on phenotypic variation.

As found when examining mating latency, variation in sperm ejection time was significantly influenced by an interaction between male and female genotype (Table 1), with mean ejection time displaying cross-specific variation in comparison to the population average for both sexes (Pembury Smith and Snook, unpublished; *Chapter I*). The timing of sperm ejection also displayed large cross-specific variation in the strength and direction of Ψ (Pembury Smith and Snook, unpublished; *Chapter I*). Together, these results show that IGEs underly variation in this phenotype. Interestingly, sperm ejection time also showed sex-specific variation in the magnitude of Ψ, but displayed the opposite pattern to mating latency, showing that the indirect genetic effect of females on focal male phenotype was significantly greater than the indirect genetic effect of males on focal female phenotype (Figure 5).

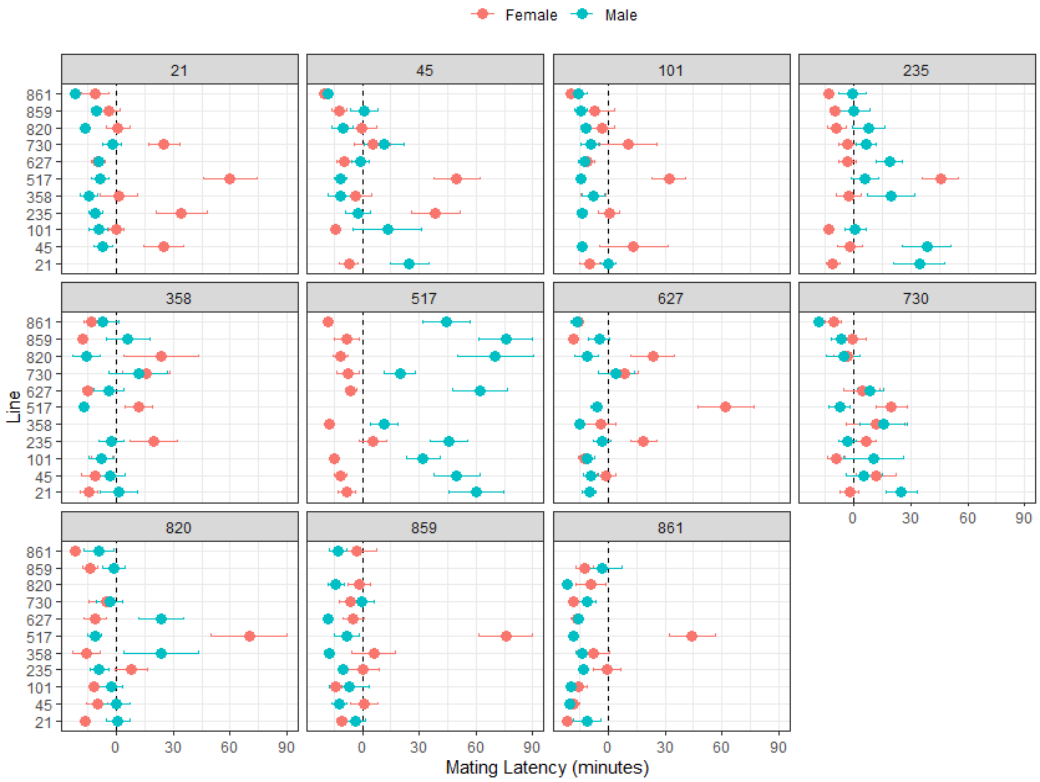


Figure 3. Variation in sex-specific mean mating latency for each focal genotype when crossed with an interacting genotype compared to the population average. Each box represents a focal genotype, denoted by the grey label above each graph. Within each box, each point represents the mean mating latency and standard deviation when each focal male (blue) or female (red) genotype is crossed with a specific interacting genotype, denoted on the y axis. The vertical dashed line represents the average mating latency of the population. A point left of the dashed line suggests that the mean mating latency for the cross involving those lines is shorter than the population average.

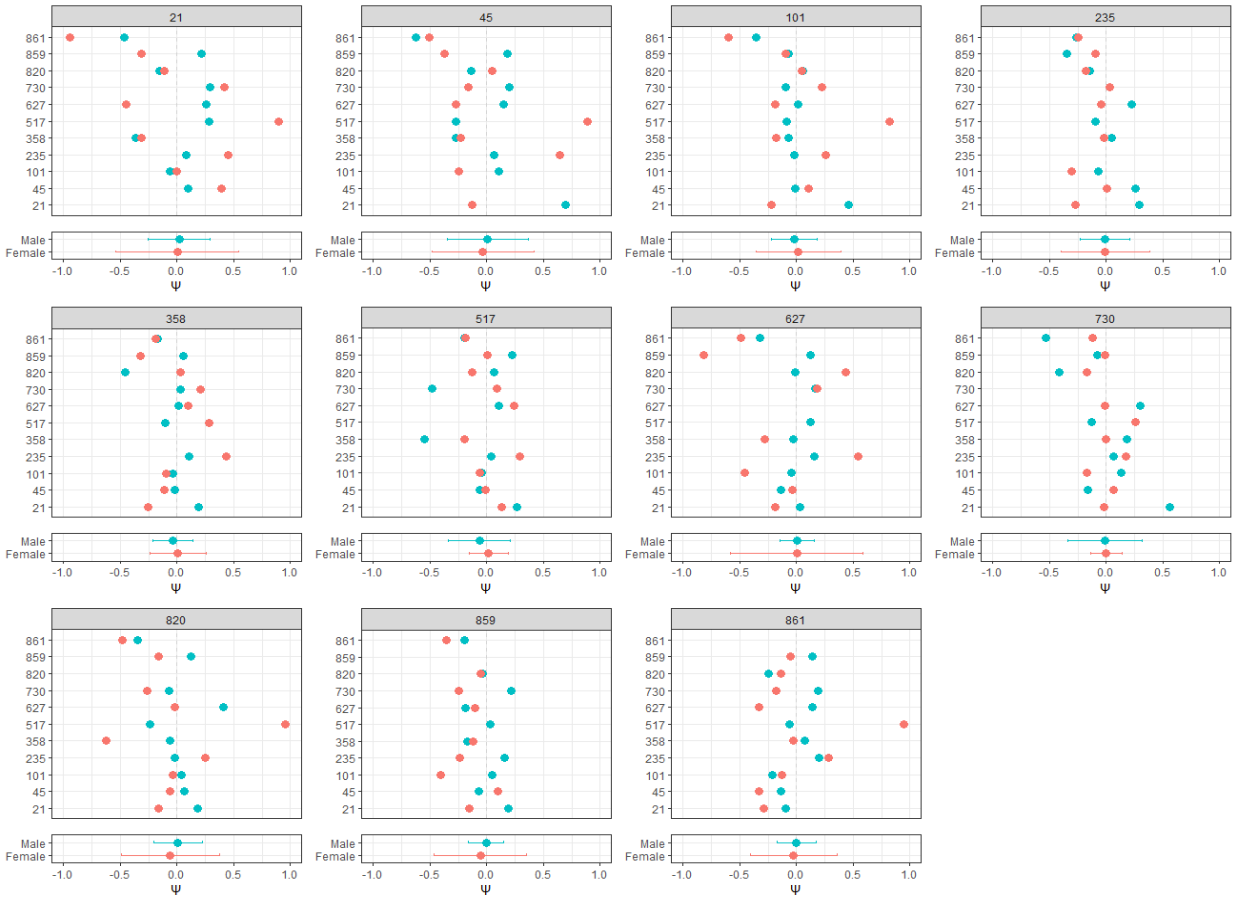


Figure 4. The sex-specific estimates of Ψ for mating latency when each focal genotypic line is paired with each interacting genotype. Each box above represents a focal genotype, denoted by the grey label above each graph. Within each box, each point represents the Ψ value when each focal genotype, male (blue) or female (red) is crossed with a specific interacting genotype, denoted on the y axis. The vertical dashed line represents the average mating latency of the focal genotype (average of the eleven isofemale lines), when male and female respectively. The further away a point is from the dashed line, the greater the phenotype deviates from the focal genotypes' phenotypic average when crossed with that specific interacting genotype: representing the strength of the IGE. A point left of the dashed line suggests that an interacting genotype drives a mating latency shorter than the focal genotypes' phenotypic average: representing the direction of the IGE. Each box below summarises the overall mean ± SD of Ψ when the focal male (blue) or female (red) genotype is crossed with all interacting genotypes of the opposite sex: representing the magnitude of IGEs. Details on how Ψ is calculated when the focal genotype is male and female can be found in Supplementary Figure S2.

