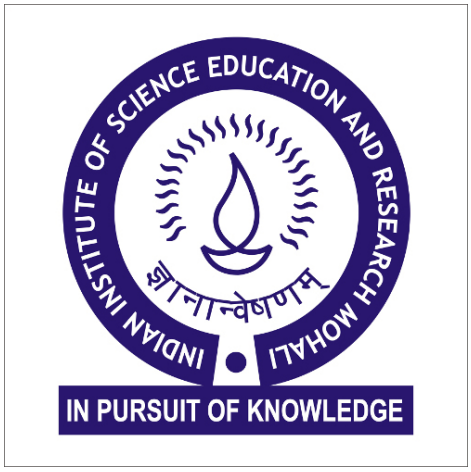
Effect of diet on mate choice in populations of *Tribolium castaneum*

Soumya Panyam

A dissertation submitted for the partial fulfilment of BS-MS dual degree in Science



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May 2021

Certificate of Examination

This is to certify that the dissertation titled "Effect of diet on mate choice in populations of *Tribolium castaneum*" submitted by Ms. Soumya Panyam (Reg. No. MS16082) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Deepa Agashe at the National Centre for Biological Sciences, Bengaluru, and Prof. N. G. Prasad at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Soumya Panyam

(Candidate)

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Prof. N. G. Prasad

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Abstract

Adaptation to novel environments can lead to reproductive isolation, one of the hallmarks of divergent evolution. This project aimed to investigate mechanisms of reproductive isolation in populations of *Tribolium castaneum*, adapted to novel diets. Founding populations from an out-bred line on wheat were used to set up adapted lines in corn, sorghum and finger millet. Earlier results on mate choice in the corn and sorghum adapted lines revealed assortative mating patterns between the adapted populations and the ancestral population. To address whether this pattern was due to reproductive isolation or plastic responses to exposure to novel environment, I assayed mate choice in individuals reared in corn or sorghum for only one generation, and found that mating was random between these populations. I also found random mating between the finger millet adapted lines and the ancestral line. My results indicated that behavioural pre-zygotic isolation had occurred in the adapted lines on corn and sorghum but not in the finger millet adapted lines. This suggests that the mechanisms of adaptation and reproductive isolation are different in different environments.

Chapter 1

Introduction

1.1 Background

1.1.1 Ecological speciation

When populations adapt to new environments, different alleles may confer fitness advantages in the different environments. Selection drives these alleles to fixation in these different populations (Kilias, Alahiotis, and Pelecanos 1980; Schluter 2009). Genic differences between populations diverging via adaptation to different diets has been studied in yeast (Dettman et al. 2007) and *Drosophila* (Kilias, Alahiotis, and Pelecanos 1980; Dodd 1989). Predation gradients can also drive ecological divergence, with antipredator traits evolving in different populations (Langerhans, Gifford, and Joseph 2007). This mechanism of divergence mainly occurs in allopatric populations of the same species, which have physical geographical barriers hindering gene flow, although it can occur in sympatric populations if there is sufficient environmental variation (Kilias, Alahiotis, and Pelecanos 1980; Schluter 2009). A characteristic of ecological speciation is lower fitness of hybrids than the average fitness of either population in their respective environments (Schluter 2009). Upon subsequent interaction, if sufficient genetic differences have arisen between the populations, they remain reproductively isolated. Over time, with reduced gene flow, reproductive isolation is completed, leading to speciation (Kilias, Alahiotis, and Pelecanos 1980).

1.1.2 Reproductive isolation

Reproductive isolation, a characteristic feature of divergent evolution, comprises the processes that reduce gene flow between populations (Mayr 1942). There are two broad categories of mechanisms of reproductive isolation: (i) post-zygotic reproductive isolation mechanisms, which operate after fertilisation takes place, and (ii) pre-zygotic reproductive isolation mechanisms, which operate prior to fertilisation (Mayr 1963; Mayr 1942; J. A. Coyne and Orr 1998). Often, more than one of these mechanisms operate simultaneously ensuring complete reproductive isolation.

Post-zygotic reproductive isolation

Post-zygotic mechanisms of isolation operate following successful fertilisation, by preventing successful hybridisation between the organisms. It has two broad categories of action: 1. **Hybrid non-viability**, by which the development of the hybrid zygote is halted at early stages in development or suffers reduced viability if it survives, and 2. **Hybrid sterility**, wherein the hybrid develops normally but is unable to reproduce (Haldane 1922; J. A. Coyne and Orr 1998).

Pre-zygotic reproductive isolation

Pre-zygotic reproductive isolation operates by creating barriers to fertilisation success. Pre-zygotic mechnanisms are the primary causes of reproductive isolation, especially when the organisms occur in sympatry following allopatric separation in *Drosophila*, as reviewed by Coyne and Orr (J. A. Coyne and Orr 1989). There are four main mechanisms by which pre-zygotic reproductive isolation occurs:

1. **Habitat isolation**

   Habitat isolation occurs by preventing potential mates from different populations from coming in contact with each other. This can take the form of physical or temporal habitat exclusion. For instance, closely related species of spadefoot toads (*Scaphiopus couchi* and *S. holbrooki hurteri*) are isolated by different soil types even when present in the same geographical region (Wasserman 1957). In other cases, there may be temporal differences in reproductive activity such as in the case of periodical cicada which have either 13 or 17 year long development periods. Thus, they have limited opportunity to hybridise (once in 221 years), causing reproductive isolation (Williams and Simon 1995; Cooley, Simon, and Marshall 2003).

2. **Mechanical isolation**

   Morphological variations can result in mating inability by causing incompatible genital structures. In land snail (genus *Euhedra*), snails have shells with either dextral or sinistral coils, which cause reversal in genital position, creating barriers to mating (Asami, Cowie, and Ohbayashi 1998; Ueshima and Asami 2003; Gittenberger, Hamann, and Asami 2012). Alternatively, some angiosperm coevolve morphological and physiological traits with specific pollinators having different body sizes, ensuring that no pollen is transferred to flowers of different species. For example, the balsams *Impatiens capensis* and *Impatiens pallida* are related species. However *I. capensis* is pollinated by hummingbirds, whereas *I. pallida* is pollinated by bumblebees (Grant 1994).

3. **Gametic isolation**

   Failure of the sperm to fertilise the egg after successful copulation can occur due to antigenic reactions in the female genital tract in organisms which have internal fertilisation (reviewed in Mayr 1963). In organisms which show external fertilisation, such as marine invertebrates, gametes are released synchronously, but heterospecific sperm is unable to fertilise eggs (Slaughter, Yund, and Rawson 2003).

4. **Behavioural isolation**

   Varying reproductive behaviour can create barriers to mating and fertilisation (Panhuis et al. 2001; West-Eberhard 1983). Since sexual selection acting on mate choice directly affects traits or signals that are involved in mate recognition, it can cause reproductive isolation by driving divergence of these traits or signals via assortating mating patterns (Panhuis et al. 2001; Mendelson and Shaw 2005; Guerra and Ron 2008; Schumer et al. 2017). Behvioural or sexual isolation resulting from incompatibilities in mating may manifest in lack of courtship, or courtship not being carried out to completion despite motivation for mating (Mayr 1963). For instance, in the *Drosophila virilis* group, males prefer to perform courtship behaviour to females of the same species over those of other related species (Spieth 1952).

1.1.3 Mate choice and sexual selection

Sexual selection, as proposed by Darwin in *The Descent of Man and Selection in Relation to Sex* (Darwin 1871), affects the reproductive success of individuals in a populations. It manifests in by 1. intra-sexual competition and 2. mate choice. The sex exhibiting mate choice is generally the females, with higher strength of sexual selection acting on the males, which compete for opportunities to reproduce (Darwin 1871; Trivers 1972), although this is

not always the case (Trivers 1972; Fitzpatrick, Berglund, and Rosenvist 1995; Jones 2001; Bonduriansky 2001).

Reproductive behaviour is modulated by visual, auditory or chemical signals. For instance, in *Drosophila melanogaster*, male courtship consists of sequential displays of visual, auditory and olfactory signals (J. Coyne, Crittenden, and Mah 1994). Cuticular hydrocarbons (CHCs), volatile compounds present on the surface of insects, play a role in communication via chemical signalling (Howard and Blomquist 2005). They facilitate recognition of conspecific individuals, potential mates and, in the case of social insects, individuals from the same colony, besides protecting against desiccation (Howard and Blomquist 2005; Thomas and Simmons 2008; Jallon 1984). They have been identified in many different species for their role in mate choice, mainly by allowing recognition of conspecifics (Curtis et al. 2013; Buellesbach, Vetter, and Schmitt 2018; Tyler et al. 2015; Zhang et al. 2014). In the cricket species *Gryllodes sigillatus* females mark mates with their CHCs to avoid remating with the same mate, facilitating polyandry (Ivy, Weddle, and Sakaluk 2005).

Olfactory signals have been identified in both males and females in closely related species of the genus *Drosophila*. Evidence from work on *D. simulans* and *D. sechellia* indicates that female CHCs play a role in reproductive isolation between these two species (Mackay et al. 2005; J. Coyne, Crittenden, and Mah 1994). In some cricket species which overlap geographically, CHCs have been found to play a role in heterospecific discrimination by females, contributing to reproductive isolation (Tyler et al. 2015; Mullen et al. 2007). CHCs and their effects can be altered by geographic distance (Jennings et al. 2014) and diet (Ng, Simpson, and Simmons 2018; Rapkin et al. 2017; Ingleby et al. 2014; Otte, Hilker, and Geiselhardt 2014). They also show plastic variation in resposnse to the social environment (Krupp et al. 2008; Petfield et al. 2005).

The aim of this project is to investigate ecological divergence in populations of *Tribolium castaneum* (the red flour beetle) adapted to novel diets. In my thesis, I addressed this by assaying mate choice in these populations as evidence of pre-zygotic reproductive isolation.

1.1.4 *Tribolium castaneum*: Mating system

Tribolium castaneum (or the red flour beetle) is a highly promiscuous grain pest found in many parts of the world (Haubruge and Arnaud 1999; Lewis and Iannini 1995), and reviewed in Sokoloff 1977). They are generalists and occupy a wide dietary niche (Sokoloff, Franklin, and Lakhanpal 1966; Gerken and Campbell 2020). Males prefer to mate with virgin females over females that had previously mated (Haubruge and Arnaud 1999; Lewis and Iannini 1995). However, males choose mates that mated with rival males over previous mates of

their own (Haubruge and Arnaud 1999). Some studies cite last male sperm precedence as a reason for this (Haubruge and Arnaud 1999), but other studies found little to no evidence of last male precedence as a general principle (Lewis 2004; Pai and Yan 2002). Virgin females tend to show little mate discrimination, but choose more attractive mates when they have the opportunity to remate (Fedina and Lewis 2007).

Male courtship in *T. castaneum* involves identifying a potential mate and initiating contact, following which the male mounts the female dorsally to attempt copulation. The duration of this can range from 30 seconds to 30 minutes, during which time the male transfers spermatheca to the female (Haubruge and Arnaud 1999). The females can store sperm and exert cryptic choice after mating.

For my thesis, I used populations of *T. castaneum* maintained on wheat flour, and adapted lines in three novel diets: corn, sorghum and finger millet flours. These flours offer a range of nutritive quality, with sorghum and finger millet being of poorer quality than wheat flour, and corn flour being the lest optimal (Agarwal and Agashe 2018; Gerken and Campbell 2020). Earlier work on this project (Rittik Deb unpublished data) addressed reproductive isolation in the populations adapted to corn and sorghum. Mate choice assays revealed assortative mating patterns in these populations, with respect to the ancestral population reared in wheat, as visualised in Fig 1.1.

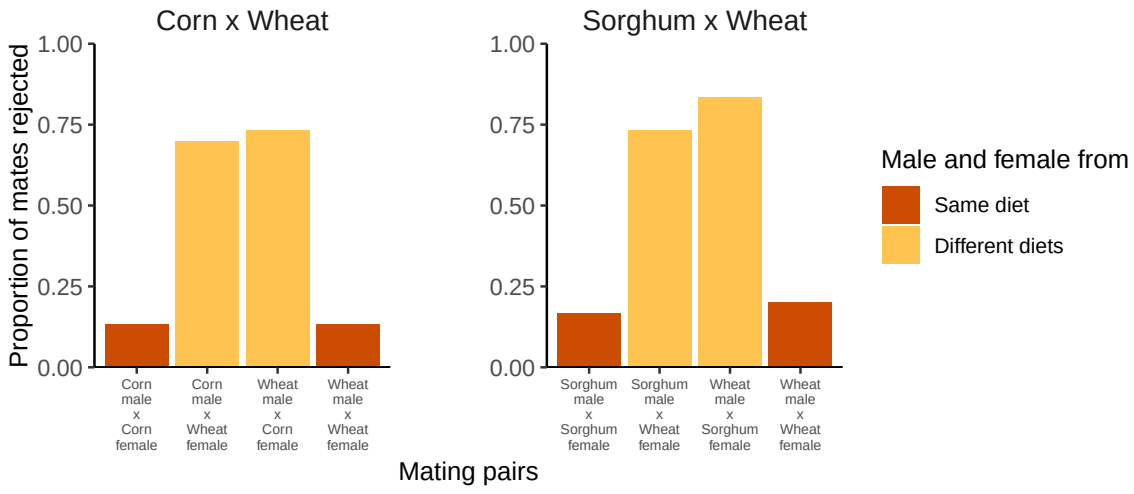


Figure 1.1: Data on mate rejection rates from one mate choice assay each of corn adapted (left) and sorghum adapted (right) populations with respect to the ancestral population in wheat. Significantly lower rates of mating failure between individuals from the same diet (GLM: binomial test: for Corn $Z = 5.885$, and Sorghum $Z = 6.072$, with df = 3, P-value < 0.001 for both cases).

There are two potential explanations for the mating patterns observed: 1. behavioural isolation by adaptation to novel resources, and 2. plastic responses to properties of the flour (such as odour, particle size, texture etc). I investigated mate choice in *T. castaneum* individuals that were allowed to develop from egg to adult for only one generation in a novel resource (corn or sorghum). If the pattern was because of flour properties that elicit a plastic response, individuals that developed in a novel resource for one generation in the novel resources would show the same response and mating patterns; this allowed me to distinguish between the two possibilities. In addition, my thesis work involved investigating behavioural isolation in the populations adapted to a third diet, finger millet.

Further, I extracted CHCs from the individuals used in mating assays to investigate the potential mechanisms underlying mate choice in these populations.

1.2 Experimental Methods

1.2.1 Beetle populations

A large out-bred population (henceforth referred to as "OBL") of *T. castaneum* was established in the laboratory by out-crossing twelve wild-collected strains from different parts of the country (Kumar, Issar, and Agashe 2018). The beetles were kept in plastic boxes containing whole wheat flour (henceforth referred to as "wheat"). The lids of the boxes had small holes to allow for airflow. They were maintained at 34°C ± 1°C. Discrete generation cycles of 4 weeks were maintained, with 1 week allowed for oviposition each generation. Adults from OBL were used for mate choice work 6 weeks post egg laying.

Flour bags were cold sterilised at -80°C to kill insect eggs that may be present in the flour, and then allowed to thaw at room temperature (25°C) prior to use. The flours I used for my experimental work are wheat (for the ancestral OBL population), and three "novel" flours: sorghum (*Sorghum bicolor*), maize (*Zea mays*) and finger millet (*Eleusine coracana*). The generation cycle period of *Tribolium* in each of these flours in given in Table 1.1.

Flour	Scientific name of flour	Length of generation cycle maintained
Wheat	*Triticum* sp.	4 weeks
Sorghum	*Sorghum bicolor*	4 weeks
Maize	*Zea mays*	6 weeks
Finger millet	*Eleusine coracana*	6 weeks

Table 1.1: Development time of *Tribolium castaneum* in different diets.

Adapted lines in novel resources

Populations adapted to novel resources were established using a founding a population of 200 or 500 individuals from the out-bred line. They were allowed to ovisposit on the novel resource (at a density of 1 gram of flour per adult), and maintained as per their respective maintenance times (Agarwal and Agashe 2018). Once the population showed signatures of adaptation, the individuals were used for mate choice assays. The indicators of adaptation used were

1. 3 successive generations of population size growth
2. Population size equal to or greater than that of the initial founding population

Single generation development in novel flour

200 adults from the out-bred line from wheat were allowed to oviposit for 1 week in either sorghum or maize. The adults were then removed and the individuals were allowed to develop for 6 weeks and 8 weeks respectively. These individuals were then used for mate choice assays.

1.2.2 Mate choice assays

No choice assays were conducted between individuals from a novel diet (reared for one generation in case of corn and sorghum; adapted in case of finger millet) and the ancestral population in wheat. Individuals for mating pairs were sexed and isolated 72 to 96 hours prior to the assays, in order to motivate mating. To separate the male and female adults, the beetles were subjected to cold shock using ice and then observed under a microscope to identify sex patches on the first pair of legs, which is a distinguishing feature of male *T. castaneum* adults (Hinton 1942). The body length of each adult along the ventral side was also noted. Females were marked with a non-toxic dye to distinguish them during the assays. The separated individuals were kept isolated in 1.5 ml Eppendorf tubes having a perforated lid, containing 1 ml of their respective flour.

72 to 96 hours following the isolation and marking of the adults, mating pairs were set up for the mate choice assays. These assays were set up in a flour-free environment. Each individual was removed from their tube, brushed off to remove flour particles and placed in a plastic petri plate at least 30 minutes prior to commencing the mate choice trial, in order to allow the beetles time to acclimatise to the flour-free environment. The mate choice assays

were set up in separate plastic petri plates lined with filter paper to provide texture for the beetles to walk, and were placed in a cardboard box to simulate a dark environment. From each pair, the female was released into the mating petri plate first. After 30 seconds, the male was placed in the petri plate and the observation began.

Mating behaviour characterised

Behaviour	Description
Interaction	Male and female come in contact with each other
Mount attempt	Male attempt to climb (mount) the female A mount that lasts 40 seconds or longer is considered a successful mating session
Avoidance behaviour	Moving away or attempting to move away from one of the individuals by the other

Table 1.2: Characterisation of mating behaviour.

The observation period for each mating pair was 30 minutes, starting from the first interaction. The data noted was mainly whether or not mating occurred (i.e., whether the mate was accepted or rejected). In addition, the following were also noted, using the characterisations of behaviour in Table 1.2:

1. Latency (time from first interaction until mating began
2. Number of interactions initiated by each individual until mating began
3. Duration of the first mating session
4. Total number of interactions during the 30 minute observation period
5. Total mating duration, including all mating sessions
6. Number of instances of avoidance behaviour by each individual
7. Number of mating sessions during the 30 minute observation period

The average mating duration was calculated as

$$Average\ mating\ duration\ =\ \frac{Total\ mating\ duration}{Number\ of\ mating\ sessions}$$

If no interaction occurred within the first 10 minutes of introducing the male into the plate, the individuals were considered unmotivated to mate, and were discarded. Only pairs that interacted were retained in the data set.

A total of nine mate choice assays were conducted, using individuals from the following populations, with OBL individuals:

1. Finger millet adapted – 3 biological replicates (referred to henceforth as FM population 1, 2 and 3)
2. Single generation corn developed – 3 biological replicates (referred to henceforth as Corn population 1, 2 and 3)
3. Single generation sorghum developed – 3 biological replicates (referred to henceforth as Sorghum population 1, 2 and 3)

Each mating assay involved four mating pair types:

1. Novel diet ♂x novel diet ♀
2. Novel diet ♂x ancestral (wheat) ♀
3. Wheat ♂x novel diet ♀
4. Wheat ♂x wheat ♀

In each of the above cases, a complete set of data of all the enlisted behaviours was collected for the first two biological replicates. In these assays, we found little to no mate rejection across different diets and types of mating pairs. Analysis of this data revealed significant correlation between behaviour variables prior to first mating and the behaviour over the 30 minute period. For the third replicate, observation for each mating pair was carried out until the first mating event, with no further data collected, although the individuals were allowed to remain in the experimental plate for the entirety of the 30 minute duration.

After the mate choice assays, the individuals were stored at -80°C to prepare them for extraction of cuticular hydrocarbons and further tests.

1.2.3 Cuticular hydrocarbon extraction

To investigate potential mechanisms underlying mate choice and mating behaviour, CHCs were extracted from the individuals taken from the different resources used in the mate choice assays. Each individual was placed in a 2 ml glass vial with 0.5 ml of n-Hexane for 19 minutes. The insect was then removed and the excess solvent was allowed to evaporate for 8-10 hours at room temperature. The extracted CHCs in the vials were maintained at -20°C until analysis by mass spectroscopy.

1.2.4 Data analysis and statistics

Statistical analysis was done using R version 3.5.3 (R Core Team 2019). The packages *emmeans* and *reshape2* were used for structuring the data (Lenth 2019; Wickham 2007), and the package *ggplots* was used for creating plots (Wickham 2016).

Mate choice or mating success was analysed using a generalised linear model in the binomial form. The number of interactions and the number of instances of avoidance were analysed using a Poisson model, calculated pairwise for each treatment, using the *emmeans* package. Analysis of Variance (ANOVA) was performed on the behaviour variables of mating duration. All tests were performed at a 95% confidence interval for each distribution. The results of these analyses are presented in the next section.

Chapter 2

Summary and Conclusions

2.1 Results

2.1.1 Single generation corn developed populations: mating assays

The no choice mating assays between individuals reared on corn for a single generation and those from the ancestral wheat population revealed no preference for mates from either diet, represented in Fig 2.1. Generalised linear models with a binomial distribution used to fit this data for each population revealed no significant effect of the type of mating pair on mating success (Corn population 1: $N = 10$ per type of mating pair, Z = 0.001, df = 39 (null model) $P = 0.99$; Corn population 2: $N = 16$ per type of mating pair, Z = -0.004, df = 63 (null model) $P = 0.997$; Corn population 3: $N = 12$ per type of mating pair, Z = -0.507, df = 47 (null model) $P = 6124$). There was no significant effect of the number of interactions or the body size of males and females in mating success (GLM: Binomial on Number of interactions: Z = 0.001, -0.615, 0.613; $P = 0.999$, 0.539 and 0.540; Body size: Z = 0.001, 1.357, 0.088; $P = 0.999$, 0.175 and 0.930; in Corn populations 1, 2 and 3 respectively). The low sample size in corn is due to the poor nutritional quality of corn, reducing fecundity in this resource, when reared for one generation, since these individuals are not adapted to corn. No post-hoc tests were done as the binomial test revealed no significant differences.

Figure 2.1: Proportion of pairs that did not successfully mate during the observation time in three populations of single generation corn-reared individuals.

Further, I analysed all the behavioural variables measured for each assay. The latency in mating is shown in Fig 2.2. Analysis of Variance indicated no significant differences in latency to mate between the different mating pair types (Corn population 1: $F = 0.32$, df = 3, $P = 0.811$; Corn population 2: $F = 0.45$, df = 3, $P = 0.688$; Corn population 3: $F = 1.38$, df = 3, $P = 0.264$). Since ANOVA returned no significant differences in the variable, no post-hoc test was done.

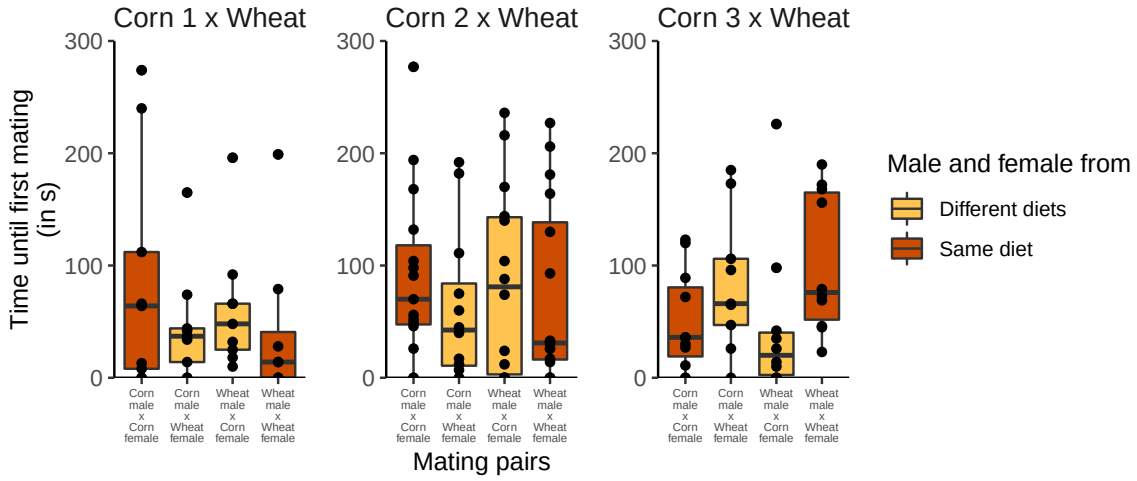


Figure 2.2: Time until first mating in three populations of individuals reared in corn for a single generation.

No significant difference in the duration of the first mating event was found between the different mating types in any of the replicates (represented in Fig 2.3), using ANOVA (Corn

population 1: F = 1.573, df = 3, P = 0.213; Corn population 2: F = 1.466, df = 3, P = 0.233; Corn population 3: F = 1.371, df = 3, P = 0.264). No post-hoc test conducted.

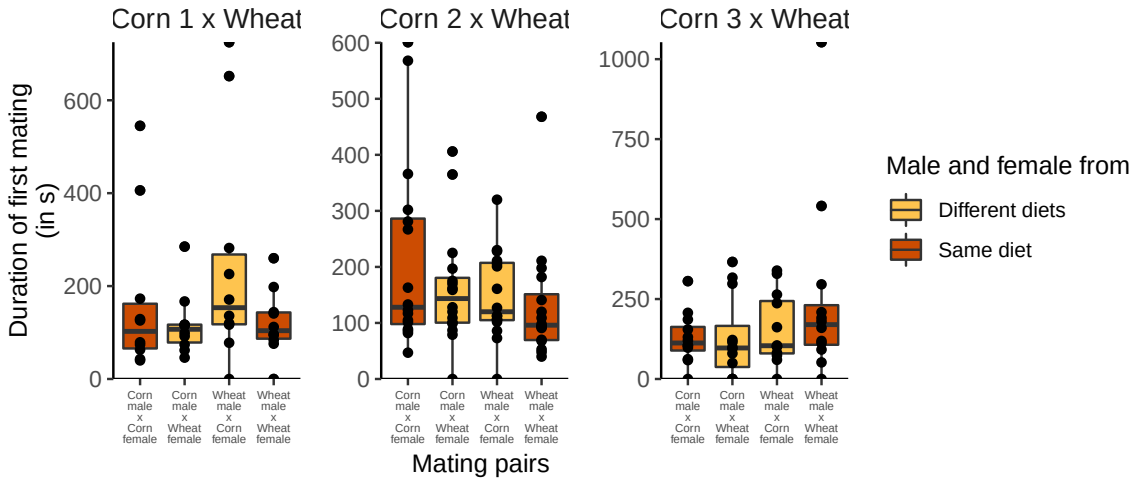


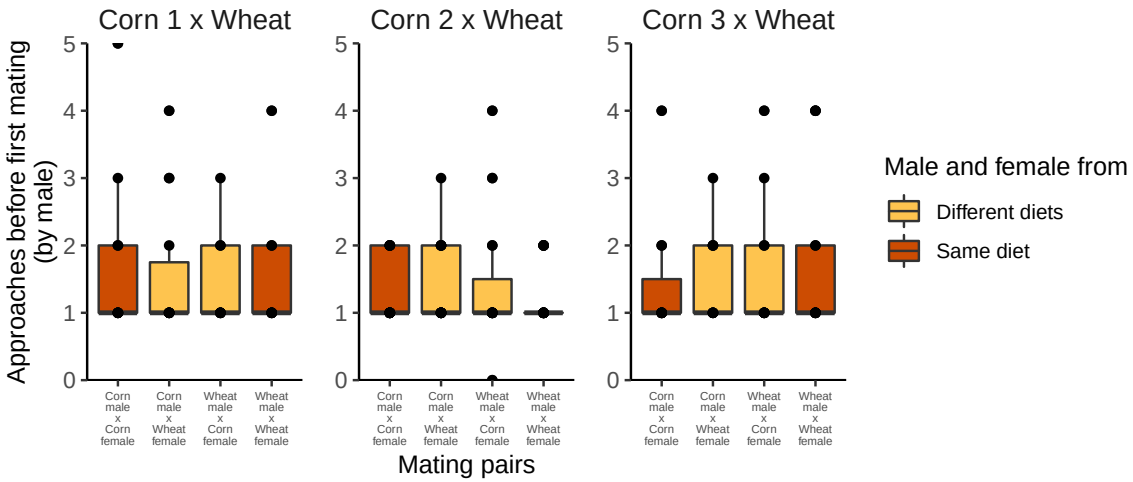
Figure 2.3: Duration of the first successful mating for each mating pair in three populations of single generation corn reared individuals.

The number of interactions prior to first mating is shown in Fig 2.4. This includes the number of approaches by either individual of the mating pair (fig 2.4a and 2.4b) as well as the total number of interactions (fig 2.4c). Table 2.1 shows the Z and P values using a GLM fit with a poisson distribution (null model df = 37, 61 and 41 for Corn populations 1, 2 and 3).

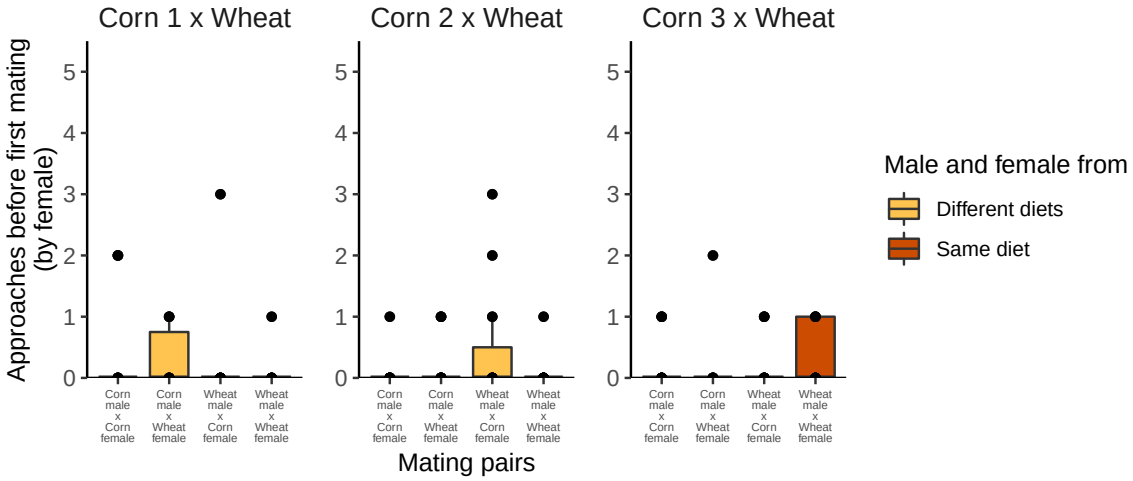
Table 2.1: Pairwise P values of the number of interactions before first mating between each mating type

	Corn population 1	Corn population 2	Corn population 3
♂approach	Z = -0.343; P = 0.732	Z = 0.209; P = 0.834	Z = 0.183; P = 0.854
♀approach	Z = -0.377, P = 0.706	Z = 0.619; P = 0.5362	Z = 0.201; P = 0.841
Total interactions	Z = -0.761, P = 0.447	Z = 0.514, P = 0.607	Z = 2.487, P = 0.013

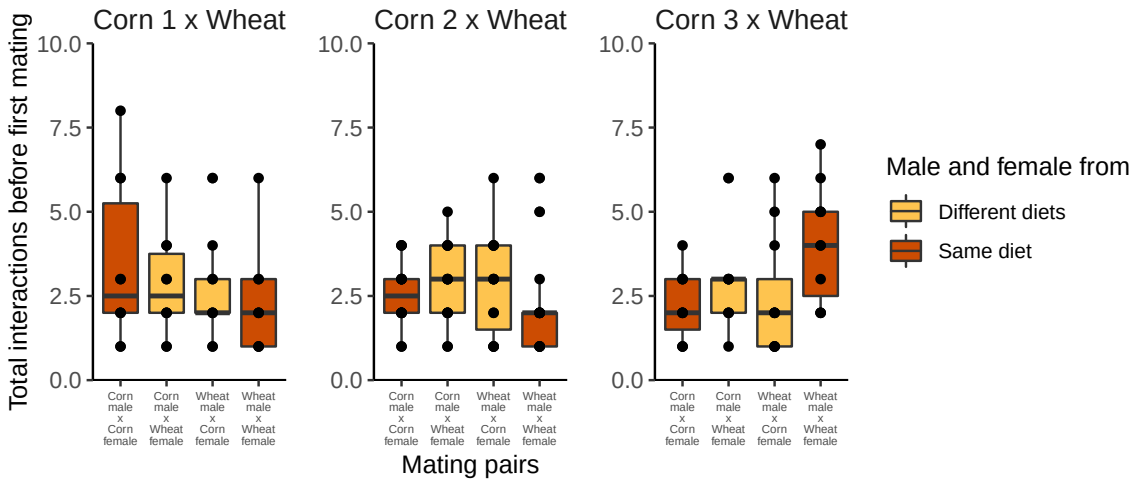
As seen in Table 2.1 none of the mating pair types show consistent significant differences in the number of interactions before the first mating (in most cases P values > 0.05).



(a) Number of approaches by males prior to the first mating event.



(b) Number of approaches by females prior to the first mating event.



(c) Total number of interactions prior to the first mating event.

Figure 2.4: Interactions before first mating in three populations of single generation corn reared individuals.

For Corn population 3, observation was only done until the first mating, however, I found significant correlations between 1. the number of interactions before the first mating and the total number of interactions, and 2. the first mount duration and the total mount duration. Since the data was not normally distributed, Spearman rank correlation was performed. Positive correlations were seen in all the cases. The correlation details are given in Table 2.2.

Table 2.2: Correlations between pre-mating and total variable values in two corn reared populations.

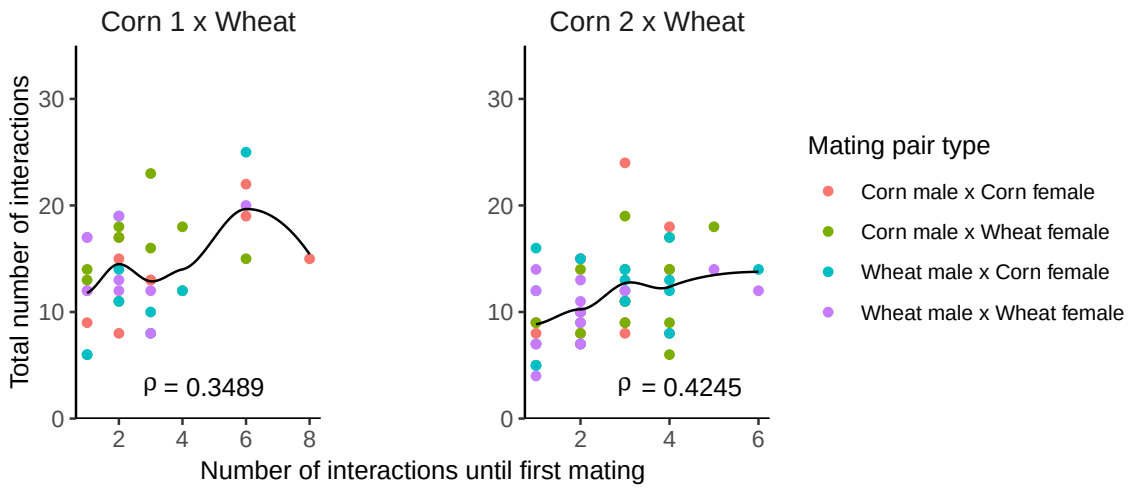
(a) Correlation between interactions before mating and total interactions.