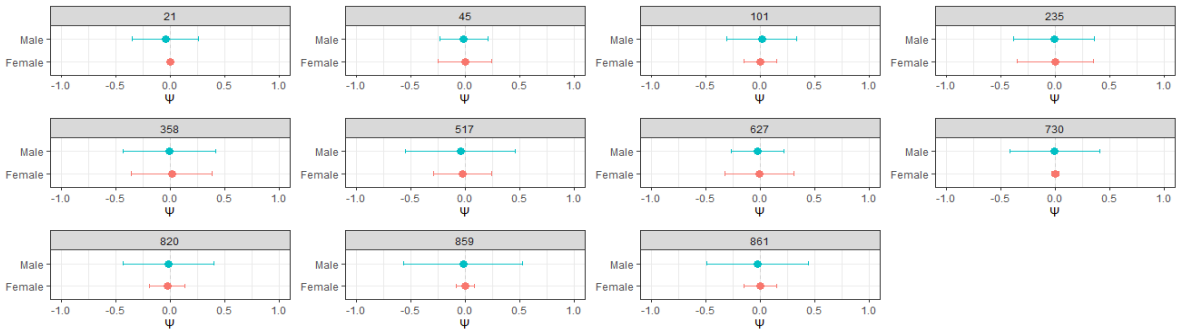


Figure 5. The magnitude of sex-specific IGEs for ejection time for each focal genotype. Each box represents a focal genotype, denoted by the grey label above each graph. Within each box, a point represents the overall mean ± SD of Ψ when the focal male (blue) or female (red) genotype is crossed with all interacting genotypes of the opposite sex: representing the magnitude of IGEs. The greater the SD, the greater Ψ varies, meaning that the phenotype of the focal genotype shows large variation depending on the interacting genotype they are crossed with. Details on how Ψ is calculated when the focal genotype is male and female can be found in Supplementary Figure S2.

3.3 The relationship between pre- and post-copulatory traits

We observed a significant positive phenotypic correlation between mating latency and copulation duration (Table 2). We also found a significant negative phenotypic correlation between mating latency and ejection time (which could also be described as a positive phenotypic correlation between the speed at which mating occurred and sperm ejection time), and a significant positive correlation between copulation duration and ejection time (Table 2). There was no significant interaction between copulation duration and mating latency on ejection time, suggesting that copulation duration does not significantly influence the relationship between mating latency and ejection time (Table 2). Additionally, when examining the genetic correlations between traits, we found no significant line-specific (additive) correlations between any of the phenotypes measured (Table S1).

Table 2. Linear mixed effects model examining the phenotypic relationships between interactive phenotypes.

Interactive Phenotype	Source of Variance	Est	Std. Error	DF	T	P	Cond. R^2
Copulation Duration	Mating Latency	0.04	0.01	2424	7.98	**< 0.001**	0.24
Ejection Time	Mating Latency	-0.04	0.01	2016	-34.1	**< 0.001**	0.26
	Copulation Duration	0.02	0.01	2013	2.2	**0.027**	
	Mating Latency:Copulation Duration	0.005	0.009	2028	0.5	0.617	

4. Discussion

We aimed to examine the quantitative genetic basis of and correlation between three interactive phenotypes that span episodes of pre- and post-copulatory sexual selection. We used isofemale lines of *D. melanogaster* which allowed us to quantify the extent to which each trait is subject to genetic influences of the focal individual and the interacting individual (the heritable environment component). By using a modified quantitative genetic framework, we found significant line-specific and sex-specific effects influence mating latency and sperm ejection, and identify the specific genotypes that drive these significant effects in the former. We also show that the magnitude with which IGEs influence variation in mating latency was genotype- and sex-specific, with focal male phenotype less affected by the genotype of the interacting female than in the opposite direction. Variation in copulation duration was also heritable, but we found limited evidence that IGEs influence phenotypic variance in this trait. We found significant phenotypic correlations between all three traits However, these relationships were absent at the additive genetic level. By using this approach, we have quantified the role of direct and IGEs on interactive antagonistic traits, identified the capacity for sexual conflict, and examined the relationship between traits that experience different episodes of sexual selection to understand the extent with which this may influence trait evolutionary potential.

A traditional quantitative genetics framework derives heritability by examining the additive influence of parent genes on offspring phenotype. Here, additive line-specific genotypic effects represent a heritability-like measure, indicating whether intrinsic effects of genotype, independent of sex, significantly contribute to phenotypic variation. By using this framework, we show that the mating latency is heritable, with direct genetic effects (line- and sex-specific) influencing phenotypic variation in mating latency. We also identify the specific genotypes that show significant line-specific and sex specific effects. In doing so we expand on previous corroborating work which has suggested that mating latency is a heritable trait and that the genotypes of both sexes contribute to phenotypic

variance (Ratterman *et al.*, 2014). These results suggest that there is a large amount of additive genetic variation available to respond to selection underlying this trait. Additionally, as inter-sexual conflict drives genotypic variance in the ability to control phenotypic outcome, significant sex-specific effects support the idea that mating latency is a sexual conflict trait, and that antagonistic interactions maintain phenotypic variation in the population and influences the evolutionary trajectories of the sexes.

In addition to direct genetic effects, IGEs influence phenotypic variation in mating latency. Within each focal genotypic line, we show that phenotypic outcome depended on the genotype of the interacting partner. Theoretical models of sexual conflict in interactive phenotypes predict fluctuating patterns of selection in each sex for multiple persistence and resistance traits (Moore and Pizzari, 2005). Taken together, these results suggest that substantial phenotypic variation in the population is maintained via antagonistic interactions between the sexes. In addition, we show that the magnitude of Ψ was sex-specific, with focal male phenotype less affected by the genotype of the interacting female than in the opposite direction. Large Ψ values have been proposed as an indicator of strong conspecific influence on focal phenotype (Moore and Pizzari, 2005). This may suggest that there is a strong effect of interacting male manipulation, with certain focal female genotypes better able to resist this manipulation than others. Alternatively, we expect females to benefit from flexibly adjusting mating latency according to partner genotype. Large variation in Ψ was predominantly driven by interacting male genotype 517 which showed significantly longer mating latencies than the population average. As increased mating latencies have negative fitness consequences in males (Holland and Rice, 1998), this result may suggest that there is strong selection in females to control phenotypic outcome and that large variation in Ψ is due to females adjusting mating latency based on interacting male genotype.

Courtship behaviour in *Drosophila* is a complex polygenic process that involves numerous visual, auditory, physical and chemical signals (Mackay *et al.*, 2005), with many of the underlying genes spanning multiple biological processes (Hall *et al.*, 1980). Although the present study does not identify the exact mechanisms underlying variation in mating latency, a number of candidate genes have been proposed. For example, *desat1* is a gene regulating the amount of 7-T, a principal cuticular hydrocarbon, in males. Males with increased levels of 7-T display reduced mating latency, suggesting that allelic variance in *desat1*, underlying variable 7-T expression, may influence mating latency in this species (Grillet, Dartevelle and Ferveur, 2005). Similarly, female mutants for the *desat1* enzyme are less able to detect variation in 7-T (Grillet, Dartevelle and Ferveur, 2005), suggesting that allelic variation at this gene in females can also influence mating latency. When examining females specifically, mutations in 7, 11-diene pheromones have been shown to alter mating latency (Marcillac and Ferveur, 2004; Ueyama *et al.*, 2005). Additionally, both the Painless (*Pain*) TRP channel (Sakai *et al.*, 2009) and dopamine regulation (Neckameyer *et al.*, 2000; Andretic, van Swinderen and Greenspan, 2005) play a critical role in mating latency with female *pain* mutants or those with reduced dopamine content reducing mating latency compared to wildtypes. Despite these strong candidates, confirmation that allelic variation has a direct influence on mating latency (and subsequent fitness) has not been tested and warrants further investigation to pinpoint the focal and interacting genetic variants underlying the substantial phenotypic variation in mating latency.