	Corn population 1	Corn population 2
ρ	0.3489	0.4245
P	0.0318 *	0.0006 ***

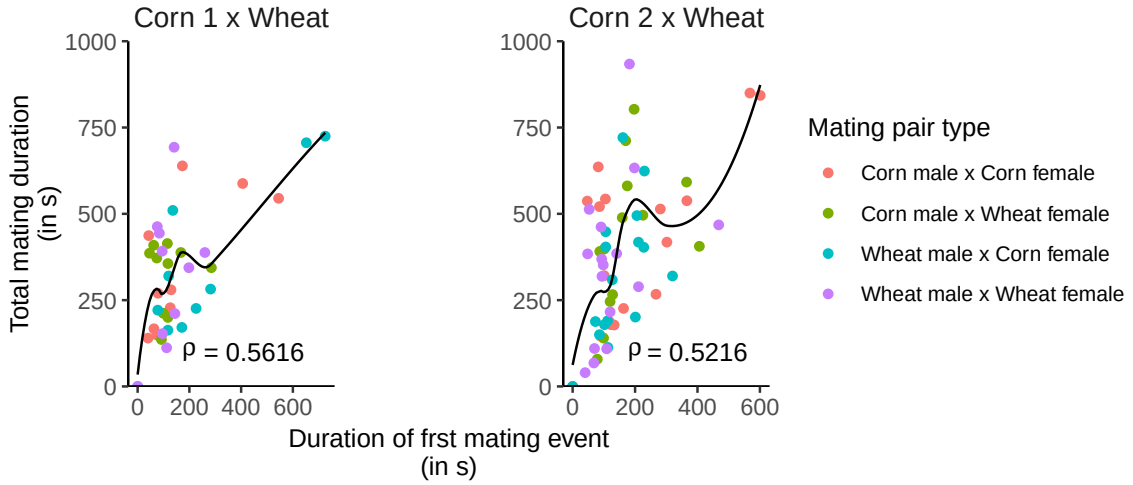
(b) Correlation between first mating duration and total mating duration.

	Corn population 1	Corn population 2
ρ	0.4563	0.4832
P	0.0031 **	0.00005 ***

Since the P values are lower than 0.05 in all the cases, and ρ is greater than 0, there is a significant positive correlation between these variables. Thus, the number of interactions until the first mating and duration of the first mating even can potentially give an estimate of the total interactions or mating duration. Figure 2.5 shows the plots of the correlated variables, with Fig 2.5a and 2.5b representing the correlation between interaction variables and mating duration variables respectively.



(a) Correlation between the total number of interactions and interactions before first mating.



(b) Correlation between the total mating duration and mounting duration of the first mating.

Figure 2.5: Correlation between variables values until first mating and total values in Corn populations 1 and 2.

Further, in the case of Corn population 1 and 2, in addition to the total mount duration (fig 2.6) and the total number of interactions (fig 2.7, with Fig 2.7a, 2.7b and 2.7c displaying the number of approaches by males, females and the total interactions respectively), I also measured avoidance behaviour (fig 2.8, with 2.8a and 2.8b displaying the instances of avoidance behaviour shown by males and females respectively), the number of mating sessions (fig 2.9) and the average mating duration (fig 2.10). There was no significant difference between the different mating pair types in regard to any of these behavioural variables, so no post-hoc test was done.

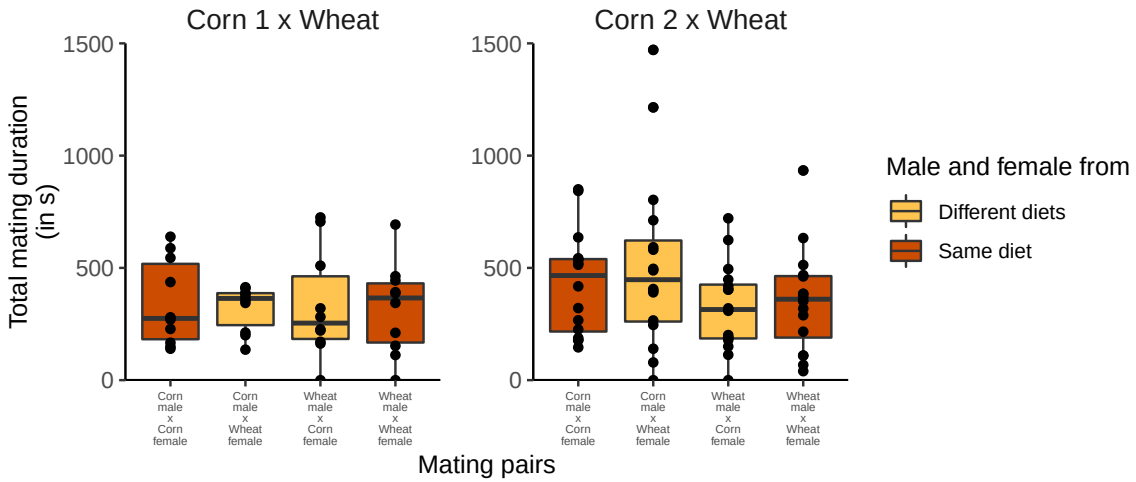
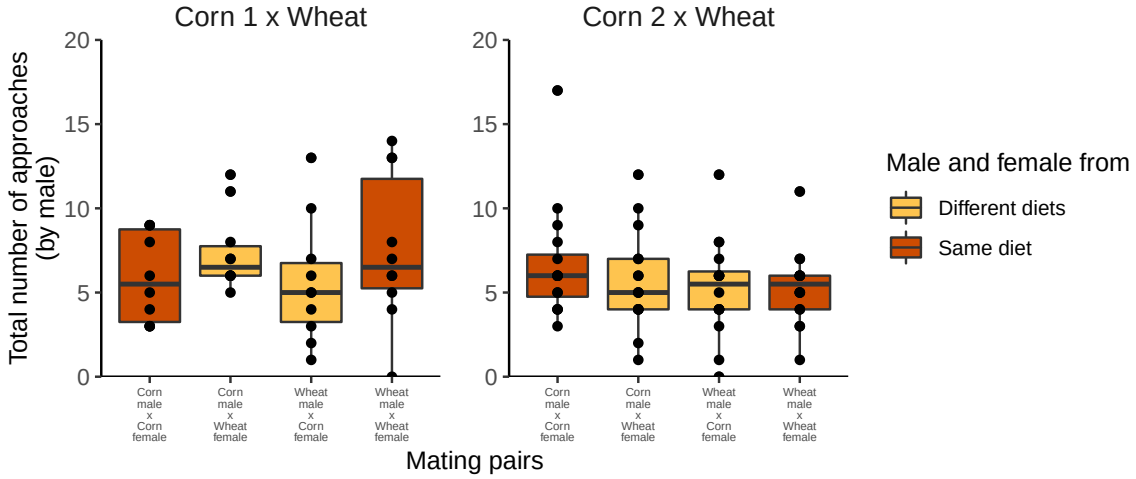
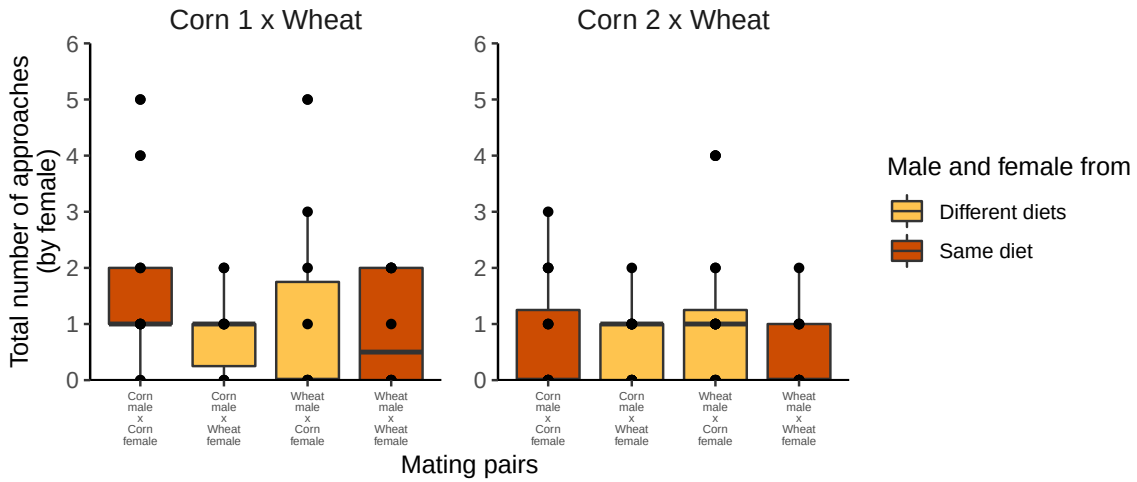


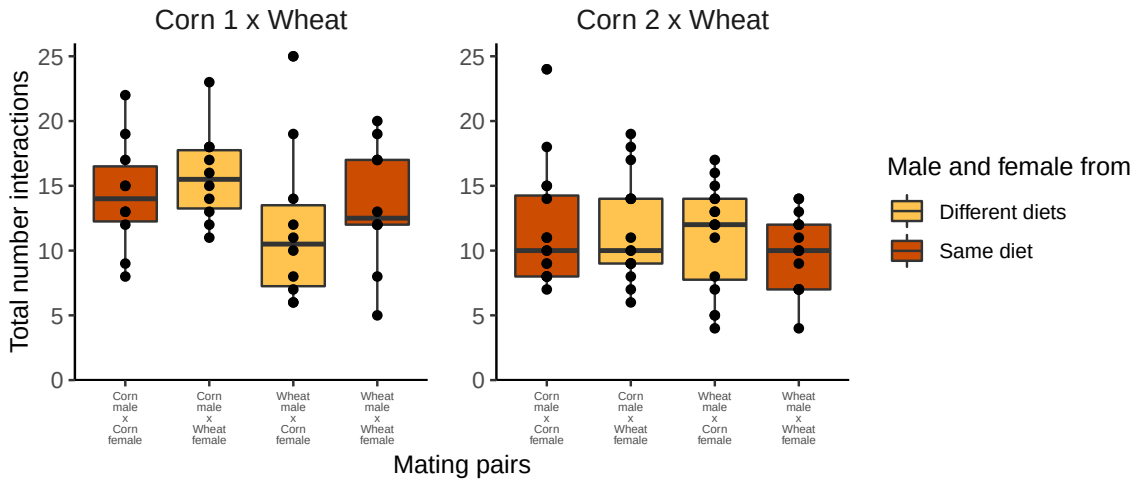
Figure 2.6: Total mating duration in Corn populations 1 and 2 (ANOVA (df = 3 for both cases): Corn population 1 $F = 0.035$, $P = 0.991$; Corn population 2 $F = 1.645$, $P = 0.188$).



(a) Total number of approaches by males in Corn populations 1 and 2 (GLM: Poisson fit: Corn population 1 $Z = 1.298$, $P = 0.194$; Corn population 2 $Z = -1.068$, $P = 0.286$).

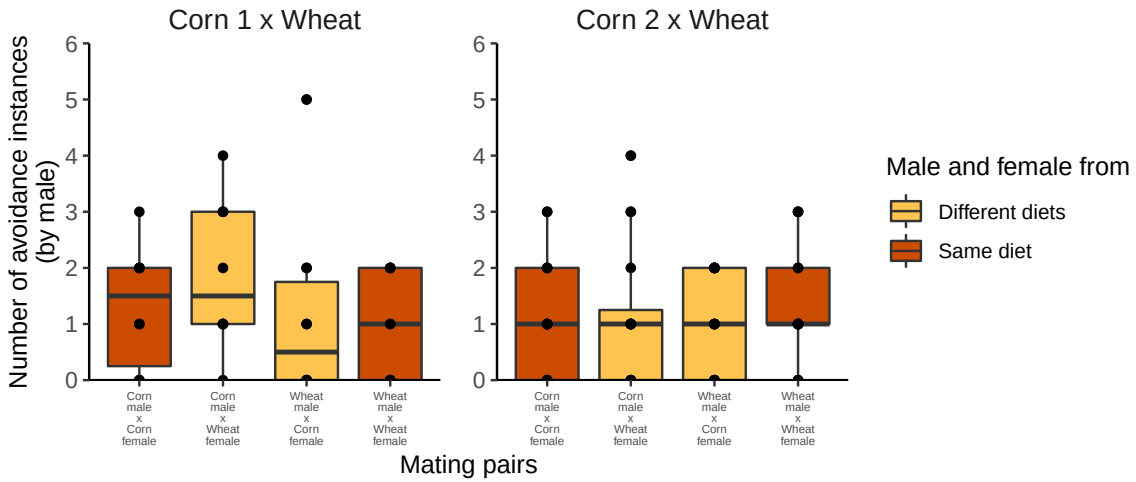


(b) Total number of approaches by females in Corn populations 1 and 2 (GLM: Poisson fit: Corn population 1 Z = -1.543, P = 0.1229; Corn population 2 Z = -0.426, P = 0.670).

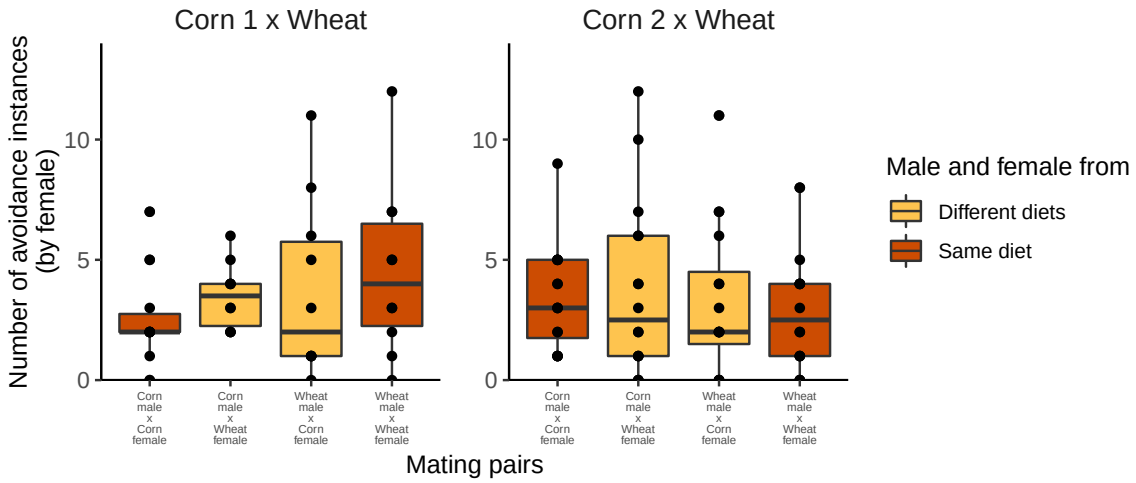


(c) Total number of interactions in Corn populations 1 and 2 (GLM: Poisson fit: Corn population 1 Z = 0.808, P = 0.419; Corn population 2 Z = -0.052 ,P = 0.161).

Figure 2.7: Total number of interactions in Corn populations 1 and 2 (df for GLM: Poisson fit = 39 and 63 for null model).



(a) Number of instances of avoidance exhibited by males in Corn populations 1 and 2 (GLM: Poisson fit: Corn population 1 $Z = 1.054$, $P = 0.344$; Corn population 2 $Z = 0.164$, $P = 0.617$).



(b) Number of instances of avoidance exhibited by females in Corn populations 1 and 2 (GLM: Poisson fit: Corn population 1 $Z = 1.148$, $P = 0.026$; Corn population 2 $Z = 0.470$, $P = 0.638$).

Figure 2.8: Number of instances of avoidance behaviours in Corn populations 1 and 2 (df for GLM: Poisson fit = 39 and 63 for null model).

Figure 2.9: Number of mating sessions in 30 min observation period in Corn populations 1 and 2 (GLM: Poisson fit: Corn population 1 Z = 0.629, df = 39 (null), P = 0.529; Corn population 2 Z = -0.404, df = 63 (null), P = 0.686).

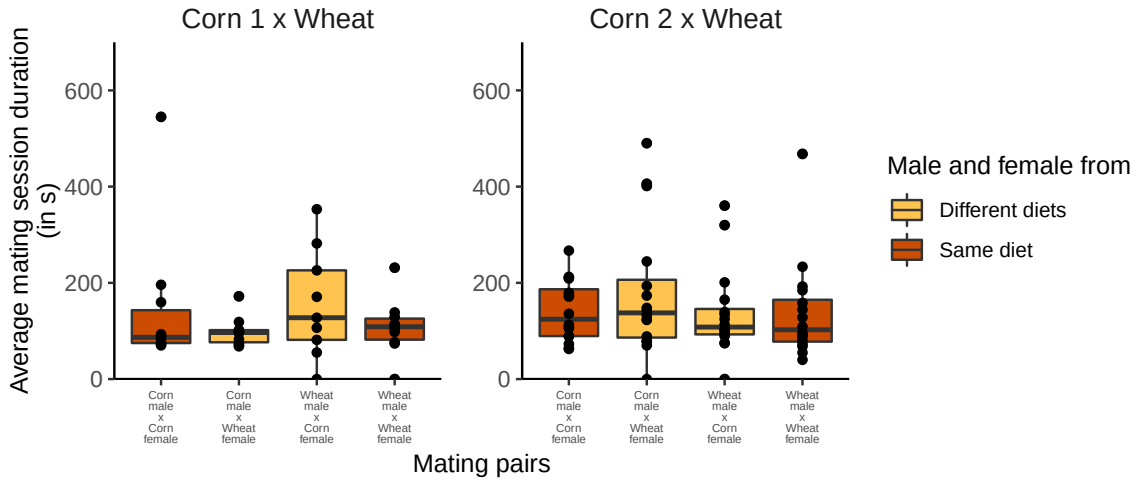


Figure 2.10: Average mating session duration in Corn populations 1 and 2 (ANOVA (df = 3 in both cases): Corn population 1 F = 1.551, P = 0.218; Corn population 2 F = 0.684, P = 0.565).

2.1.2 Single generation sorghum developed populations: mating assays

Similar to the mating assays using single-generation corn reared individuals, sorghum reared individuals were subject to the same treatments. The sample size was 20 pairs for each mating pair type, in each of the three biological replicates (Sorghum populations 1, 2 and 3). The rate of mating failure is shown in Fig 2.11. Generalised linear models with a binomial fit showed no significant difference between the mating types in any of

the replicates (Sorghum population 1: Z = 0.003, P = 0.998; Sorghum population 2: Z = -0.003, P = 0.998; Sorghum population 3: Z = -0.012, P = 0.990; df = 79 in all cases). Since there was no significant difference in any of the replicates, no post-hoc tests were carried out. There was no significant effect of the number of interactions or the body size of males and females in mating success (Binomial tests Number of interactions: P = 0.156, 0.848 and 0.996; Body size: P = 0.236, 0.278 and 0.929; in Sorghum populations 1, 2 and 3 respectively).

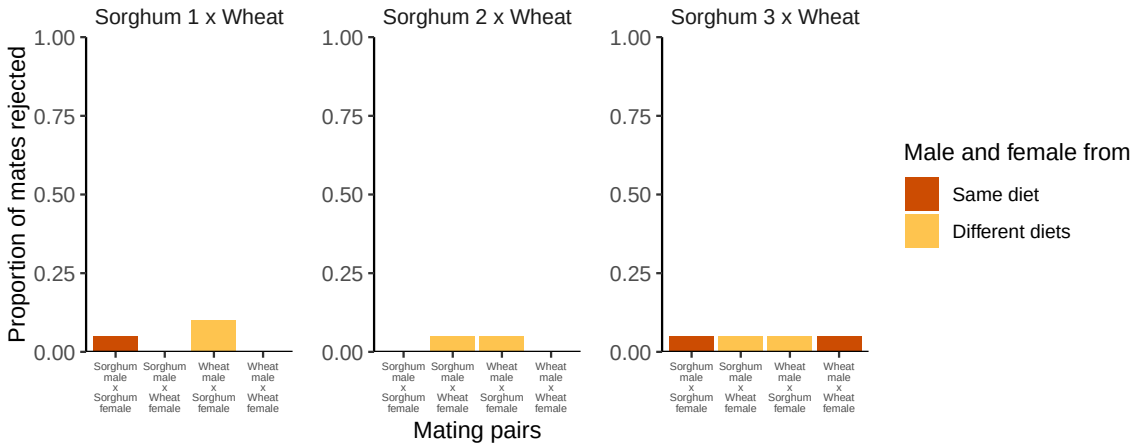


Figure 2.11: Rate of mating failure in three populations of beetles reared for a single generation in sorghum.

The detailed behaviour analysis was carried out, the results of which are presented here. The time until the first mating event (fig 2.12), showed a significant difference between the mating pair types in the Sorghum population 1 (ANOVA F = 3.809, df = 3, P = 0.0135). Post-hoc TukeyHSD revealed that the difference was between the Sorghum ♂x Sorghum ♀and the Wheat ♂x Wheat ♀mating pair. Since this difference is not consistent across the other replicates (Sorghum population 2: F = 1.351, P = 0.265; Sorghum population 3: F = 0.799, P = 0.498; df = 3 for both), and further, occurs between the two mating types from the same resource, this difference is unlikely to have any meaningful impact on our results.

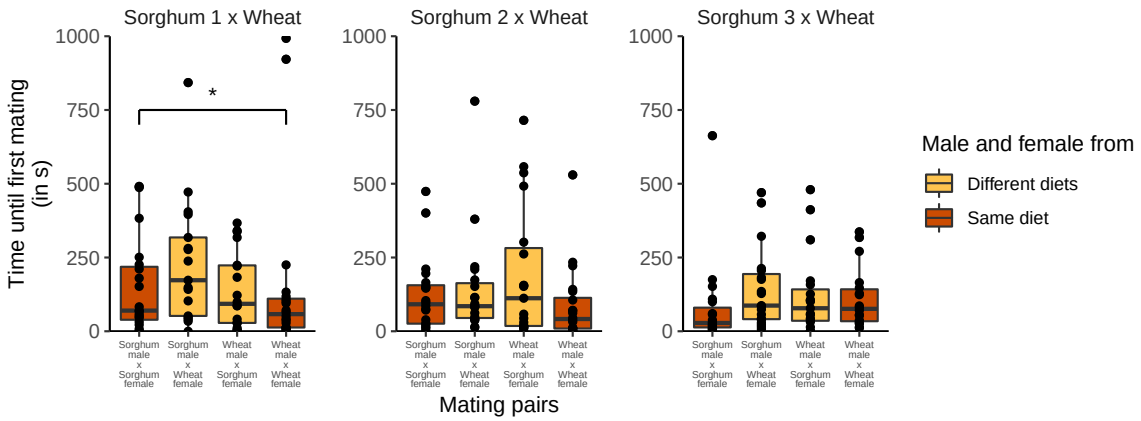


Figure 2.12: Time until the first mating in three populations of beetles reared for a single generation in sorghum.

The duration of the first mating session (fig 2.13) was analysed using ANOVA, which did not reveal any significant differences between the mating types (Sorghum population 1: F = 2.225, $P = 0.092$; Sorghum population 2: $F = 0.92$, $P = 0.435$; Sorghum population 3: F = 2.029 $P = 0.117$, df = 3 in all cases).

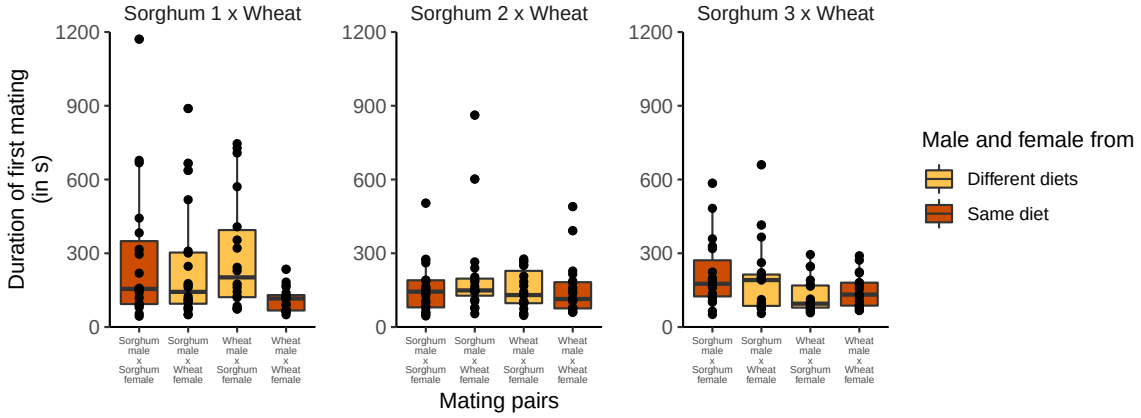


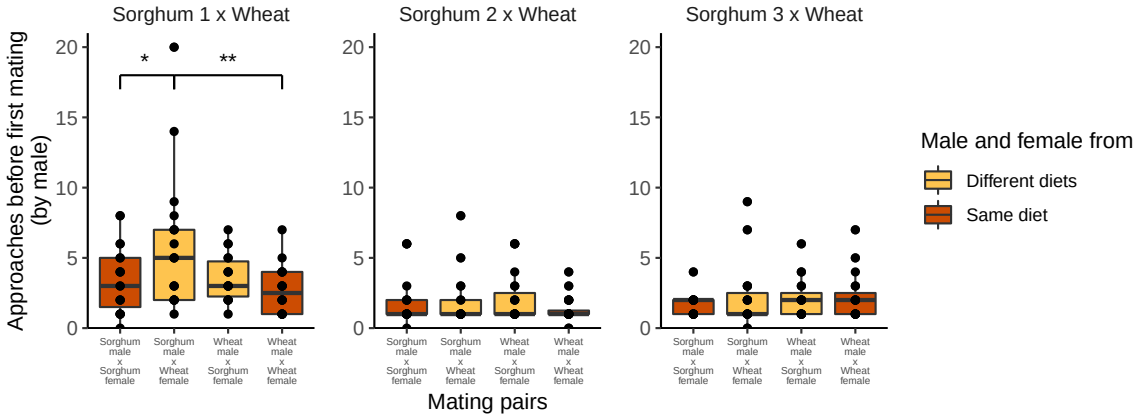
Figure 2.13: Duration of the first mating session in three populations of beetles reared for a single generation in sorghum.

The number of interactions before the first mating is shown in Fig 2.14, with Fig 2.14a, 2.14c, 2.14d corresponding to the number of approaches made by males, and females, and the total number of interactions. There are significant differences between different mating pair types in the number of approaches before mating males make in one replicate (GLM: Poisson fit: Sorghum population 1 Z = 3.140, df = 76 (null), $P = 0.0017$; Sorghum population 2: Z = 3.311, df = 77 (null), $P = 0.738$; Sorghum population 3: Z = 1.493, df = 79 (null), P = 0.135). Post-hoc pairwise comparison revealed differences between Sorghum ♂x Wheat

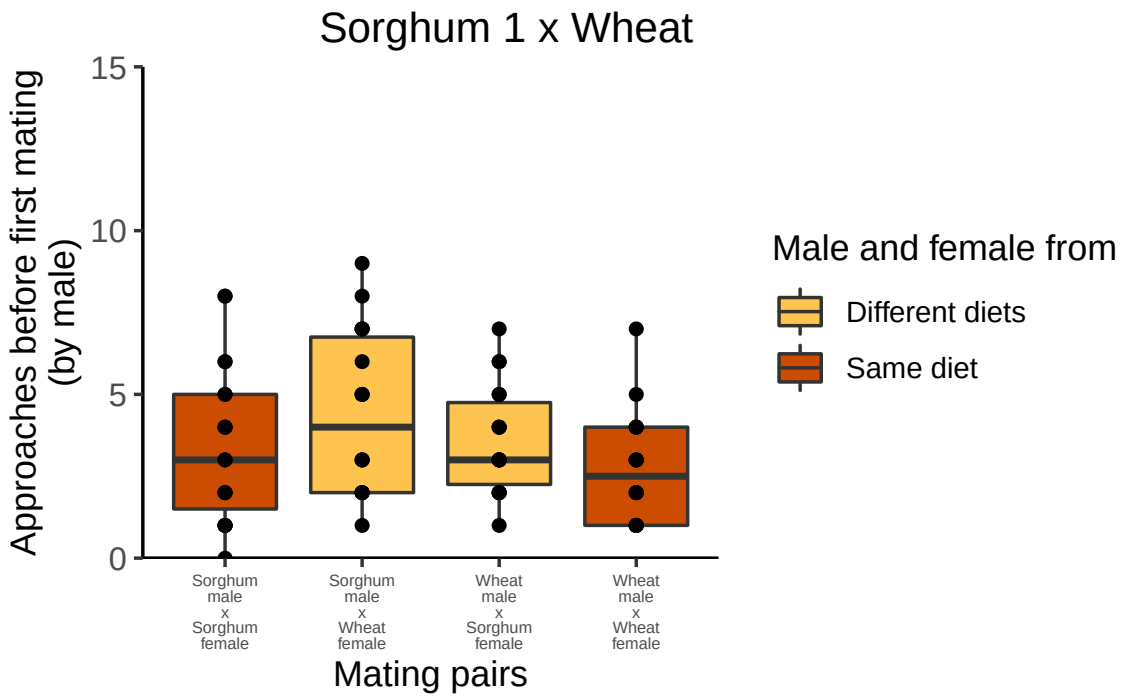
♀pairs and other types of mating pairs (Sorghum ♂x Sorghum ♀: $P = 0.0092$; Wheat ♂x Sorghum ♀: $P = 0.021$; Wheat ♂x Wheat ♀: $P = 0.0001$). It is likely this difference is due to the presence of an outlier. When Poisson test was run again, after removing the outlier (fig 2.14b), there was no significant difference (Z = 1.402, df = 74 (null), $P = 0.105$).

Similarly, in case of the total number of interactions before first mating (fig 2.14d), there are significant differences in replicate populations 1 and 3, due to the presence of outliers (Sorghum population 1: Z = 3.461, df = 76 (null), $P = 0.0005$; Sorghum population 3: Z = 2.516, df = 79 (null), $P = 0.012$). After removing outliers (figure 2.14e), there were no significant pairwise differences found in Sorghum population 1 (Z = -1.856, df = 71 (null), $P = 0.068$); for Sorghum population 3: Z = 2.428, df = 74 (null), $P = 0.0177$). Poisson fit on population 2 did not indicate any significant differences at the 95% confidence level (Z = 0.642, df = 77 (null), $P = 0.521$).

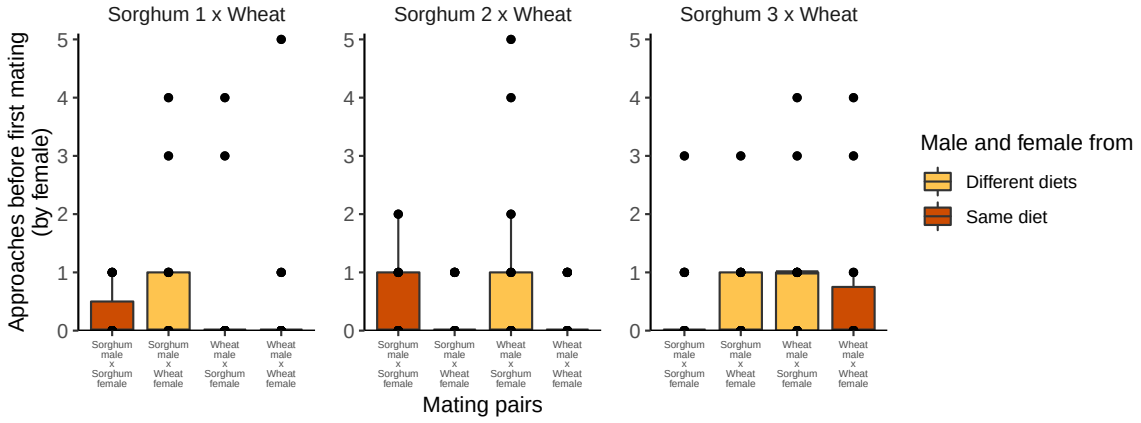
There is no significant pairwise difference in the number of approaches made by females in any of the replicates (Sorghum population 1: Z = 1.548, df = 77 (null), $P = 0.122$; Sorghum population 2: Z = -1.264, df = 77 (null), $P = 0.2062$; Sorghum population 3: Z = 1.493, df = 79 (null), $P = 0.135$)



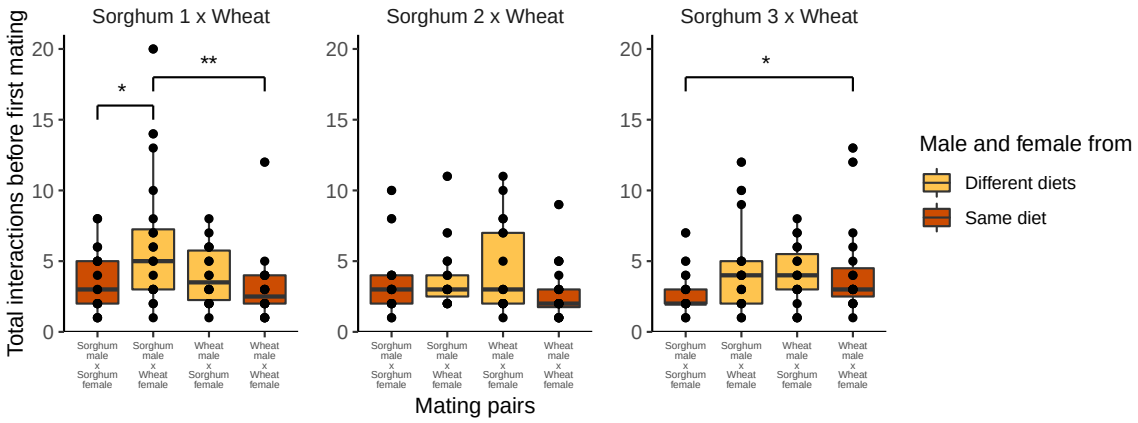
(a) Number of approaches by males prior to the first mating event.



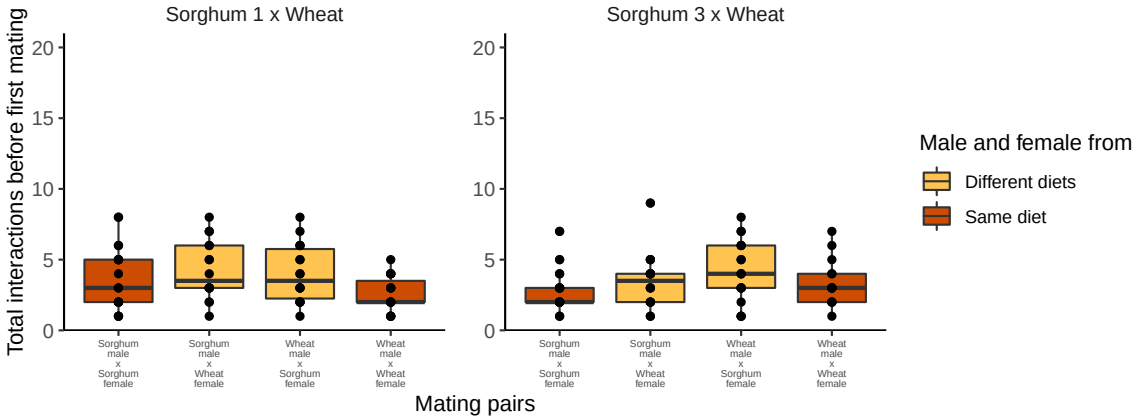
(b) Total number of interactions prior to the first mating event in Sorghum population 1 after removing outliers.