Similar to mating latency, significant narrow-sense heritability, male line and female line contributed to phenotypic variance in copulation duration. This result is in line with work showing significant

broad-sense heritability (Gaertner *et al.*, 2015), but counters others (Taylor, Evans and Garcia-Gonzalez, 2013). As the latter study used a random sample of females, this emphasises the importance of accounting for female genotype when estimating trait heritability. Despite finding significant narrow-sense heritability, no specific genotypes showed significant line-specific or sex-specific effects. In our analysis, measurements of heritability are prospective and suggest that direct genetic effects would likely impact future experiments. Subsequently, this result suggests that narrow-sense heritability in this phenotype is driven by deviations between individual genotypes rather than the population average. Unlike mating latency, the interaction between male line and female line did not significantly influence phenotypic variation. This result also corroborates existing work in *D. melanogaster* (Ratterman *et al.*, 2014) which also used individuals from the DGRP. As this study and our own utilised different isofemale lines, this provides substantial evidence that copulation duration is influenced by the genotype of both sexes, but that IGEs have limited influence on phenotypic variation across a large variety of genotypes. Early work has shown that copulation duration is less sensitive to environmental variation in comparison to mating latency (MacBean and Parsons, 1967). Our results expand on this and show that, despite being an interactive phenotype, copulation duration is also less sensitive to the heritable environmental component. Taken together, these results provide limited evidence that copulation duration is a sexual conflict trait. This is unsurprising as sperm delivery does not occur continuously throughout copulation in *D. melanogaster* (Gilchrist and Partridge, 2000), with limited evidence that it influences reproductive success and fitness (Fricke *et al.*, 2009; Dore, Bretman and Chapman, 2020). Instead, copulation duration may be influenced by sex-specific traits that evolve independently in the absence of sexually antagonistic coevolution, as it is in the interest of both sexes that copulation is successful once it has begun (Tennant, Sonser and Long, 2014).

A number of candidate genes associated with copulation duration have been identified in *D. melanogaster*. Most candidates to date have been shown to disrupt the physical interaction between the sexes, stimulating (Baba *et al.*, 1999) or preventing termination (Kuniyoshi *et al.*, 2002). For example, *fru*-mutant males display significantly longer copulations due to defective abdomen muscles that make them unable to disengage from the female (Lee *et al.*, 2000; Jois *et al.*, 2018, 2022). Although variation in these candidates could contribute to the significant male genotypic effect observed in our study, it is more likely that allelic variation in other candidates are involved as we observed no obvious defects in the act of copulation. For example, significantly longer copulations have been observed in males that lack the functioning clock genes *per* and *tim* (Beaver and Giebultowicz, 2004). Despite a few named candidates, the underlying genetic architecture of copulation duration remains largely unresolved, with work examining candidate genes in females particularly lacking. As we observed that the genotype of both males and females independently contribute to phenotypic variation, future work looking at the underlying genetic basis of copulation durations in both sexes would improve understanding on the genetic architecture of this trait.

Significant narrow-sense heritability, sex specific effects and IGEs contributed to variance in the timing of sperm ejection (Pembury Smith and Snook, unpublished; *Chapter I*). In addition, the magnitude of IGEs were sex-specific, with females displaying consistently smaller variance in Ψ than males for the same focal genotype, suggesting that there is strong selection in females to counter male manipulation. Interestingly, this was opposite to the sex-specific pattern observed in mating latency, with females displaying consistently larger variance in Ψ than males. It has been suggested that limited variation in Ψ may represent reduced influence of manipulation by interacting genotypes (Moore and

Pizzari, 2005). Based on this, our results may suggest that pre-copulatory sexual selection may be more strongly driven by male genotype and post-copulatory sexual selection by female genotype. However, when examining mating latency, we show that large variation in Ψ is predominantly due to focal females consistently displaying a longer mating latency when paired with specific interacting male genotypes. As a long mating latency reduces male fitness, we provide more support for the idea that variation in Ψ, when examining mating latency, is driven by the focal female rather than the interacting male genotype. However, the patterns observed need to be tested across more traits and in different taxa in order to draw accurate conclusions on the extent to which the influence of interacting genotypes differs between episodes of selection.

When examining the phenotypic relationship between traits, there was a significant positive phenotypic correlation between the speed at which mating occurred and sperm ejection time. This result describes a positive correlation for fitness with individuals with shorter mating latencies (attractive males) displaying longer ejection times (resulting in more time to retain his ejaculate). This positive correlation between pre- and post-copulatory fitness is in line with previous work on *Drosophila* species (Hosken *et al.*, 2008), and supports the phenotype-linked fertility hypothesis as we would expect females to retain sperm for longer from desirable males. Although the underlying mechanism behind this specific relationship is not known, in *D. melanogaster* courtship can trigger genotypic variation before copulation has begun, influencing post-copulatory trait expression in the opposite sex (Immonen and Ritchie, 2011). For example, the expression of Glucose dehydrogenase, a protein that facilitates sperm storage in mated females, increases in response to song stimulation (Iida and Cavener, 2004; Immonen and Ritchie, 2011). The connection between mate recognition and downstream post-copulatory traits has been implicated as a way in which females distinguish between males via cryptic female choice (Immonen and Ritchie, 2011), and could therefore contribute to the phenotypic relationship between pre- and post-copulatory traits observed.

We also observed a significant phenotypic relationship between mating latency and copulation duration, showing that individuals that had a longer mating latency (unattractive males) copulated for a longer duration. This result suggests a potential trade-off between pre-copulatory traits and traits that increase copulation duration. This result is surprising as we found limited evidence that copulation duration is a sexual conflict trait and the association between copulation duration and reproductive success in this species is often weak or absent (Pitnick, 1991; Fricke *et al.*, 2009; Price *et al.*, 2012; Dore, Bretman and Chapman, 2020). Although our results indicate that there is also a positive relationship between copulation duration and ejection time, suggesting that males may be selected to increase copulation duration in order to prolong sperm retention, this was only marginally significant. Additionally, we observed no significant interactive effect between copulation duration and mating latency on ejection time, suggesting that extended copulation durations do not significantly influence the relationship between mating latency and ejection time. This suggests that mating latency, independent of copulation duration, has the strongest influence on the timing of sperm ejection. As a result, we provide limited evidence that there is selection in males to increase copulation duration following a long mating latency in order to increase sperm ejection time. Subsequently, the mechanism underlying an adjustment in copulation duration in response to mating latency remains unknown, and warrants further investigation.

Despite observing significant phenotypic relationships between traits, we found limited evidence of underlying additive genetic correlations. This suggests that although the traits involved in both

episodes of selection are heritable and can evolve, they do so independently and do not have a common genetic basis. However, it should be noted that only genotypic correlations between additive line-specific effects were examined, meaning that a large proportion of the genotype-by-genotype variation is masked from the analysis. Future work examining how these relationships genetically covary with sex and cross would provide a more comprehensive understanding as to whether the phenotypic correlations between the interactive phenotypes observed have a genetic basis.

In conclusion, using an interactive phenotypic framework we show that both mating latency and ejection time are strongly influenced by direct and IGEs, and that sexual conflict likely drives considerable phenotypic variation in these traits. Furthermore, the relationship between mating latency and ejection time suggests that pre-copulatory traits are indicative of genetic quality in males and are subsequently favoured via cryptic female choice mechanisms. In comparison, despite being an interactive phenotype, there was limited evidence to suggest that variation in copulation duration is influenced by IGEs, and that there is (ongoing) antagonistic selection for this trait. While we did observe significant phenotypic correlations between traits there was limited evidence that this had an underly genetic basis. By examining three interactive phenotypes that represent sequential stages of the reproductive process, we provide a better understanding of trait evolutionary dynamics. In addition, by using sequenced isofemale lines, this work contributes to future research that can pinpoint new candidate genes and the role of allelic variation in existing candidates that underly phenotypic variation, and how this may influence the relationship between pre- and post-copulatory fitness.

5. References

Alcock, J. (1994) 'Postinsemination Associations Between Males and Females in Insects: The Mate-Guarding Hypothesis', *Annual Review of Entomology*, 39(1), pp. 1–21. Available at: https://doi.org/10.1146/annurev.en.39.010194.000245.

Andersson, M. (1994) *Sexual Selection*. Princeton University Press.

Andretic, R., van Swinderen, B. and Greenspan, R.J. (2005) 'Dopaminergic Modulation of Arousal in Drosophila', *Current Biology*, 15(13), pp. 1165–1175. Available at: https://doi.org/10.1016/j.cub.2005.05.025.

Arnqvist, G. and Rowe, L. (2005) *Sexual Conflict*. Princeton University Press.

Baba, K. *et al.* (1999) 'The Drosophila Bruton's Tyrosine Kinase (Btk) Homolog Is Required for Adult Survival and Male Genital Formation', *Molecular and Cellular Biology*, 19(6), pp. 4405–4413. Available at: https://doi.org/10.1128/MCB.19.6.4405.

Bates, D. *et al.* (2014) 'Fitting Linear Mixed-Effects Models using lme4'. arXiv. Available at: https://doi.org/10.48550/arXiv.1406.5823.

Beaver, L.M. and Giebultowicz, J.M. (2004) 'Regulation of Copulation Duration by period and timeless in Drosophila melanogaster', *Current Biology*, 14(16), pp. 1492–1497. Available at: https://doi.org/10.1016/j.cub.2004.08.022.