(c) Number of approaches by females prior to the first mating event.



(d) Total number of interactions prior to the first mating event.



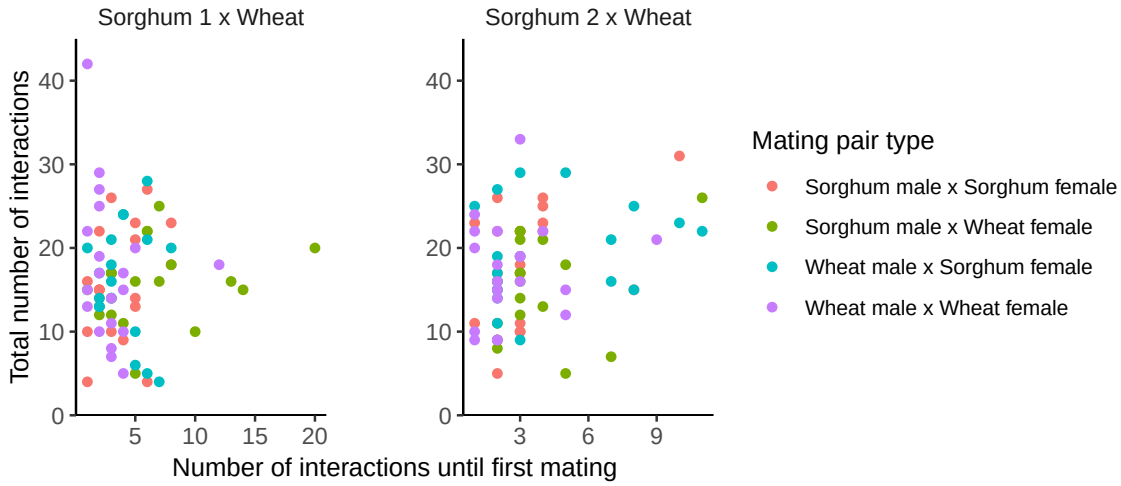
(e) Total number of interactions prior to the first mating event after removing outliers in Sorghum populations 1 and 2.

Figure 2.14: Interactions before first mating in three populations of single generation sorghum reared individuals.

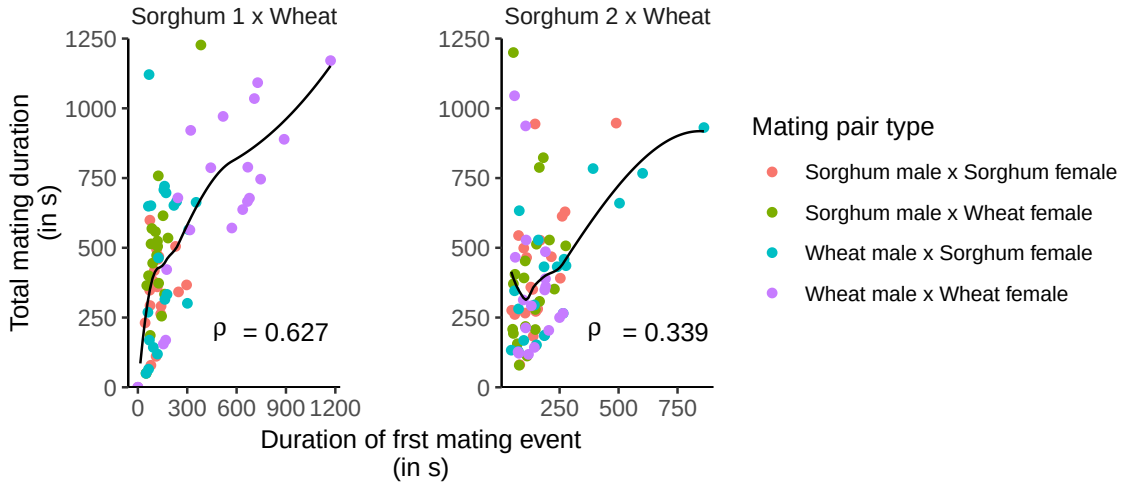
In the case of sorghum as well, the population 3 was observed only until first mating. I looked for correlations between the variables measured until first mating and the total values in the mating assays conducted using Sorghum populations 1 and 2 (figure 2.15). In the case of Sorghum population 1, I did not find a correlation between the interaction variables (P value from Spearman rank correlation = 0.54). Sorghum population 2 showed a significant correlation between the interaction variables, with P values from spearman rank correlation = 0.04 (fig 2.15a). However, there was a significant positive correlation between the first mating duration and the total mating duration (fig 2.15b) in both populations (Spearman rank correlation on mating duration variables given in table 2.3). So, duration of the first mating event can potentially give us information about the total mating duration.

Table 2.3: Correlation between first mating duration and total mating duration in Sorghum populations 1 and 2 mating assays.

	Sorghum population 1	Sorghum population 2
ρ	0.627	0.339
P	4.97e-10 ***	0.0024 **



(a) Correlation between the total number of interactions and the number of interactions prior to the first mating event.



(b) Correlation between the total mating duration and the duration of the first mating event.

Figure 2.15: Correlation between variables values until first mating and total values in sorghum populations 1 and 2.

The total mating duration and the total number of interactions are shown in figures 2.16 and 2.17 respectively. The number of instances of avoidance behaviour are shown in figure 2.18,

with figures 2.18a and 2.18b displaying male avoidance and female avoidance behaviours respectively. The number of mating sessions and the average mating session duration are also shown in figures 2.19 and 2.20 respectively for the mating assays using Sorghum populations 1 and 2.

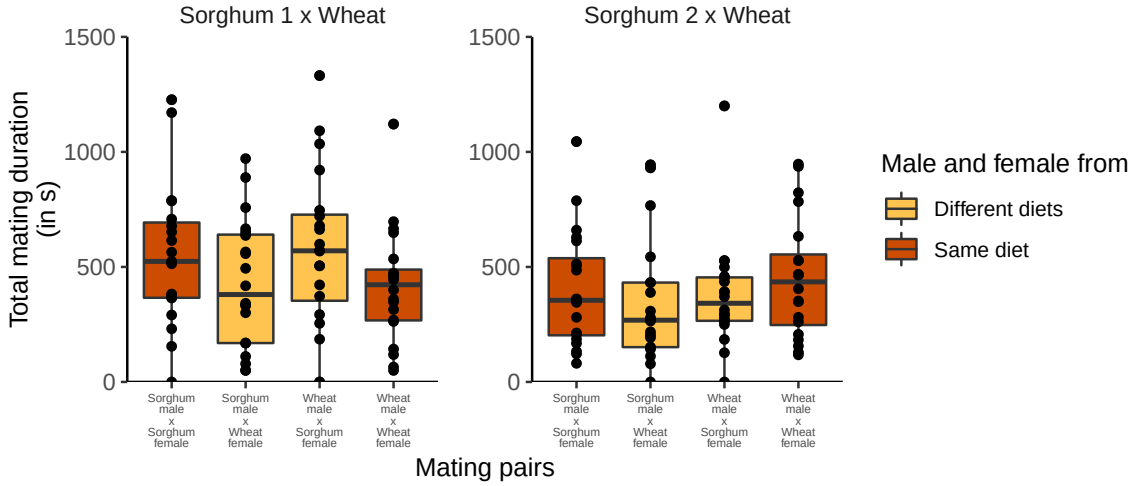
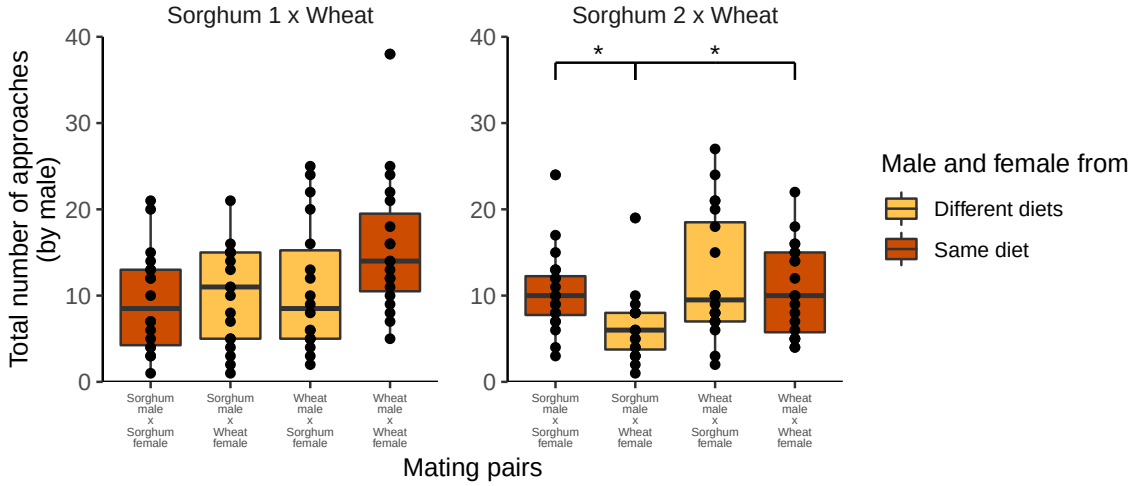
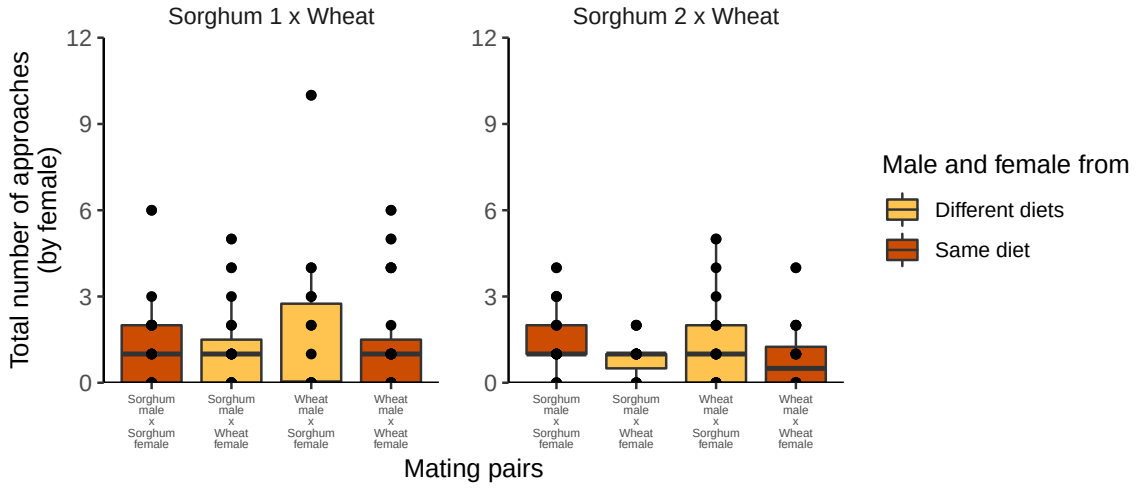


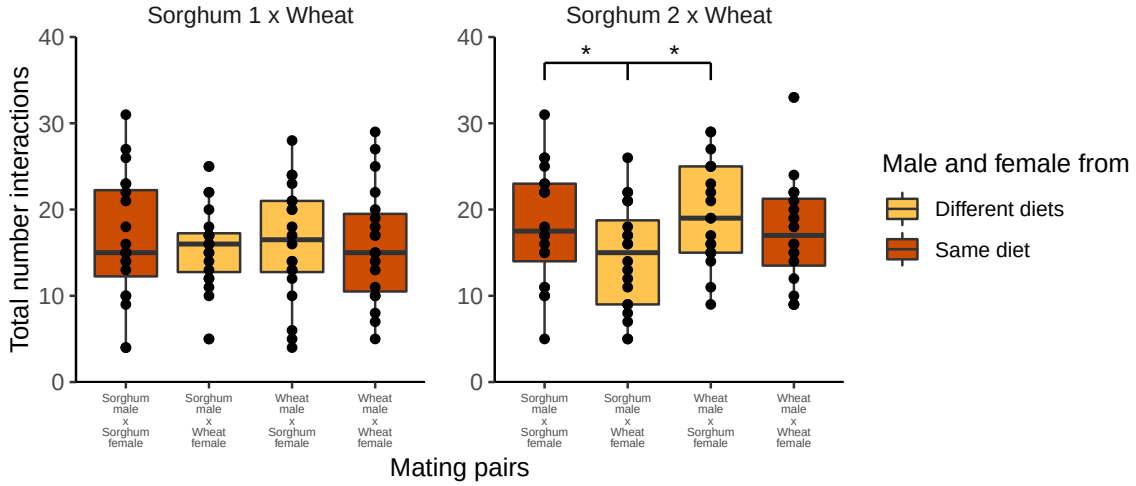
Figure 2.16: Total mating duration in Sorghum populations 1 and 2 (ANOVA (df = 3 for both): Sorghum population 1 $F = 1.815$, $P = 0.151$; Sorghum population 2 $F = 0.664$, $P = 0.577$).



(a) Total number of approaches by males in Sorghum population 1 (GLM: Poisson fit: Sorghum population 1 $Z = 2.549$, df = 70 (null), $P = 0.0528$ after removing one outlier), and Sorghum population 2 (significant difference, pairwise Z and P values in table 2.4).



(b) Total number of approaches by females in Sorghum populations 1 and 2 (GLM: Poisson fit: Sorghum population 1 Z = -0.622, df = 73 (null), $P = 0.534$; Sorghum population 2 Z = 0.132, df = 79 (null), $P = 0.895$).



(c) Total number of interactions in Sorghum population 1 (Poisson test: Sorghum population 1 Z = -670, df = 79 (null), $P = 0.503$) and Sorghum population 2 (significant difference, Z and P values in table 2.5).

Figure 2.17: Total number of interactions in Sorghum populations 1 and 2.

Table 2.4: Number of approaches by males in Sorghum population 2 mating assay: GLM (Poisson) Z and P values.

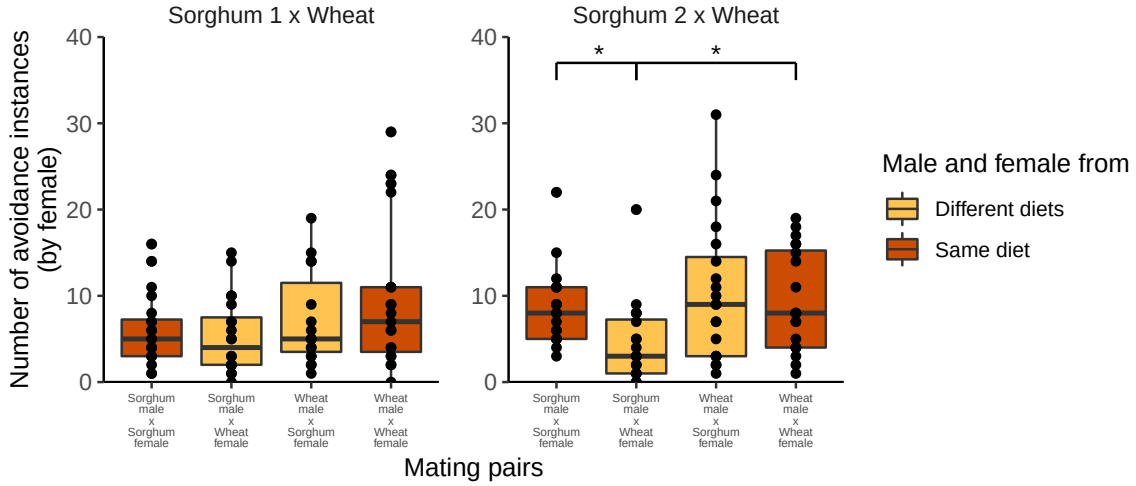
Mating pair types		Z, P
Sorghum ♂x Sorghum ♀	Sorghum ♂x Wheat ♀	4.44, 0.0001 ***
Sorghum ♂x Sorghum ♀	Wheat ♂x Sorghum ♀	-1.56, 0.4041
Sorghum ♂x Sorghum ♀	Wheat ♂x Wheat ♀	-0.34, 0.9864
Sorghum ♂x Wheat ♀	Wheat ♂x Sorghum ♀	-5.90, <0.0001 ***
Sorghum ♂x Wheat ♀	Wheat ♂x Wheat ♀	-4.76, <0.0001 ***
Wheat ♂x Sorghum ♀	Wheat ♂x Wheat ♀	1.22 0.6161

Table 2.5: Total number of interactions in Sorghum population 2 mating assay: GLM (Poisson) Z and P values.

Mating pair types		Z, P
Sorghum ♂x Sorghum ♀	Sorghum ♂x Wheat ♀	2.96, 0.0160 *
Sorghum ♂x Sorghum ♀	Wheat ♂x Sorghum ♀	-0.98, 0.7602
Sorghum ♂x Sorghum ♀	Wheat ♂x Wheat ♀	0.75, 0.8765
Sorghum ♂x Wheat ♀	Wheat ♂x Sorghum ♀	-3.93, 0.0005 **
Sorghum ♂x Wheat ♀	Wheat ♂x Wheat ♀	-2.22, 0.1175
Wheat ♂x Sorghum ♀	Wheat ♂x Wheat ♀	1.73 0.3080

The males from Sorghum population 2 show lower number of approaches (resulting in lower total interactions for this mating pair type) when mating with females from wheat populations (figures 2.17a and 2.17, and tables 2.4 and 2.5). However, this has no effect on the mating success of the pairs (GLM (Binomial) with df = 79 for null model: Number of approaches by males Z = -0.89, P = 0.373; Total number of interactions Z = -0.192, P = 0.848).

(a) Number of instances of avoidance exhibited by males in Sorghum populations 1 and 2 (GLM: Poisson: Sorghum population 1 Z = -0.46, df = 78 (null), $P = 0.647$; Sorghum population 2 Z = -1.37, df = 79 (null), $P = 0.170$).



(b) Number of instances of avoidance exhibited by females in Sorghum populations 1 and 2. No significant difference between mating types in Sorghum population 1 after removing an outlier (GLM: Poisson Z = -0.931, df = 73 (null), $P = 0.251$). Pairwise Z and P values for Sorghum population 2 in table 2.6.

Figure 2.18: Number of instances of avoidance behaviours in Sorghum populations 1 and 2.

Table 2.6: Female avoidance instances in Sorghum population 2 mating assay: GLM (Poisson) Z and P values.

Mating pair types		Z, P
Sorghum ♂x Sorghum ♀	Sorghum ♂x Wheat ♀	4.93, <0.0001 ***
Sorghum ♂x Sorghum ♀	Wheat ♂x Sorghum ♀	-1.90, 0.229
Sorghum ♂x Sorghum ♀	Wheat ♂x Wheat ♀	-0.84, 0.8327
Sorghum ♂x Wheat ♀	Wheat ♂x Sorghum ♀	-6.64, <0.0001 ***
Sorghum ♂x Wheat ♀	Wheat ♂x Wheat ♀	-5.70, <0.0001 ***
Wheat ♂x Sorghum ♀	Wheat ♂x Wheat ♀	1.06, 0.7162

The number of instances of avoidance behaviour shown by females from the wheat population when mating with a male from a sorghum population is significantly lower than avoidance shown by other females in Sorghum population 2. However, since there was no significant difference in the mating success or failure (fig 2.11) between the mating pairs, the avoidance behaviours exhibited may not be successful in preventing mating. This difference may also be due to lower number of approaches by the males in this mating pair type (figure 2.17a and table 2.4).

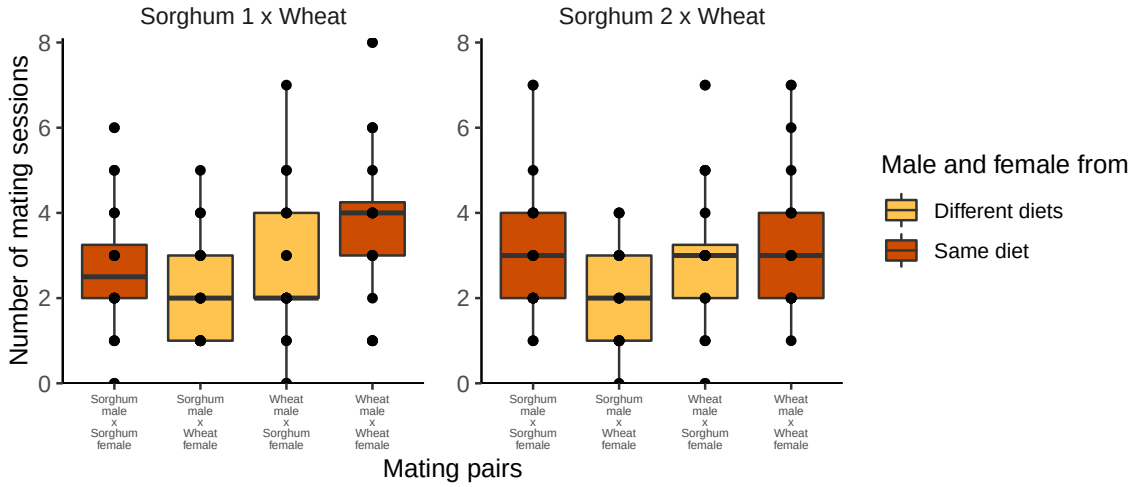


Figure 2.19: Number of mating sessions in 30 min observation period in Sorghum populations 1 and 2 (GLM: Poisson fit pairwise: Sorghum population 1 Z = -1.126, df = 79 (null), P = 0.0673; Sorghum population 2 Z = -2.455, df = 79 (null), P = 0.0671.

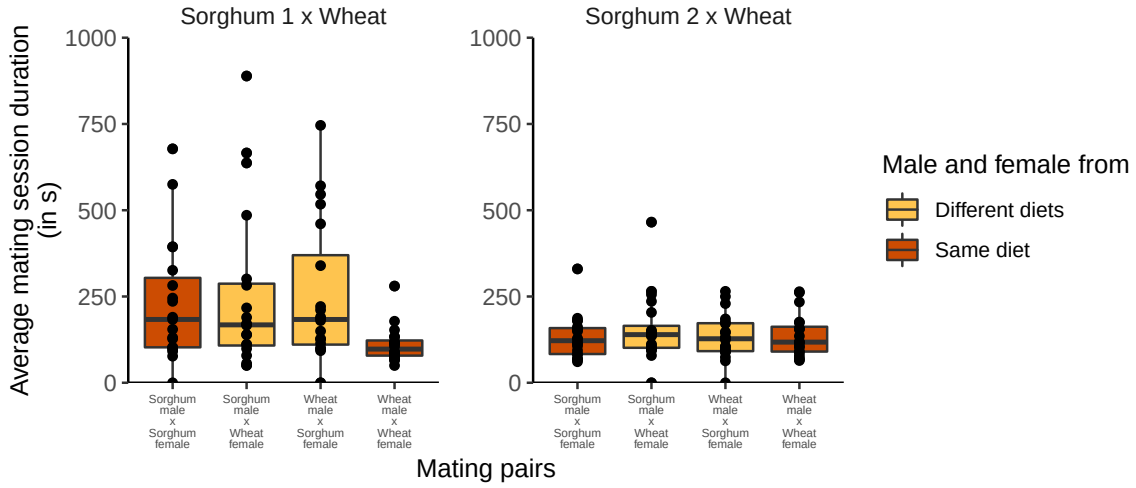


Figure 2.20: Average mating session duration in Sorghum populations 1 and 2 (ANOVA (df = 3 in both cases): Sorghum population 1 F = 2.62, P = 0.057; Sorghum population 2 F = 0.493, P = 0.688).

2.1.3 Populations adapted to finger millet: mating assays

For these assays, individuals from three populations adapted to finger millet (referred to henceforth as FM populations 1, 2 and 3) were used, with the ancestral wheat population. There were 30 pairs of each mating type in FM population 1, and 20 of each in FM populations 2 and 3. Mating was random between these individuals (Fig 2.21); GLM (binomial) between the different mating pair types did not reveal any significant differences between the different mating pair types (FM population 1: Z = -1.174, df = 119 (null), P = 0.240; FM population 2: Z = -0.007, df = 79 (null), P = 0.994; FM population 1: Z = 0.002, df = 79 (null), P = 0.998). There was a significant effect of the number of interactions on mating success in FM population 1 (Z = 2.638, P = 0.008), which is likely due to the low number of pairs that failed to mate, which happened to have fewer interactions (5 pairs failed to mate out of a total of 120). There was no significant effect of the number of interactions on mating success in FM populations 2 and 3 (Z = 0.648 and 0.001; P = 0.517 and 0.999), nor of the body size of males and females in any of the three biological replicates (Z = 0.232, 544 and -0.003; P = 0.817, 0.587 and 0.998; in FM populations 1, 2 and 3 respectively).

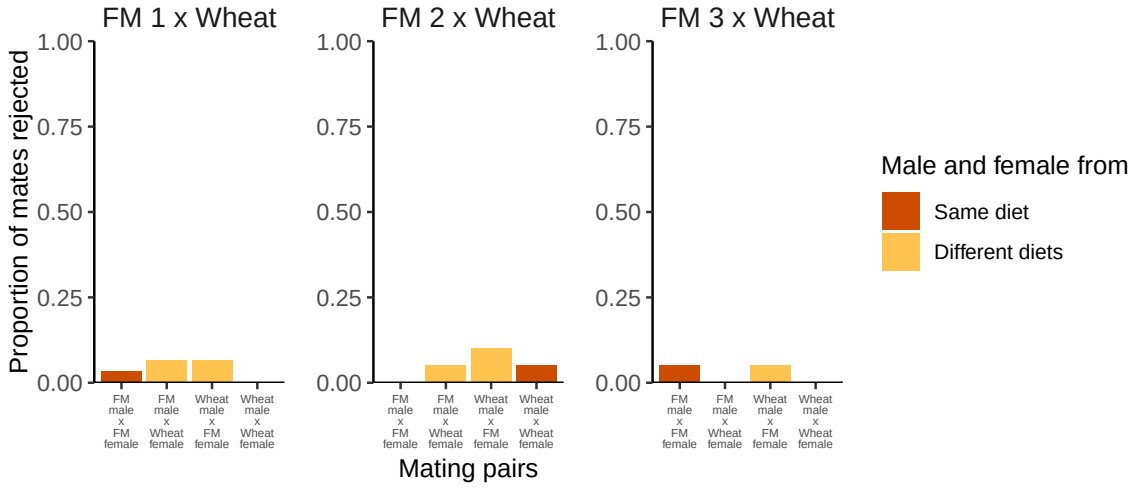


Figure 2.21: Proportion of pairs that did not successfully mate during the observation time in three populations adapted to finger millet.

The same behaviour variables as noted in the previous sections was also recorded here. The time until the first mating is shown in figure . ANOVA performed on this data in FM population 1 returned $F = 2.702$, df = 3, P value of 0.049, but a post-hoc TukeyHSD test did not reveal any significant differences (Table 2.7). In FM populations 2 and 3, the F values were 0.191 and 0.69, and the P values were 0.902 and 0.561 respectively (df = 3).

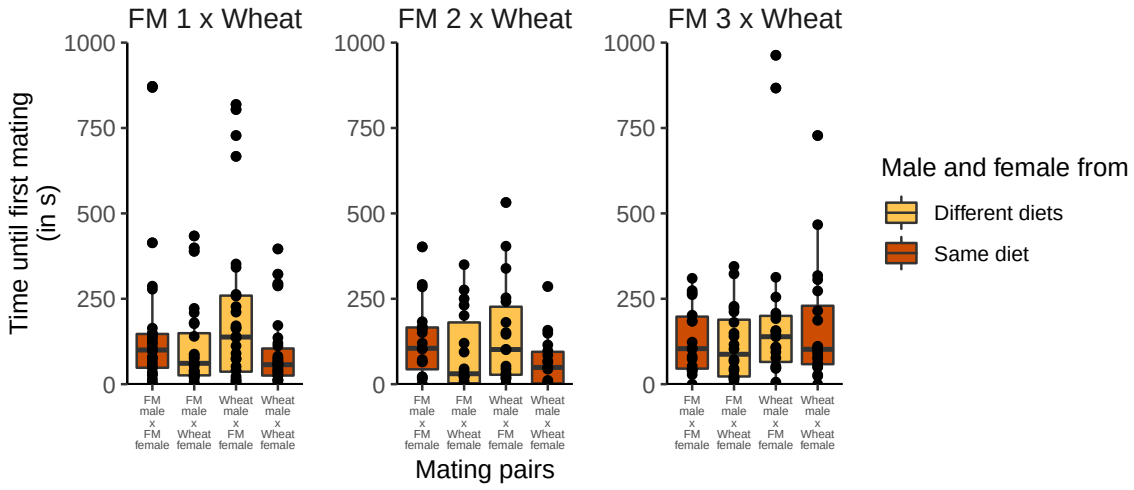


Figure 2.22: Time until the first mating event in three populations adapted to finger millet.

Table 2.7: Time until first mating in FM population 1 mating assay: ANOVA *Difference* and *P* values.

Mating pair types		*Difference*, *P*
FM ♂x FM ♀	FM ♂x Wheat ♀	-54.60, 0.673
FM ♂x FM ♀	Wheat ♂x FM ♀	52.15, 0.704
FM ♂x FM ♀	Wheat ♂x Wheat ♀	-71.57, 0.438
FM ♂x Wheat ♀	Wheat ♂x FM ♀	106.75, 0.133
FM ♂x Wheat ♀	Wheat ♂x Wheat ♀	-16.98, 0.985
Wheat ♂x FM ♀	Wheat ♂x Wheat ♀	-123.73, 0.054

The duration of the first mating event (Fig 2.23) was not significantly different at the 95% confidence level (ANOVA (df = 3): FM population 1: $F = 0.848$, $P = 0.471$; FM population 2: $F = 2.034$, $P = 0.116$; FM population 3: $F = 0.424$, $P = 0.736$).

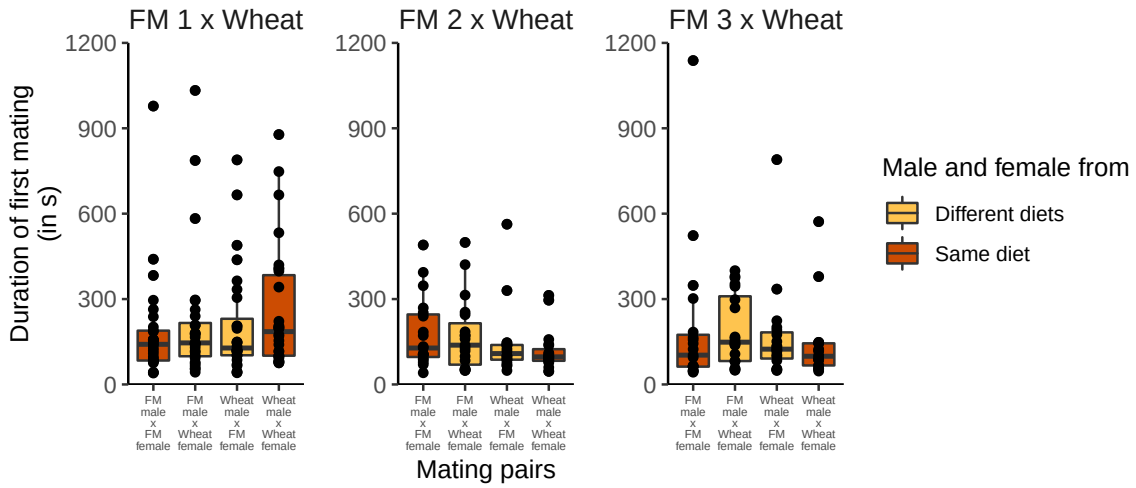
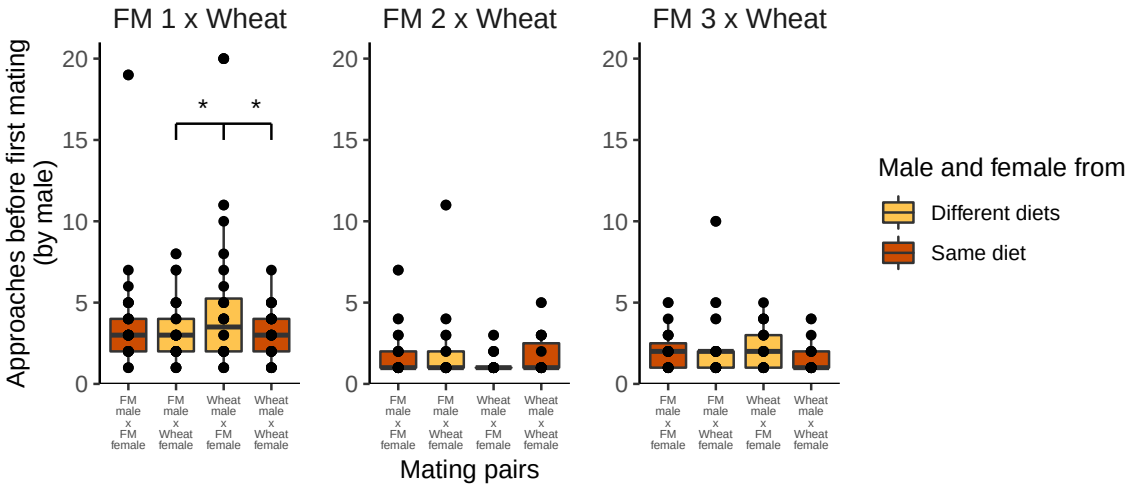


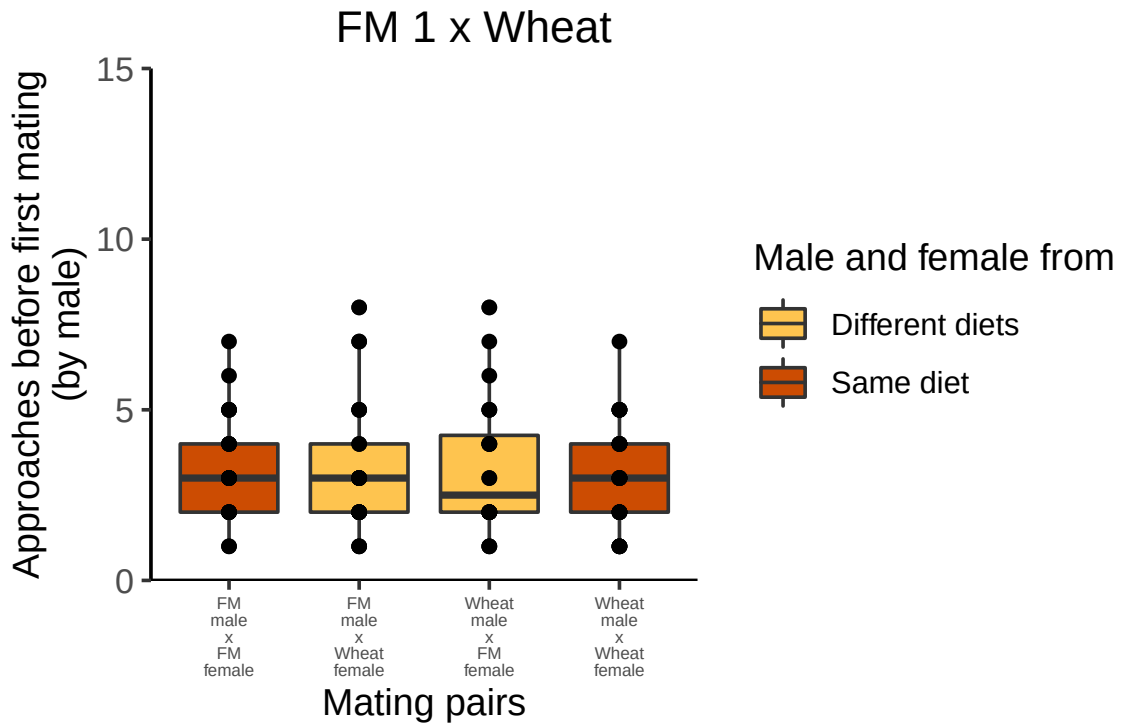
Figure 2.23: Duration of the first mating in three populations adapted to finger millet.