Birkhead, T.R. (1998) 'CRYPTIC FEMALE CHOICE: CRITERIA FOR ESTABLISHING FEMALE SPERM CHOICE', *Evolution*, 52(4), pp. 1212–1218. Available at: https://doi.org/10.1111/j.1558-5646.1998.tb01848.x.

Bleakley, B.H. and Brodie III, E.D. (2009) 'Indirect Genetic Effects Influence Antipredator Behavior in Guppies: Estimates of the Coefficient of Interaction Psi and the Inheritance of Reciprocity', *Evolution*, 63(7), pp. 1796–1806. Available at: https://doi.org/10.1111/j.1558-5646.2009.00672.x.

Bretman, A. *et al.* (2023) 'Chapter One - Playing to the crowd: Using Drosophila to dissect mechanisms underlying plastic male strategies in sperm competition games', in J. Podos and S. Healy (eds) *Advances in the Study of Behavior*. Academic Press, pp. 1–35. Available at: https://doi.org/10.1016/bs.asb.2022.11.001.

Chapman, T. *et al.* (1995) 'Cost of mating in Drosophila melanogaster females is mediated by male accessory gland products', *Nature*, 373(6511), pp. 241–244. Available at: https://doi.org/10.1038/373241a0.

Cockerham, C.C. and Weir, B.S. (1977) 'Quadratic Analyses of Reciprocal Crosses', *Biometrics*, 33(1), pp. 187–203. Available at: https://doi.org/10.2307/2529312.

Collet, J.M. and Sztepanacz, J.L. (2022) 'Mating traits are phenotypically but not genetically correlated to fitness', *Behavioral Ecology*, 33(4), pp. 833–843. Available at: https://doi.org/10.1093/beheco/arac047.

Crowley, J.J. *et al.* (2014) 'Genetics of Adverse Reactions to Haloperidol in a Mouse Diallel: A Drug–Placebo Experiment and Bayesian Causal Analysis', *Genetics*, 196(1), pp. 321–347. Available at: https://doi.org/10.1534/genetics.113.156901.

Dapper, A.L. and Wade, M.J. (2016) 'The evolution of sperm competition genes: The effect of mating system on levels of genetic variation within and between species', *Evolution*, 70(2), pp. 502–511. Available at: https://doi.org/10.1111/evo.12848.

Dingemanse, N.J. and Araya-Ajoy, Y.G. (2015) 'Interacting personalities: behavioural ecology meets quantitative genetics', *Trends in Ecology & Evolution*, 30(2), pp. 88–97. Available at: https://doi.org/10.1016/j.tree.2014.12.002.

Dore, A.A., Bretman, A. and Chapman, T. (2020) 'Fitness consequences of redundant cues of competition in male Drosophila melanogaster', *Ecology and Evolution*, 10(12), pp. 5517–5526. Available at: https://doi.org/10.1002/ece3.6293.

Eberhard, W. (1996) *Female Control: Sexual Selection by Cryptic Female Choice*. Princeton University Press.

Edvardsson, M. and Canal, D. (2006) 'The effects of copulation duration in the bruchid beetle Callosobruchus maculatus', *Behavioral Ecology*, 17(3), pp. 430–434. Available at: https://doi.org/10.1093/beheco/arj045.

Evans, J.P. (2010) 'Quantitative genetic evidence that males trade attractiveness for ejaculate quality in guppies', *Proceedings of the Royal Society B: Biological Sciences* [Preprint]. Available at: https://doi.org/10.1098/rspb.2010.0826.

Firman, R.C. *et al.* (2017) 'Postmating Female Control: 20 Years of Cryptic Female Choice', *Trends in Ecology & Evolution*, 32(5), pp. 368–382. Available at: https://doi.org/10.1016/j.tree.2017.02.010.

Fricke, C. *et al.* (2009) 'The benefits of male ejaculate sex peptide transfer in Drosophila melanogaster', *Journal of Evolutionary Biology*, 22(2), pp. 275–286. Available at: https://doi.org/10.1111/j.1420-9101.2008.01638.x.

Fulker, D.W. (1966) 'Mating Speed in Male Drosophila melanogaster: A Psychogenetic Analysis', *Science*, 153(3732), pp. 203–205. Available at: https://doi.org/10.1126/science.153.3732.203.

Gaertner, B.E. *et al.* (2015) 'Heritable Variation in Courtship Patterns in Drosophila melanogaster', *G3 Genes|Genomes|Genetics*, 5(4), pp. 531–539. Available at: https://doi.org/10.1534/g3.114.014811.

Gilchrist, A.S. and Partridge, L. (2000) 'Why It Is Difficult to Model Sperm Displacement in Drosophila Melanogaster: The Relation Between Sperm Transfer and Copulation Duration', *Evolution*, 54(2), pp. 534–542. Available at: https://doi.org/10.1111/j.0014-3820.2000.tb00056.x.

Grillet, M., Dartevelle, L. and Ferveur, J.-F. (2005) 'A Drosophila male pheromone affects female sexual receptivity', *Proceedings of the Royal Society B: Biological Sciences*, 273(1584), pp. 315–323. Available at: https://doi.org/10.1098/rspb.2005.3332.

Hall, J.C. *et al.* (1980) 'Neurogenetics of courtship in Drosophila', *Proceedings - Stadler Genetic Symposium (USA)* [Preprint]. Available at: https://scholar.google.com/scholar_lookup?title=Neurogenetics+of+courtship+in+Drosophila&author=Hall%2C+J.C.&publication_year=1980 (Accessed: 23 January 2023).

Hoffmann, A.A. (1999) 'Is the heritability for courtship and mating speed in Drosophila (fruit fly) low?', *Heredity*, 82(2), pp. 158–162. Available at: https://doi.org/10.1038/sj.hdy.6884640.

Holland, B. and Rice, W.R. (1998) 'Perspective: Chase-Away Sexual Selection: Antagonistic Seduction Versus Resistance', *Evolution*, 52(1), pp. 1–7. Available at: https://doi.org/10.1111/j.1558-5646.1998.tb05132.x.

Hopkins, B.R. *et al.* (2019) 'BMP signaling inhibition in Drosophila secondary cells remodels the seminal proteome and self and rival ejaculate functions', *Proceedings of the National Academy of Sciences*, 116(49), pp. 24719–24728. Available at: https://doi.org/10.1073/pnas.1914491116.

Hosken, D.J. *et al.* (2008) 'Attractive males have greater success in sperm competition', *Current Biology*, 18(13), pp. R553–R554. Available at: https://doi.org/10.1016/j.cub.2008.04.028.

Huang, W. *et al.* (2014) 'Natural variation in genome architecture among 205 Drosophila melanogaster Genetic Reference Panel lines', *Genome Research*, 24(7), pp. 1193–1208. Available at: https://doi.org/10.1101/gr.171546.113.

Iida, K. and Cavener, D.R. (2004) 'Glucose dehydrogenase is required for normal sperm storage and utilization in female Drosophila melanogaster', *Journal of Experimental Biology*, 207(4), pp. 675–681. Available at: https://doi.org/10.1242/jeb.00816.

Immonen, E. and Ritchie, M.G. (2011) 'The genomic response to courtship song stimulation in female Drosophila melanogaster', *Proceedings of the Royal Society B: Biological Sciences*, 279(1732), pp. 1359–1365. Available at: https://doi.org/10.1098/rspb.2011.1644.

Jennions, M.D. and Petrie, M. (1997) 'VARIATION IN MATE CHOICE AND MATING PREFERENCES: A REVIEW OF CAUSES AND CONSEQUENCES', *Biological Reviews*, 72(2), pp. 283–327. Available at: https://doi.org/10.1017/S0006323196005014.

Johnstone, R.A. (1995) 'Sexual Selection, Honest Advertisement and the Handicap Principle: Reviewing the Evidence', *Biological Reviews*, 70(1), pp. 1–65. Available at: https://doi.org/10.1111/j.1469-185X.1995.tb01439.x.

Jois, S. *et al.* (2018) 'Characterization of the Sexually Dimorphic fruitless Neurons That Regulate Copulation Duration', *Frontiers in Physiology*, 9. Available at: https://www.frontiersin.org/articles/10.3389/fphys.2018.00780 (Accessed: 11 April 2023).

Jois, S. *et al.* (2022) 'Sexually dimorphic peripheral sensory neurons regulate copulation duration and persistence in male Drosophila', *Scientific Reports*, 12(1), p. 6177. Available at: https://doi.org/10.1038/s41598-022-10247-3.

Kuniyoshi, H. *et al.* (2002) 'lingerer, a Drosophila Gene Involved in Initiation and Termination of Copulation, Encodes a Set of Novel Cytoplasmic Proteins', *Genetics*, 162(4), pp. 1775–1789. Available at: https://doi.org/10.1093/genetics/162.4.1775.

Kvarnemo, C. and Simmons, L.W. (2013) 'Polyandry as a mediator of sexual selection before and after mating', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 368(1613), p. 20120042. Available at: https://doi.org/10.1098/rstb.2012.0042.

Lee, G. *et al.* (2000) 'Spatial, temporal, and sexually dimorphic expression patterns of the fruitless gene in the Drosophila central nervous system', *Journal of Neurobiology*, 43(4), pp. 404–426. Available at: https://doi.org/10.1002/1097-4695(20000615)43:4<404::AID-NEU8>3.0.CO;2-D.

Lenarcic, A.B. *et al.* (2012) 'A General Bayesian Approach to Analyzing Diallel Crosses of Inbred Strains', *Genetics*, 190(2), pp. 413–435. Available at: https://doi.org/10.1534/genetics.111.132563.

Lung, O. and Wolfner, M.F. (2001) 'Identification and characterization of the major Drosophila melanogaster mating plug protein', *Insect Biochemistry and Molecular Biology*, 31(6), pp. 543–551. Available at: https://doi.org/10.1016/S0965-1748(00)00154-5.

Lüpold, S. *et al.* (2012) 'How Multivariate Ejaculate Traits Determine Competitive Fertilization Success in Drosophila melanogaster', *Current Biology*, 22(18), pp. 1667–1672. Available at: https://doi.org/10.1016/j.cub.2012.06.059.

Lüpold, S. *et al.* (2013) 'Female mediation of competitive fertilization success in *Drosophila melanogaster*', *Proceedings of the National Academy of Sciences*, 110(26), pp. 10693–10698. Available at: https://doi.org/10.1073/pnas.1300954110.

Lüpold, S. *et al.* (2014) 'Female monopolization mediates the relationship between pre- and postcopulatory sexual traits', *Nature Communications*, 5(1), p. 3184. Available at: https://doi.org/10.1038/ncomms4184.

Lüpold, S. *et al.* (2020) 'How female × male and male × male interactions influence competitive fertilization in Drosophila melanogaster', *Evolution Letters*, 4(5), pp. 416–429. Available at: https://doi.org/10.1002/evl3.193.

MacBean, I.T. and Parsons, P.A. (1967) 'Directional Selection for Duration of Copulation in DROSOPHILA MELANOGASTER', *Genetics*, 56(2), pp. 233–239.

Mackay, T.F.C. *et al.* (2005) 'Genetics and genomics of Drosophila mating behavior', *Proceedings of the National Academy of Sciences*, 102(suppl_1), pp. 6622–6629. Available at: https://doi.org/10.1073/pnas.0501986102.

Manier, M.K. *et al.* (2010) 'Resolving Mechanisms of Competitive Fertilization Success in Drosophila melanogaster', *Science*, 328(5976), pp. 354–357. Available at: https://doi.org/10.1126/science.1187096.

Marcillac, F. and Ferveur, J.-F. (2004) 'A set of female pheromones affects reproduction before, during and after mating in Drosophila', *Journal of Experimental Biology*, 207(22), pp. 3927–3933. Available at: https://doi.org/10.1242/jeb.01236.

Maurizio, P.L. *et al.* (2017) 'Bayesian Diallel Analysis Reveals Mx1-Dependent and Mx1-Independent Effects on Response to Influenza A Virus in Mice', *G3: Genes|Genomes|Genetics*, 8(2), pp. 427–445. Available at: https://doi.org/10.1534/g3.117.300438.

McDonough-Goldstein, C.E., Pitnick, S. and Dorus, S. (2022) 'Drosophila female reproductive glands contribute to mating plug composition and the timing of sperm ejection', *Proceedings of the Royal Society B: Biological Sciences*, 289(1968), p. 20212213. Available at: https://doi.org/10.1098/rspb.2021.2213.

McGlothlin, J.W. *et al.* (2010) 'Interacting Phenotypes and the Evolutionary Process. Iii. Social Evolution', *Evolution*, 64(9), pp. 2558–2574. Available at: https://doi.org/10.1111/j.1558-5646.2010.01012.x.

Moore, A.J., Brodie III, E.D. and Wolf, J.B. (1997) 'Interacting Phenotypes and the Evolutionary Process: I. Direct and Indirect Genetic Effects of Social Interactions', *Evolution*, 51(5), pp. 1352–1362. Available at: https://doi.org/10.1111/j.1558-5646.1997.tb01458.x.

Moore, A.J. and Pizzari, T. (2005) 'Quantitative Genetic Models of Sexual Conflict Based on Interacting Phenotypes.', *The American Naturalist*, 165(S5), pp. S88–S97. Available at: https://doi.org/10.1086/429354.

Neckameyer, W.S. *et al.* (2000) 'Dopamine and senescence in Drosophila melanogaster☆', *Neurobiology of Aging*, 21(1), pp. 145–152. Available at: https://doi.org/10.1016/S0197-4580(99)00109-8.

Nelson, W.A. and Crone, E.E. (1999) '*Genetics and Analysis of Quantitative Traits* . Michael Lynch , Bruce Walsh', *The Quarterly Review of Biology*, 74(2), pp. 225–225. Available at: https://doi.org/10.1086/393101.

Parker, G.A. (1970) 'Sperm Competition and Its Evolutionary Consequences in the Insects', *Biological Reviews*, 45(4), pp. 525–567. Available at: https://doi.org/10.1111/j.1469-185X.1970.tb01176.x.

Parker, G.A. and Pizzari, T. (2010) 'Sperm competition and ejaculate economics', *Biological Reviews*, 85(4), pp. 897–934. Available at: https://doi.org/10.1111/j.1469-185X.2010.00140.x.

Pembury Smith, M. Q. R. and Snook. R. The impact of indirect genetic effects on sperm ejection: how partner genotype influences shared sexually selected traits. *Manuscript.*

Pennell, T.M. and Morrow, E.H. (2013) 'Two sexes, one genome: the evolutionary dynamics of intralocus sexual conflict', *Ecology and Evolution*, 3(6), pp. 1819–1834. Available at: https://doi.org/10.1002/ece3.540.

Pitnick, S. (1991) 'Male size influences mate fecundity and remating interval in Drosophila melanogaster', *Animal Behaviour*, 41(5), pp. 735–745. Available at: https://doi.org/10.1016/S0003-3472(05)80340-9.

Pitnick, S. (1996) 'Investment in Testes and the Cost of Making Long Sperm in Drosophila', *The American Naturalist*, 148(1), pp. 57–80. Available at: https://doi.org/10.1086/285911.

Price, T.A.R. *et al.* (2012) 'Experience of mating rivals causes males to modulate sperm transfer in the fly Drosophila pseudoobscura', *Journal of Insect Physiology*, 58(12), pp. 1669–1675. Available at: https://doi.org/10.1016/j.jinsphys.2012.10.008.

Ratterman, N.L. *et al.* (2014) 'Genetic Variation and Covariation in Male Attractiveness and Female Mating Preferences in Drosophila melanogaster', *G3 Genes|Genomes|Genetics*, 4(1), pp. 79–88. Available at: https://doi.org/10.1534/g3.113.007468.

R Core Team (2016) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Remick, D. (1992) 'Measuring the costs of reproduction', *Trends in Ecology & Evolution*, 7(2), pp. 42–45. Available at: https://doi.org/10.1016/0169-5347(92)90104-J.

Rice, W. and Holland, B. (1997) 'The enemies within: Intergenomic conflict, interlocus contest evolution (ICE), and the intraspecific Red Queen', *Behavioral Ecology and Sociobiology*, 41, pp. 1–10. Available at: https://doi.org/10.1007/s002650050357.

Rowe, L. and Houle, D. (1997) 'The lek paradox and the capture of genetic variance by condition dependent traits', *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 263(1375), pp. 1415–1421. Available at: https://doi.org/10.1098/rspb.1996.0207.

Sakai, T. *et al.* (2009) 'The Drosophila TRPA channel, Painless, regulates sexual receptivity in virgin females', *Genes, Brain and Behavior*, 8(5), pp. 546–557. Available at: https://doi.org/10.1111/j.1601-183X.2009.00503.x.

Schneider, M.R., Mangels, R. and Dean, M.D. (2016) 'The molecular basis and reproductive function(s) of copulatory plugs', *Molecular Reproduction and Development*, 83(9), pp. 755–767. Available at: https://doi.org/10.1002/mrd.22689.

Sheldon, Ben.C. (1994) 'Male phenotype, fertility, and the pursuit of extra-pair copulations by female birds', *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 257(1348), pp. 25–30. Available at: https://doi.org/10.1098/rspb.1994.0089.