There was also no significant difference found between the different mating pair types in regard to the number of interactions before the first mating (Fig 2.24). The number of male approaches before mating (Fig 2.24a) was analysed using a Generalised Linear Model with a Poisson fit at the 95% confidence level. After conducting an outlier analysis using the inter-quartile range method (2.24b), the *Z* and *P* values of the distribution in FM population 1 were -0.222 and 0.825, df = 109 (null). FM populations 2 and 3 had $Z = 0.338$ and 0.315, and *P* values of 0.736 and 0.753, with df (null model) = 75 and 77 respectively. The number of approaches by females before the first mating (Fig 2.24c) did not differ significantly between the mating pair types at the 95% confidence level in any of the populations (GLM: Poisson FM population 1: Z = -1.437, df = 114 (null), $P = 0.151$; FM population 2: Z

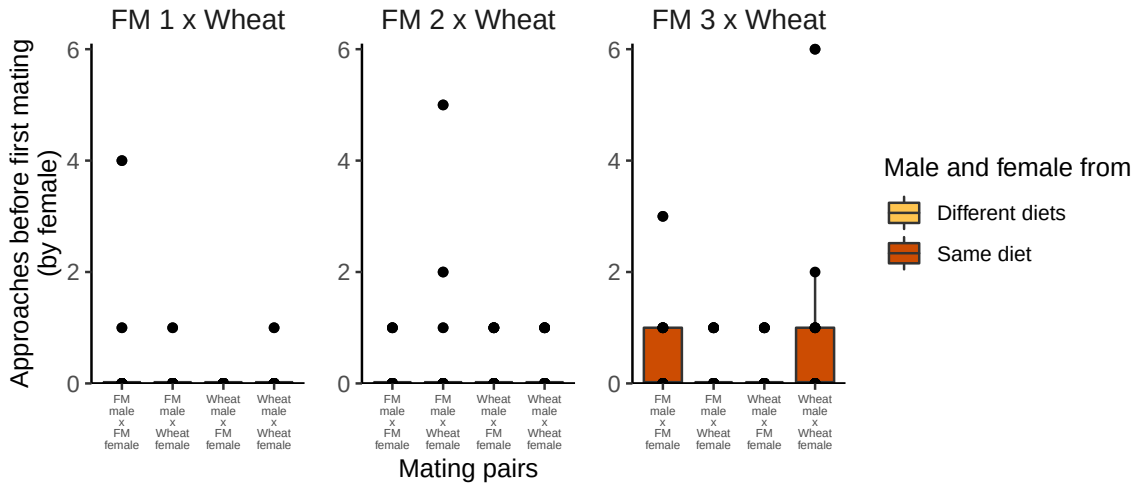
= 1.818, df = 75 (null), $P = 0.069$; FM population 3: Z = -1.525, df = 77, $P = 0.127$). The total number of interactions before mating (Fig 2.24d) did not vary either between the mating pair types (GLM: Poisson FM population 1: Z = -1.547, df = 114 (null), $P = 0.122$; FM population 2: Z = 0.375, df = 75 (null), $P = 0.708$; FM population 3: Z = -0.464, df = 77 (null), $P = 0.642$).



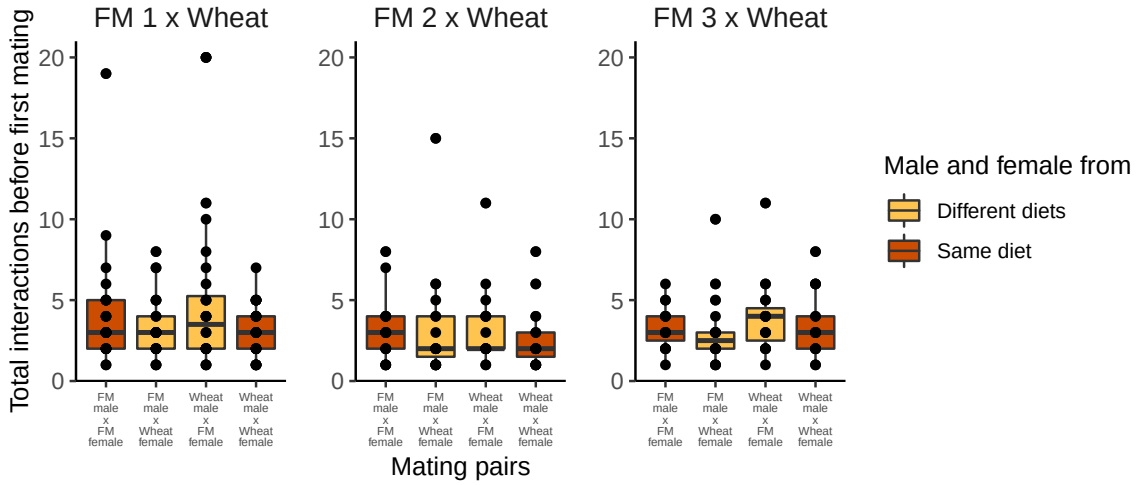
(a) Number of approaches by males prior to the first mating event.



(b) Number of approaches by males prior to the first mating event in the FM 1 x Wheat assays.



(c) Number of approaches by females prior to the first mating event.



(d) Total number of interactions prior to the first mating event.

Figure 2.24: Interactions before first mating in three populations adapted to finger millet.

Similar to the previous assays, in this case as well, for the assays conducted on the third population adapted to finger millet (FM population 3), data was recorded only until the first mating of each pair, although the individuals were allowed to remain in the experimental environment for the entire 30 minutes. Significant correlations were found between the interaction variables and the duration variables until the first mating and the total value measured. The correlations are shown in figure 2.25 (with Fig 2.25a showing the correlation between the interaction variables and Fig 2.25b the correlation between the mating duration variables); the correlation coefficient ρ calculated by Spearman rank correlation are given in table 2.8.

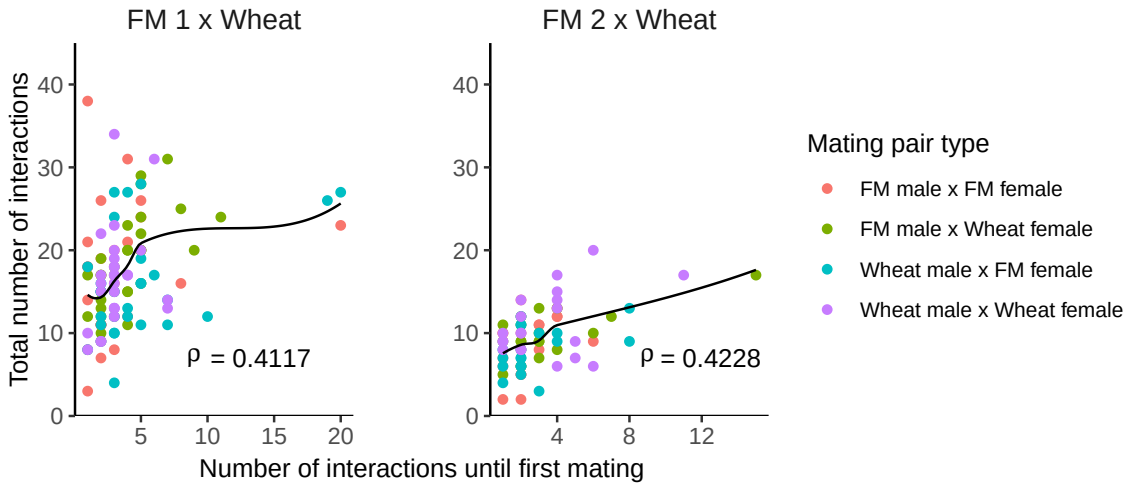
Table 2.8: Correlations between pre-mating and total variable values in two corn reared populations.

(a) Correlation between interactions before mating and total interactions

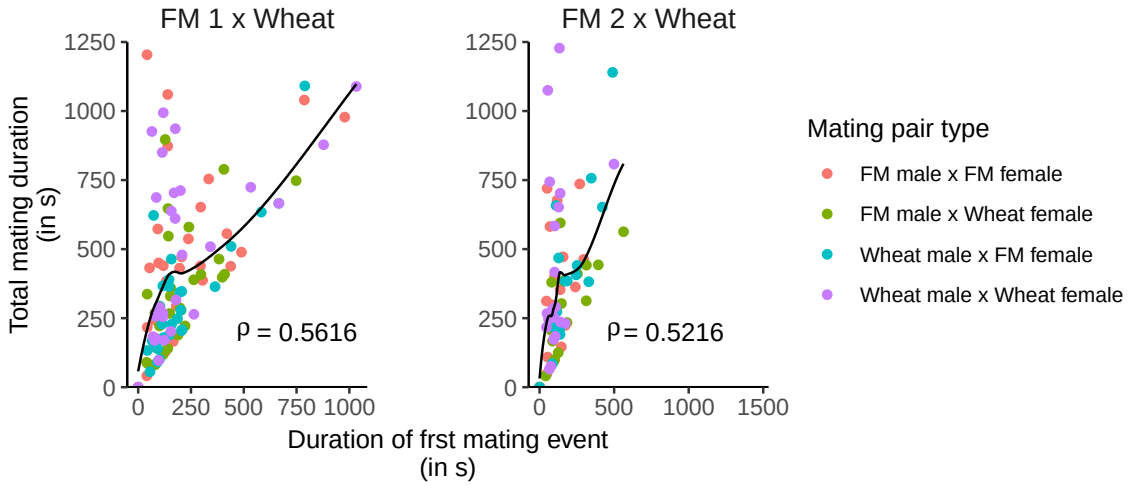
	FM population 1	FM population 2
ρ	0.4117	0.4228
P	<0.0001 ***	0.00014 ***

(b) Correlation between first mating duration and total mating duration

	Corn population 1	Corn population 2
ρ	0.5616	0.5216
P	<0.0001 ***	<0.0001 ***



(a) Correlation between the total number of interactions and the number of interactions prior to the first mating event.



(b) Correlation between the total mating duration and the duration of the first mating event.

Figure 2.25: Correlation between variables values until first mating and total values in FM populations 1 and 2.

Further, in FM populations 1 and 2, the remaining behaviour variables (total mating duration, total number of interactions, instances of avoidance behaviour, number of mating sessions and average duration of mating sessions) were recorded and analysed, as shown in figures 2.26 to 2.30.

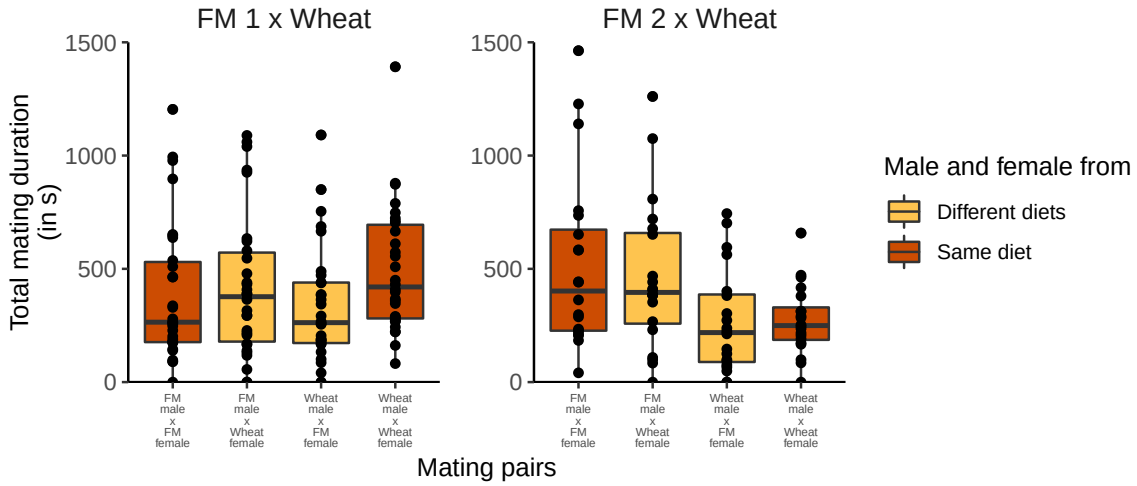
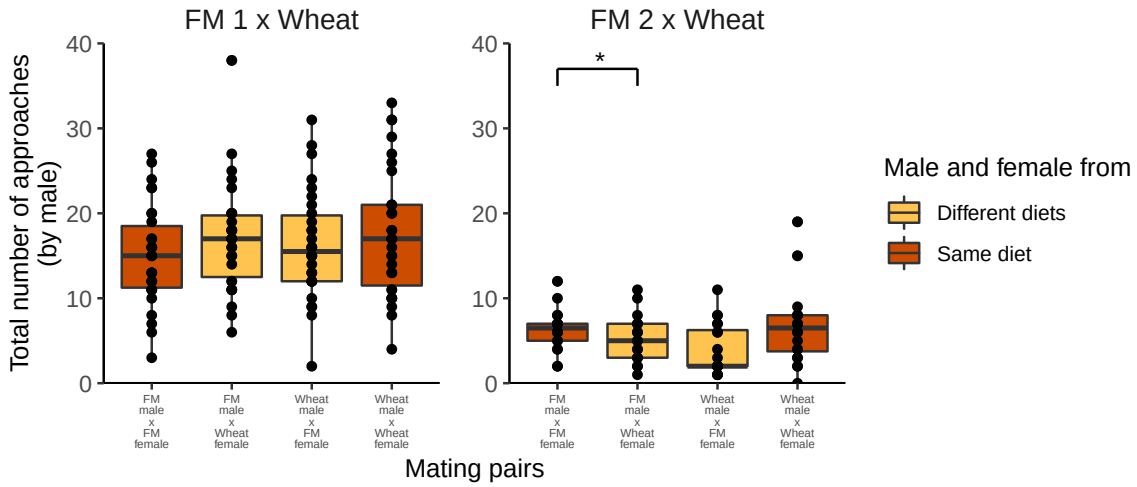
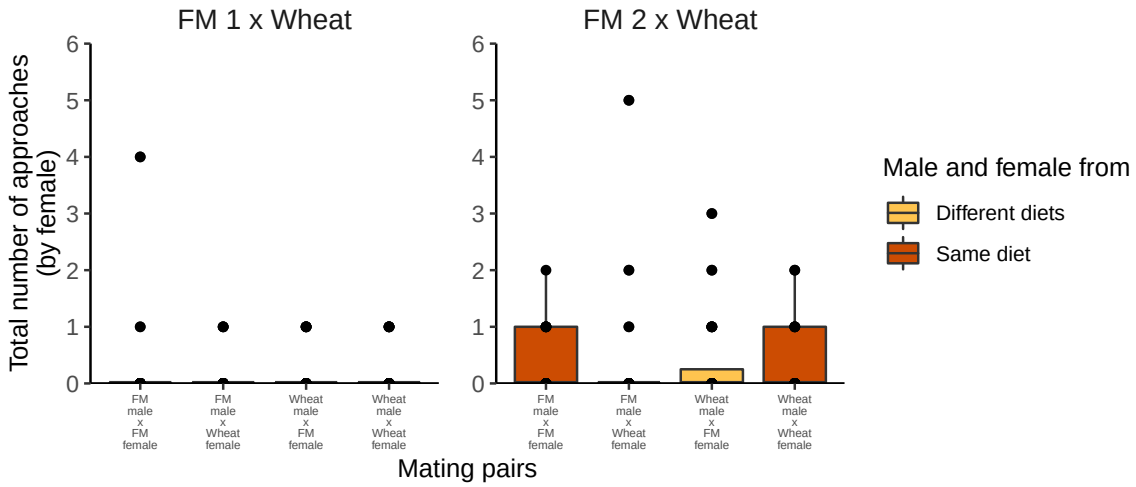


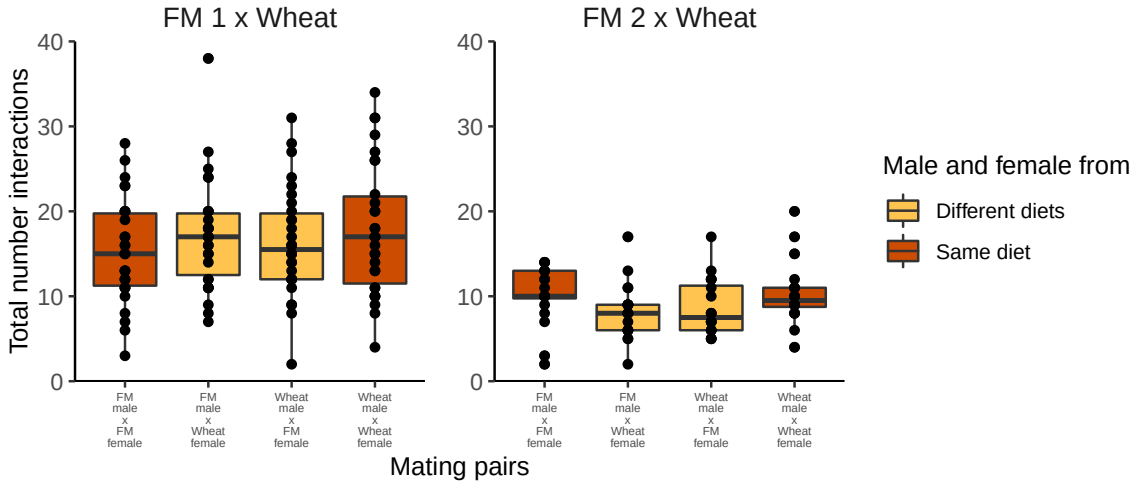
Figure 2.26: Total mating duration in the assays using FM populations 1 and 2. No significant difference in the total mating duration in FM population 1 (ANOVA F = 1.537, P = 0.209). In FM population 2, significant difference in between FM ♂x FM ♀mating pair, and Wheat ♂x FM ♀and Wheat ♂x Wheat ♀(ANOVA F = 3.981, P = 0.011, TukeyHSD P = 0.036 and 0.046), due to one outlier in the FM ♂x FM ♀pairs; after removing the outlier, ANOVA F = 1.831, P = 0.149 (df = 3 in all cases).