Simmons, L.W. and Kotiaho, J.S. (2002) 'EVOLUTION OF EJACULATES: PATTERNS OF PHENOTYPIC AND GENOTYPIC VARIATION AND CONDITION DEPENDENCE IN SPERM COMPETITION TRAITS', *Evolution*, 56(8), pp. 1622–1631. Available at: https://doi.org/10.1111/j.0014-3820.2002.tb01474.x.

Simmons, L.W., Lüpold, S. and Fitzpatrick, J.L. (2017) 'Evolutionary Trade-Off between Secondary Sexual Traits and Ejaculates', *Trends in Ecology & Evolution*, 32(12), pp. 964–976. Available at: https://doi.org/10.1016/j.tree.2017.09.011.

Simmons, L.W., Tinghitella, R.M. and Zuk, M. (2010) 'Quantitative genetic variation in courtship song and its covariation with immune function and sperm quality in the field cricket Teleogryllus oceanicus', *Behavioral Ecology*, 21(6), pp. 1330–1336. Available at: https://doi.org/10.1093/beheco/arq154.

Singh, B.N. and Singh, S.R. (1999) 'Female remating inDrosophila ananassae: shorter duration of copulation during second mating as compared to first mating', *Journal of Biosciences*, 24(4), pp. 427–431. Available at: https://doi.org/10.1007/BF02942652.

Snook, R.R. and Hosken, D.J. (2004) 'Sperm death and dumping in Drosophila', *Nature*, 428(6986), pp. 939–941. Available at: https://doi.org/10.1038/nature02455.

Taylor, M.L., Evans, J.P. and Garcia-Gonzalez, F. (2013) 'No Evidence for Heritability of Male Mating Latency or Copulation Duration across Social Environments in Drosophila melanogaster', *PLOS ONE*, 8(10), p. e77347. Available at: https://doi.org/10.1371/journal.pone.0077347.

Tennant, H.M., Sonser, E.E. and Long, T.A. (2014) 'Hemiclonal analysis of interacting phenotypes in male and female Drosophila melanogaster', *BMC Evolutionary Biology*, 14(1), p. 95. Available at: https://doi.org/10.1186/1471-2148-14-95.

Thornhill, R. (1983) 'Cryptic Female Choice and Its Implications in the Scorpionfly Harpobittacus nigriceps', *The American Naturalist*, 122(6), pp. 765–788. Available at: https://doi.org/10.1086/284170.

Travers, L.M., Garcia-Gonzalez, F. and Simmons, L.W. (2016) 'Genetic variation but weak genetic covariation between pre- and post-copulatory episodes of sexual selection in Drosophila melanogaster', *Journal of Evolutionary Biology*, 29(8), pp. 1535–1552. Available at: https://doi.org/10.1111/jeb.12890.

Turner, S.D. *et al.* (2018) 'Dissecting the Genetic Architecture of Shoot Growth in Carrot (Daucus carota L.) Using a Diallel Mating Design', *G3 Genes|Genomes|Genetics*, 8(2), pp. 411–426. Available at: https://doi.org/10.1534/g3.117.300235.

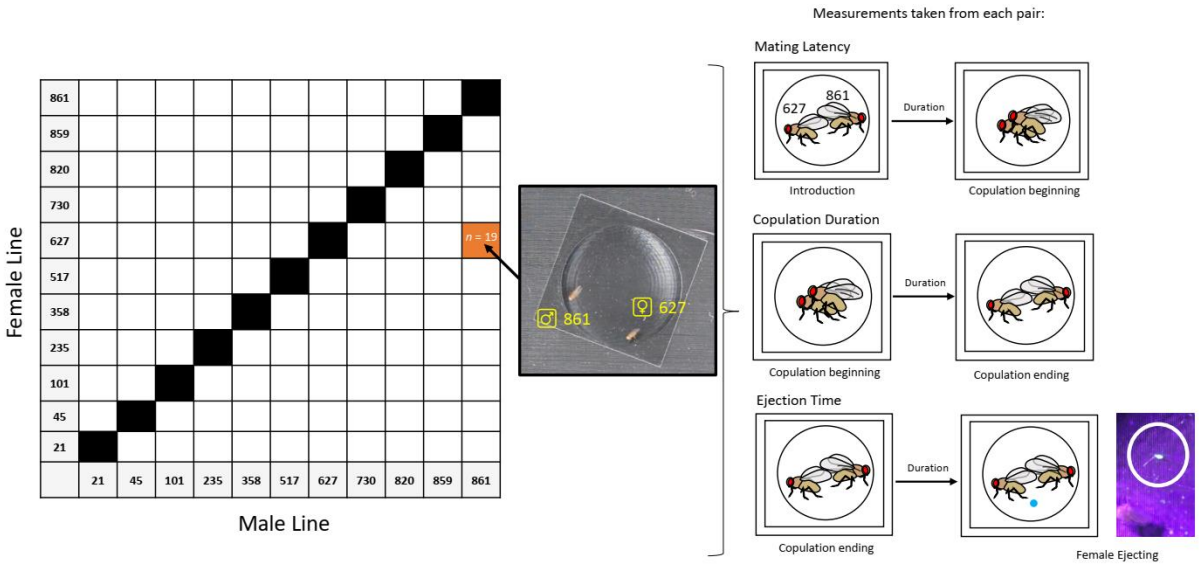
Ueyama, M. *et al.* (2005) 'Mutations in the desat1 gene reduces the production of courtship stimulatory pheromones through a marked effect on fatty acids in Drosophila melanogaster', *Insect Biochemistry and Molecular Biology*, 35(8), pp. 911–920. Available at: https://doi.org/10.1016/j.ibmb.2005.03.007.

Wolf, J.B., Brodie III, E.D. and Moore, A.J. (1999) 'Interacting Phenotypes and the Evolutionary Process. II. Selection Resulting from Social Interactions.', *The American Naturalist*, 153(3), pp. 254–266. Available at: https://doi.org/10.1086/303168.

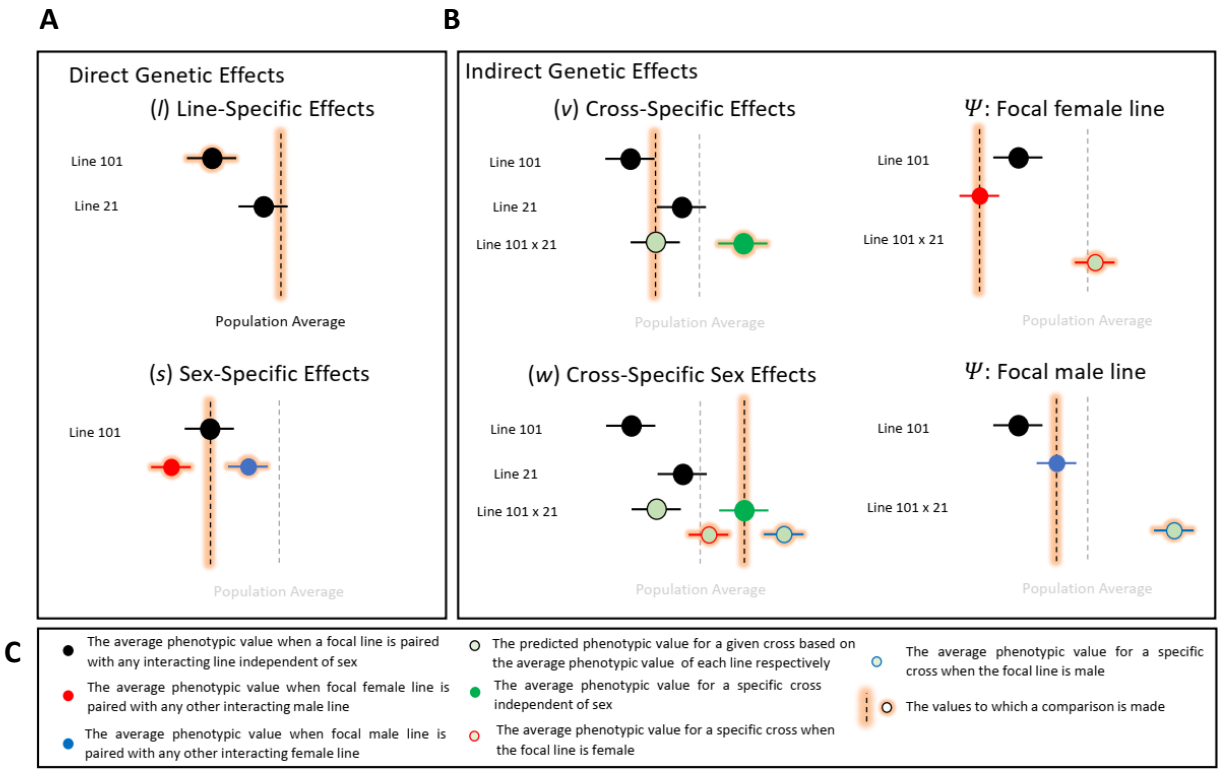
6. Chapter 2 - Supplementary Material

Supplementary Table S1. Pearson's correlation coefficient for Line-Specific (additive) effects from BayesDiallel model between interactive phenotypes. How Line-Specific effects were calculated is outlines in Supplementary Figure S2.

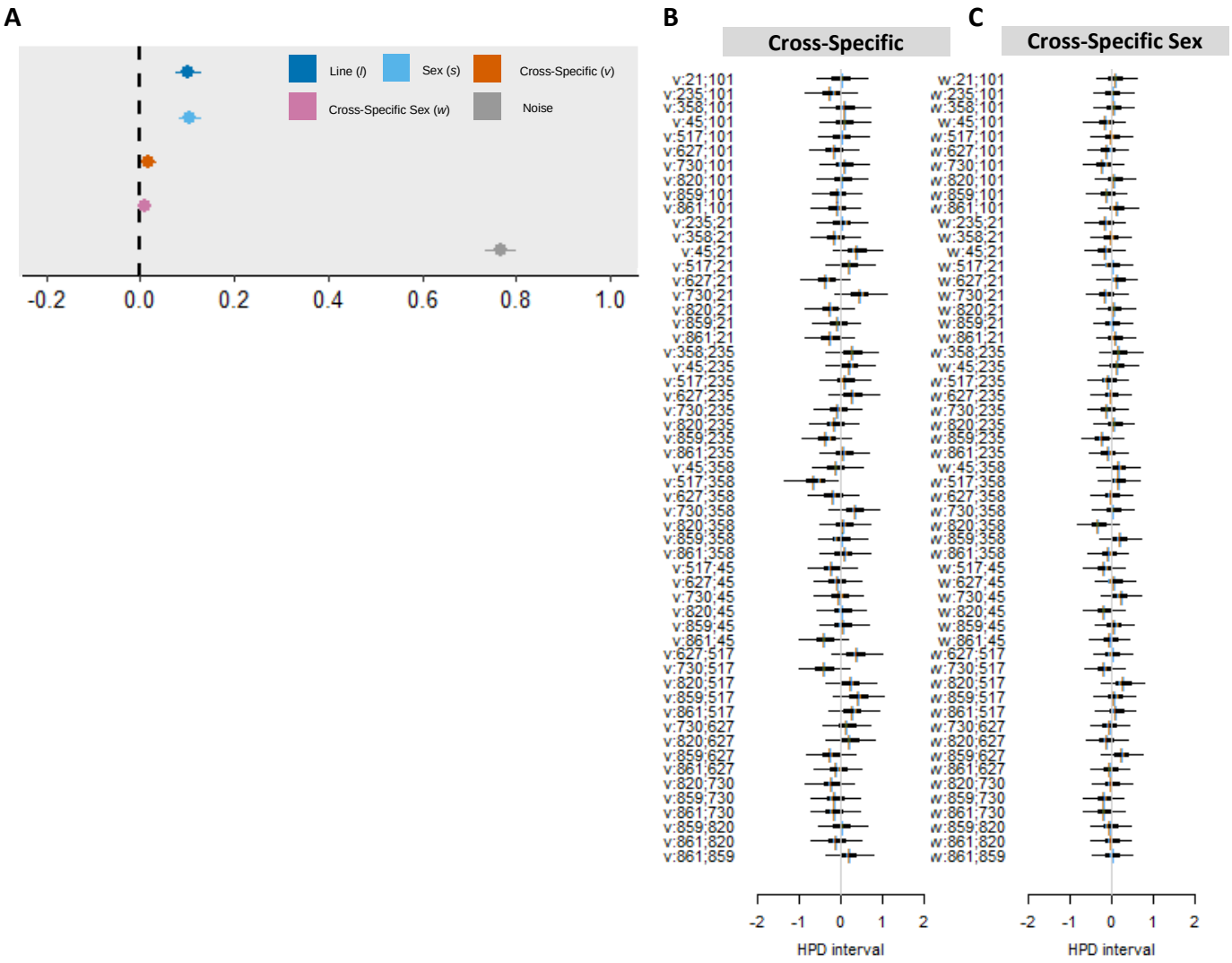
Interactive Phenotype	Source of Variance	Coefficient	P
Copulation Duration	Mating Latency	0.26	0.45
Ejection Time	Mating Latency	0.13	0.70
	Copulation Duration	0.43	0.19



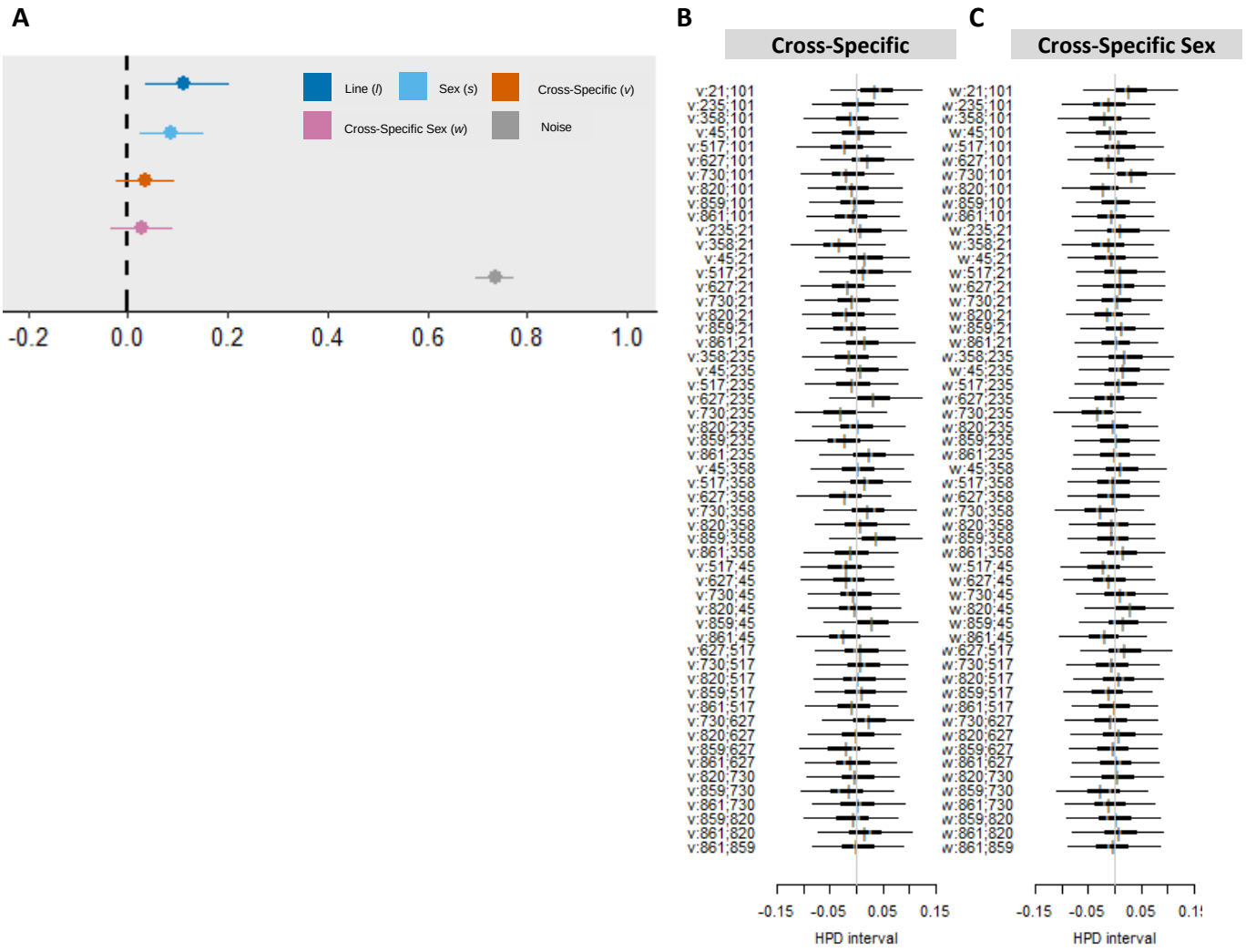
Supplementary Figure S1. A schematic of the diallel mating design. Eleven isofemale lines were crossed in a full diallel mating design excluding reciprocal crosses (represented as a black square), representing 110 possible male-by-female genotypic crosses. For each genotypic cross, one male and one female from different lines were introduced into a chamber. Mating latency, copulation duration and ejection time were recorded for each pair. A photo of the latter is shown, with the ejection mass highlighted by a white circle. A pairing between male 861 and female 627 is illustrated in the figure. A pairing between these two lines was conducted 19 times.