(a) Total number of approaches by males in FM population 1 (GLM: Poisson Z = -2.520, df = 119 (null), P = 0.05, with no pairwise differences), and Sorghum population 2 (significant difference, Z = -3.253, df = 79 (null), P = 0.001, with significant difference between FM ♂x FM ♀mating pairs and FM ♂x Wheat ♀mating pairs).

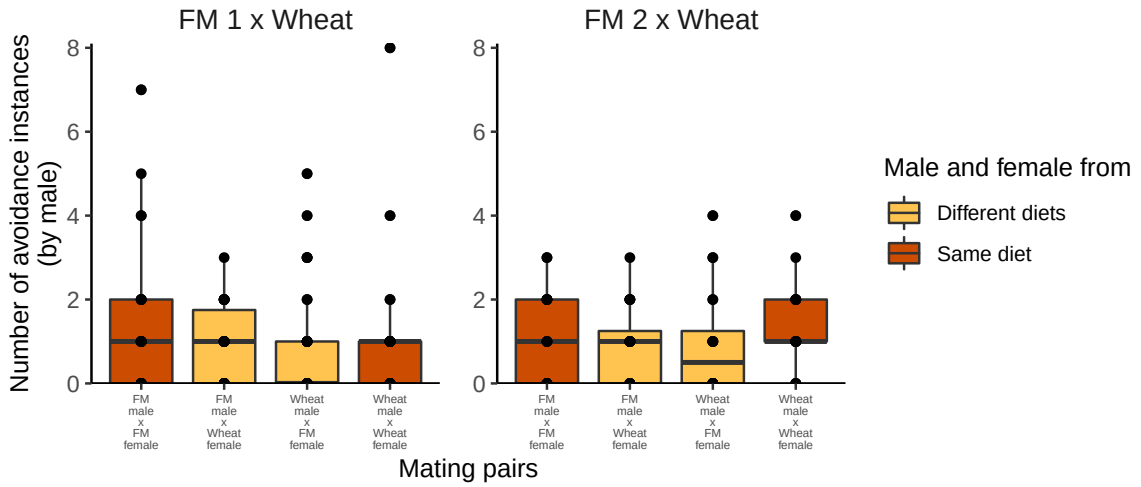


(b) Total number of approaches by females in FM populations 1 and 2 (GLM: Poisson FM population 1 Z = -1.095, df = 119 (null), $P = 0.273$; FM population 2 $Z = 0.258$, df = 79 (null), $P = 0.796$).

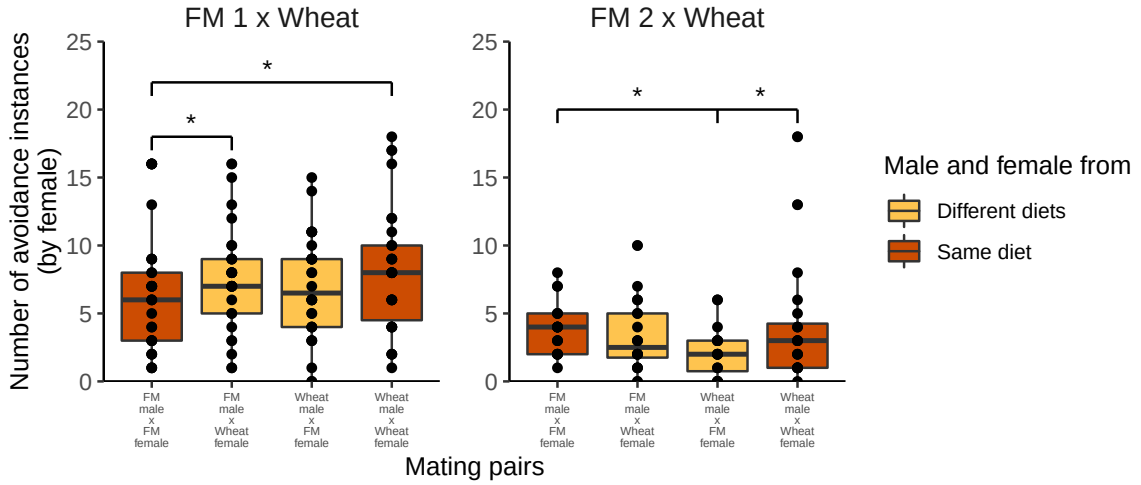


(c) Total number of interactions in FM population 1 (GLM: Poisson Z = 2.446, df = 119 (null), $P = 0.014$, but post-hoc test revealed no significant differences at 95% confidence level) and FM population 2 (GLM: Poisson Z = -2.130, df = 79 (null), $P = 0.03$, but post-hoc test revealed no significant differences at 95% confidence level).

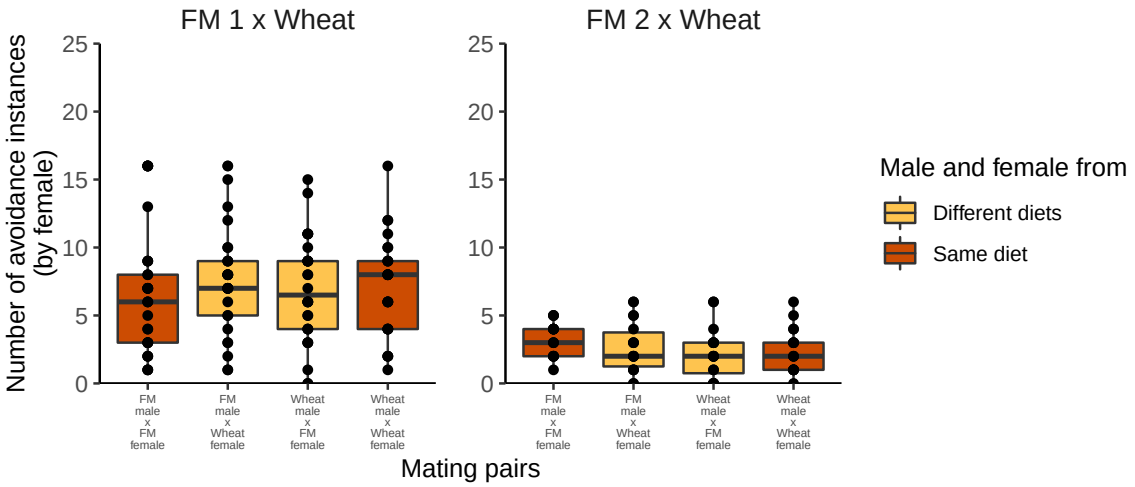
Figure 2.27: Total number of interactions in Corn populations 1 and 2.



(a) Number of instances of avoidance exhibited by males in FM populations 1 and 2 (GLM: Poisson Sorghum population 1 Z = -1.710, df = 119 (null), $P = 0.0873$; Sorghum population 2 Z = -0.160, df = 79 (null), $P = 0.873$).



(b) Number of instances of avoidance exhibited by females in Sorghum populations 1 and 2. Significant difference between mating types in FM populations 1 and 2 (GLM: Poisson gave Z values 2.976 and -3.060, and P values 0.003 and 0.002), due to presence of one outlier (df = 119 and 79 for FM populations 1 and 2).



(c) Outlier analysis by inter-quartile range method, GLM: Poisson for FM population 1 $Z = 1.531$, df = 115 (null), and $P = 0.126$. For FM population 2 $Z = -2.008$, df = 71 (null), $P = 0.045$, but post-hoc pairwise comparison did not return any significant differences at the 95% confidence level.

Figure 2.28: Number of instances of avoidance behaviours in FM populations 1 and 2.

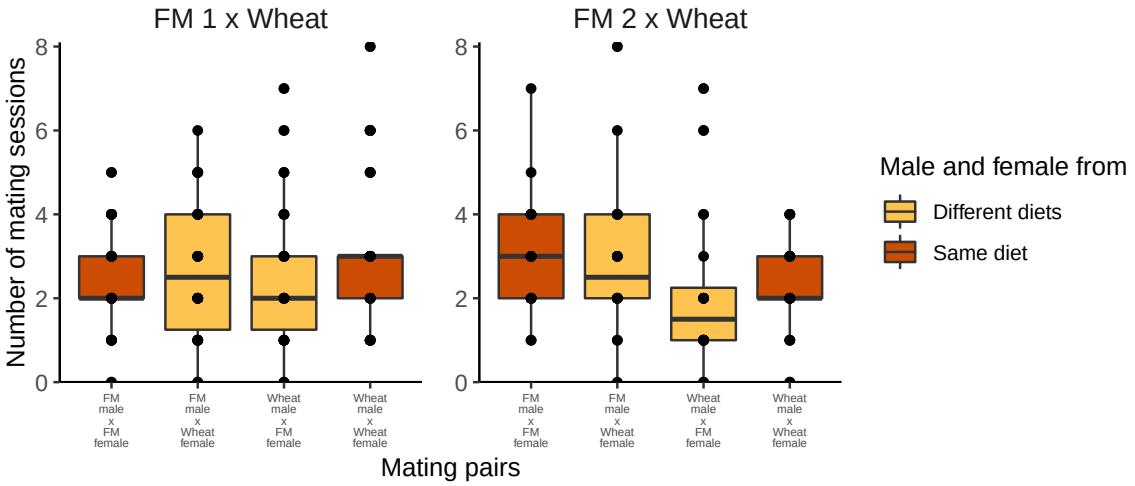


Figure 2.29: Number of mating sessions in the assays using FM populations 1 and 2. No significant differences at 95% confidence interval (GLM: Poisson FM population 1: $Z = 0.158$, df = 119 (null), $P = 0.874$; FM population 2: $Z = -0.092$, df = 79 (null), $P = 0.926$).

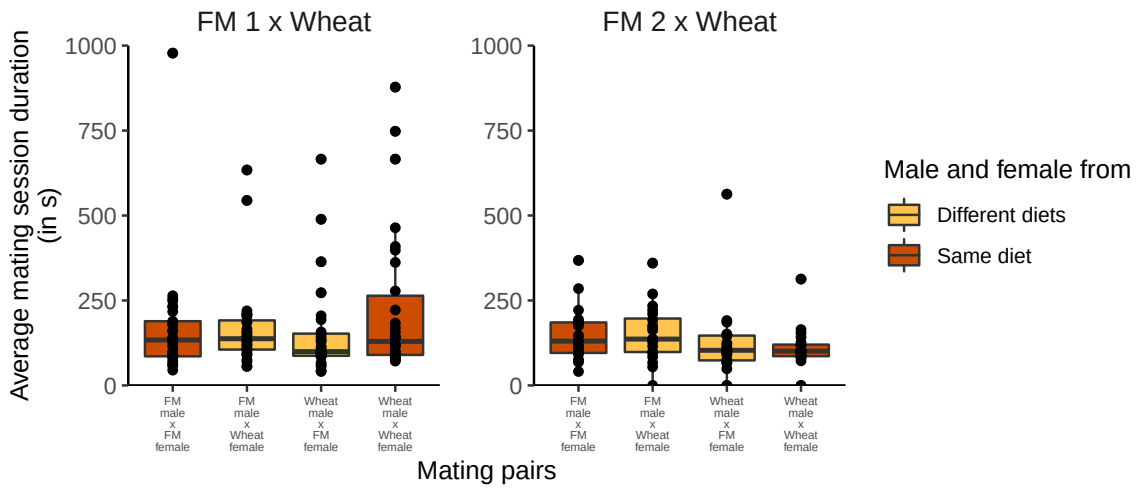


Figure 2.30: Average mating duration in the assays using FM populations 1 and 2. No significant difference in either case (ANOVA (df = 3 in both cases) FM population 1: F = 1.056, $P = 0.371$; FM population 2: F = 1.429, $P = 0.241$).

2.2 Discussion

2.2.1 Populations reared in novel diets for one generation

The earlier work on the populations adapted to corn and sorghum (Rittik Deb, unpublished data), found assortative mating patterns when the individuals from these adapted lines were allowed to interact with their ancestral (wheat reared) population; individuals that developed on either novel diet preferred mates from the same diet over potential mates from the ancestral wheat population. My results showed that the populations reared for a single generation on either of these resources showed random mating with the wheat population, indicating that the mating patterns seen in the adapted lines is evidence of behavioural reproductive isolation.

The diet on which the individuals develop does not appear to affect mate choice; rather it is adaptation to the diet that causes changes in mate choice. Development in a suboptimal resource does not affect mating success. A similar study on *T. castaneum* by Ming and Cheng 2012 showed that diet quality did not affect male attractiveness, in agreement with the results I obtained. In addition I also found that female development diet has no effect on mating success. Some response variables (such as the number of interactions) showed significant male diet x female diet interaction effects but these were not consistent across the biological replicates. One possible explanation is higher relative investment in reproduction

by individuals reared in poorer diets; for instance, in the Hawaiian species of *Drosophila*, *D. grimshawi*, males reared in poorer diets show higher relative investment in testes compared to those reared in high quality diets (Droney 1998). If this is the case in this study, increased reproductive investment by males reared in the suboptimal diets did not increase their mating success. However, mating success was quite high in these assays, so a small increase may not have been detected.

Alternatively, Ming and Lewis 2010 found that *Tribolium castaneum* males reared on a high quality diet invest more in reproduction, as they produce larger amounts of the aggregate pheromone 4,8-dimethyldecanal (DND). This study found no variation in female choice in response to male diet quality or pheromone production. This can potentially be explained by the action of other cuticular chemicals which may interact with DMD in different ways. There may also be differences in how females use CHCs to identify potential mates. For instance, in the decorated cricket *Gryllodes sigillatus* it has been found that females choose mates based on male CHC profiles, but not their own (Steiger et al. 2015), whereas in the field cricket *Teleogryllus oceanicus*, females prefer mates with CHCs dissimilar to their own (Thomas and Simmons 2011). Analysing the CHC profiles of the *T. castaneum* individuals used in these mate choice assays can tell us about the extent of plastic responses in the expression of cuticular chemicals, and their role in mate attraction.

2.2.2 Finger millet adapted lines

Contrary to the results found regarding mate choice in the corn and sorghum adapted populations, the mating pattern between individuals from the finger millet lines and individuals maintained in wheat was random, indicating no behavioural isolation having occurred in these populations. This indicates that divergence, if occurring in these populations, is mediated by other mechanisms of reproductive isolation. Development time is longer than in wheat, but it is unlikely that this plays a role in mate choice as the development time in corn is comparable to that of finger millet. The mechanisms of mate choice and adaptation to finger millet may be different from how adaptation occurs in corn and sorghum, which show genotype x genotype effects. It is possible that genotype x environment interactions may affect mate choice in the finger millet adapted populations.

Not much is known about the nutritional physiology of *Tribolium* in different diets. A few studies have found that fecundity and egg viability does not vary significantly between millets, sorghum and corn (Gerken and Campbell 2020; Naseri et al. 2017). However, Naseri et al. 2017 found that starch and protein contents of corn and sorghum were significantly different from that of wheat and millets (no significant differences in these nutrient contents

between wheat and millets), with individuals reared on sorghum having significantly different levels of enzymatic activity from that of individuals reared on wheat, corn or millets. The mechanisms of adaptation are likely to involve changes in gut enzyme composition and activity. If these changes affect other mating cues, the similarity in the nutrient composition between millet and wheat flours could potentially explain why we do not find mate discrimination between the finger millet adapted populations and the ancestral population. However, the millet used in these studies was *Panucum miliaceum*. Characterising the composition of finger millet (*Eleusine coracana*) and the nutritional physiology of the adapted populations in this resource will give us a clearer understanding of the role of gut enzyme physiology in adaptation.

The microbiome of the gut and environment is closely linked with diet and nutrient acquisition, in addition to a number of other functions in hosts, including development, reproduction and metabolism (McFall-Ngai et al. 2013; Moran, Ochman, and Hammer 2019; Leftwich, Hutchings, and Chapman 2018). Agarwal and Agashe 2018 suggested that the microbiome plays a role in host adaptation to novel resources. In *Drosophila*, diet and the associated bacteria have been found to influence mate choice, with populations adapted to different diets showing assortative mating (Dodd 1989; Sharon et al. 2010; Sharon et al. 2013, but see Leftwich, Clarke, et al. 2017). The stability of the association between microbes and hosts depends on the mode of acquisition (Leftwich, Edgington, and Chapman 2020). In *T. castaneum*, symbiotic gut microbes are largely acquired from the environment (Agarwal and Agashe 2020), and thereby changing the diet is likely to disrupt these microbial associations (Agarwal and Agashe 2018). Assaying how these microbial associations change in each diet can shed light on the role of the microbiome in determining mating success, and how the mechanisms of adaptation to these different diets varies.

Much of the work that investigated reproduction in *T. castaneum* used post-copulatory mechanisms such as offspring numbers and sperm precedence as indicators of reproductive success rather than pre-copulatory mechanisms (Pai and Yan 2002; Ming and Lewis 2010; Attia and Tregenza 2004). Attia and Tregenza 2004 found that progeny counts are higher when females mate with genetically distinct males (inter-population mating), indicating sexual conflict or adaptations to avoid inbreeding. However, Pai and Yan 2002 found that sperm defence capacity (fertilisation success by the first mate) is higher when mating occurs between genotypically similar individuals. This corroborates the results presented here, in that there is higher reproductive success when mating occurs between individuals from the same environment. However, post-copulatory reproductive success is not necessarily correlated to mating success (Pischedda and Rice 2012). Investigating post-copulatory reproductive success can give us a better understanding of how adaptation and reproductive isolation has occurred in these populations.

Using a promiscuous system, this study found that mate choice is dependent on genotype x genotype interactions in populations adapted to two novel diets: corn and sorghum. Mate choice contributes to the reproductive isolation between the ancestral line and these adapted lines, but not in populations adapted to finger millet. Further work on the molecular mechanisms underlying mate choice can help elucidate how adaptation occurs and why this pattern arises.

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