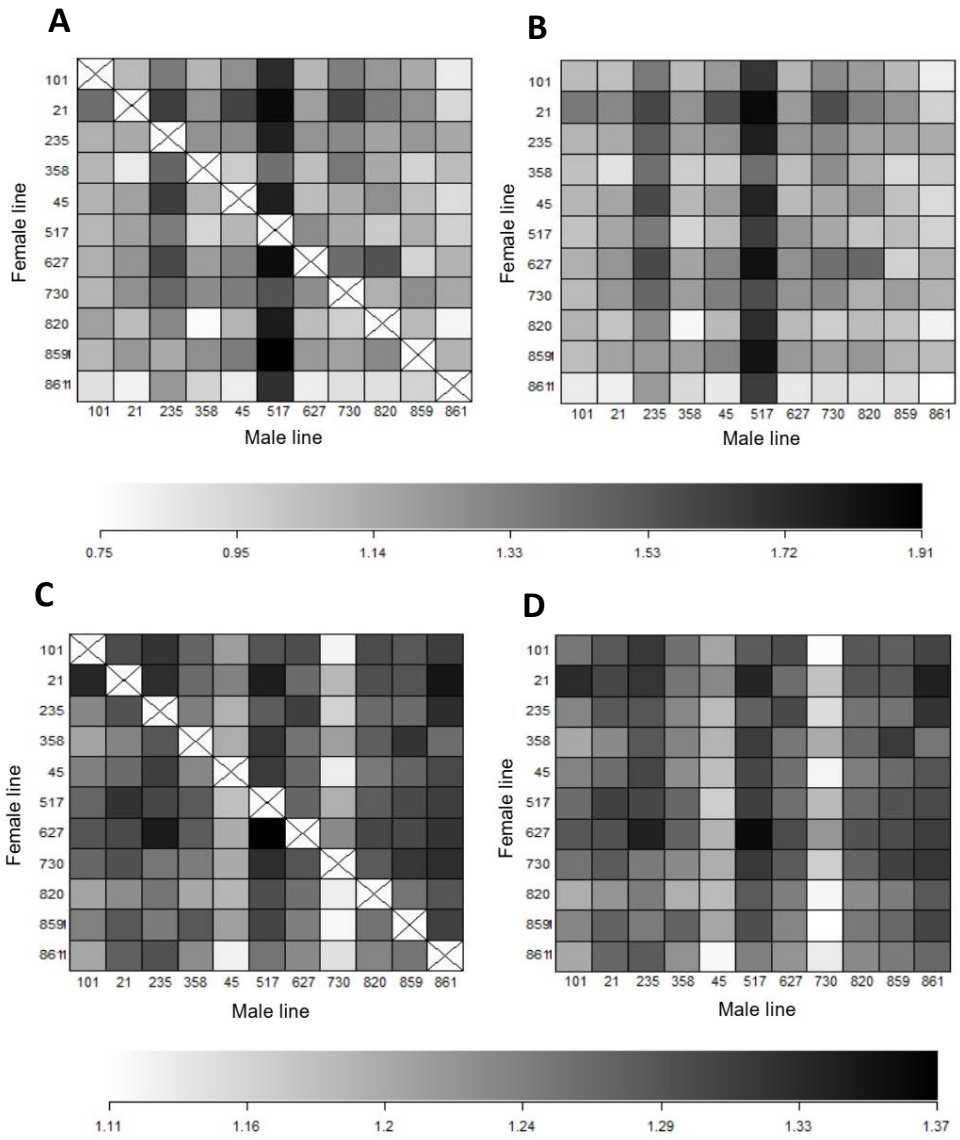
Supplementary Figure S2. A schematic to describe how Direct Genetic Effects and Indirect Genetic Effects were quantified for each phenotype measured. Direct genetic effects (A) are composed of Line-Specific Effects (*l*) and Sex-Specific Effects (*s*). Line-Specific Effects are derived by comparing the average phenotypic value when a focal line is paired with all other interacting genotypic lines, independent of sex, to the population average. Sex-Specific Effects (*s*) are derived by comparing the average phenotypic value deviation when the focal line is female, with male as a baseline, compared to the overall average phenotypic value of that line. Indirect Genetic Effects (B) are composed of Cross-Specific Effects (*v*), Cross-Specific Sex Effects (*w)*, Ψ: when the focal line is female and Ψ: when the focal line is male. Cross-Specific Effects (*v*) are derived by comparing the predicted phenotype for a given cross (between a focal genotype and an interacting genotype) based on the average phenotype of each line respectively to the actual average phenotypic value of that specific cross, independent of sex. Cross-Specific Sex Effects (*w*) are derived by comparing the average phenotypic deviation when the focal line is female for a given cross to the average phenotypic value of that specific cross (with the focal line when male as a baseline; i.e. 101 x 21 vs 21 x 101). Ψ: Focal female line is quantified by identifying the extent to which the average phenotypic value for a given focal line when female (101) differs from the average phenotypic value when that focal female line is paired to a specific interacting male genotype (21). Ψ: Focal male line is quantified by identifying the extent to which the average phenotypic value for a given focal line when male (101) differs from the average phenotypic value when that focal male line is paired to a specific interacting female genotype (21). A legend (C) describes each component presented in figures (A) and (B).



Supplementary Figure S3. BayesDiallel analysis of mating latency. (A) VarP Plot describing the variance projection of each diallel class. It predicts how much of the total phenotypic sum of squares is explained by each component for mating latency. The percentage of the variance in mating latency explained by diallel effects, which represents a broad-sense heritability like measure is 20%. Genotypic line effects, which represent a narrow-sense heritability like measure explain 10% ± 1%, sex-specific effects account for 10% ± 1%, and both cross-specific interactions (v and w) account for 3% ± 1% respectively. The black dotted line represents the significance threshold, so any model component that overlaps with the dotted line does not explain a significant proportion of the phenotypic variance. (B) Highest posterior density (HPD) intervals of mating latency based on the cross-specific effects (v) from the $lsvw$ model. (C) Highest posterior density (HPD) intervals of mating latency based on the cross-specific sex effects (w) from the $lsvw$ model. For each effect, thin and thick horizontal lines show the 95 and 50% HPD intervals, respectively, with short vertical bars indicating the posterior mean. HPD intervals that do not include zero we consider to be statistically significant at 95% credibility. Overall there was no significant cross-specific or cross-specific sex effects. Details on how model effects (l , s, v, w) are calculated can be found in Supplementary Figure S2.



Supplementary Figure S4. BayesDiallel analysis of copulation duration. (A) VarP Plot describing the variance projection of each diallel class. It predicts how much of the total phenotypic sum of squares is explained by each component for copulation duration. The percentage of the variance in copulation duration explained by diallel effects, which represents a broad-sense heritability like measure is 20%. Genotypic line effects, which represent narrow-sense heritability like measure explain 11% ± 4%, sex-specific effects accounting for 9% ± 3%, and cross-specific interactions (v and w) both accounting for 7% ± 3% respectively. The black dotted line represents the significance threshold, so any model component that overlaps with the dotted line does not explain a significant proportion of the phenotypic variance. (B) Highest posterior density (HPD) intervals of copulation duration based on the cross-specific effects (v) from the $lsvw$ model. (C) Highest posterior density (HPD) intervals of copulation duration based on the cross-specific sex effects (w) from the $lsvw$ model. For each effect, thin and thick horizontal lines show the 95 and 50% HPD intervals, respectively, with short vertical bars indicating the posterior mean. HPD intervals that do not include zero we consider to be statistically significant at 95% credibility. Overall there was no significant cross-specific or cross-specific sex effects. Details on how model effects (l , s, v, w) are calculated can be found in Supplementary Figure S2.



Supplementary Figure S5. Observed (A & C) and Predicted (B & D) means from the BayesDiallel model. The first heatmap on each row represents the observed phenotypic variation. Each cell represents the average mating latency (A) and copulation duration (C) for each pairing, where darker shading represents a longer duration. Duration (minutes) is presented on the log scale for both phenotypes. The second heatmap on each row represents the predicted phenotypic variation. Each cell represents the average mating latency (B) and copulation duration (D) for each pairing that would be expected in a future experiment based on the model and the observed data, incorporating all uncertainty due to finite sampling and prior uncertainty about the parameters. Crossed boxes indicate the absence of pairings. The observed and predicted mean phenotypes were largely similar, suggesting that the distributions predicted by the model largely represents our raw data. Note that the order of the lines is different from the other heatmaps presented and is not in numerical order.