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Life history and reproductive fitness variation associated with the Y chromosome in *Callosobruchus maculatus*

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Abstract

In the seed beetle *Callosobruchus maculatus*, the female is the larger sex and the male is the smaller sex. However, males that are almost as large as females can also occur, which is due to a specific Y chromosome haplotype. This Y chromosome polymorphism is not expected since the Y chromosome does not recombine and has lost genetic variation as a consequence. Nevertheless, the Y chromosome manages to maintain this polymorphism. Thus, the questions asked are how this occurs and how the large male Y haplotype persist to exist since previous studies have shown how small males have the higher fitness. In this study, large males are from line SL3b Y and small males are from line SL1b Y.

To answer the questions, two important measures of fitness were conducted in this study, mating- and lifetime reproductive success, as well as lifetime-history traits of the SL1b Y and SL3b Y males. Males from line SL3b Y turned out to have a faster growth rate and a shorter development time compared to the SL1b Y males. Both the SL3b Y males with a shorter development time and the SL1b Y males with a longer development time had larger body sizes. Large males also showed to have heavier ejaculate weight and produced more offspring compared to the other male Y haplotype. However, neither of the males had higher pre-mating success. In conclusion, the two male Y haplotypes must coexist in nature since their traits are beneficial in different environments and circumstances.

Table of Contents

1. Introduction

Sexes within the same species can have a different expression of phenotypical traits. This phenomenon is termed sexual dimorphism and occurs in traits such as colour, body size and shape (Frayer & Wolpoff 1985). The differences between the sexes can be caused by the sex chromosomes carried in the genome of the species (Mank 2009). Numerous species, ranging from humans to insects, such as the study organism, the seed beetle *Callosobruchus maculatus*, have X and Y chromosomes (Angus *et al.* 2011, Bachtrog *et al.* 2014). These two chromosomes are sex chromosomes that determine the biological sex of the individual and carry genetic information that can give different phenotypical traits in the sexes. However, the Y chromosome does not recombine with the X chromosome outside the pseudoautosomal regions (PAR) (Helena Mangs & Morris 2007). Recombination is the phenomenon in which chromosomes exchange segments of genetic material. When these segments have exchanged positions, a new combination is established and this increases the genetic variation (Charlesworth *et al.* 2009). The recombination of the Y chromosome with the X chromosome stopped in a slow and stepwise manner. Regions on the chromosomes stopped recombining a few at a time and these regions are called evolutionary strata (Lahn 1999). Loss of recombination caused the Y chromosome to degenerate which in turn leads to loss of functional and ancestral genes. Over evolutionary time, the Y chromosome has lost these genes and what remained was repetitive sequences, non-functional DNA and a few genes with male-related functions (Bachtrog 2013). Since in many species a large proportion of the Y chromosome does not recombine with the X, it should therefore reduce in size, lose genetic variation and gene function over time (Charlesworth & Charlesworth 2000). Yet there is new evidence from *C. maculatus* (Kaufmann *et al.* manuscript submitted) that the Y chromosome can somehow maintain its genetic variation despite being degenerated (Angus *et al.* 2011). In *Drosophila* and guppy *Poecilia reticulata* (Postma *et al.* 2011) it has also been found that the Y chromosome affects gene expression elsewhere in the genome. Genetic variation linked to the Y chromosome affects the individual's lifespan, development time, reproductive traits, mitochondrial function and more (Archer *et al.* 2017, Bachtrog 2013).

Additionally, lack of recombination does also reduce nucleotide diversity and effective population size, thus reducing the efficacy of natural selection according to the population genetics theory (Casillas & Barbadilla 2017). Natural selection acts on mutations and causes beneficial mutations to go to fixation and deleterious mutations to be selected against. However, when natural selection is reduced, drift dominates. Thus theoretically, the Y chromosome is considered to have a decreased level of natural selection and this leads to an accumulation of deleterious mutations and a low frequency of advantageous mutations. A low frequency of advantageous mutations does further lead to a lower capacity of adaptation and adaptive evolution. However, new evidence from *Drosophila melanogaster* (Griffin *et al.* 2015) shows how the Y chromosome does take part in adaptive evolution. Accumulation of mutations, even deleterious ones, can also occur since selection against them is inefficient and because they might sweep to fixation if they are close by i.e. linked with alleles that are strongly favoured by selection (Charlesworth & Charlesworth 2000). With time, the Y chromosome has accumulated plentiful of harmful mutations, lost good mutations and

segments because of deletions (Bachtrog 2013, Charlesworth & Charlesworth 2000). Genetic variation i.e. new mutations do not persist for long, as they are either quickly lost or become fixed because of drift (Whitlock 2000). This implies that there would be less genetic variation since the same mutations are kept in the genome. Therefore, segregating polymorphism i.e. genetic variation present in the population is not expected to be observed on a heteromorphic Y chromosome that affects body size, and yet it is. The recent work done by Kaufmann *et al.* (manuscript submitted) has discovered that the Y chromosome in *C. maculatus* explains as much of male body size variation as the rest of the chromosomes.

Sex chromosomes have derived from autosomes (Charlesworth 1996) and have through evolutionary processes established sexually antagonistic (SA) mutations on the sex that it benefits (Rice 1984). Physical linkage and accumulation of SA loci to the sex-determining region (SDR) are believed to be a major driver in the evolution of sex chromosomes (Abbott *et al.* 2017). Since the setup of the sex chromosomes differs in the two biological sexes, they can carry different genes and reach their optimal fitness. The Y chromosome is passed down from father to son and does not go through females. This genetic male-limited transmission makes it possible for males to contain genes that are only beneficial to males and which would increase male fitness and decrease sexual conflict (Charlesworth 1996). This asymmetrical inheritance of sex chromosomes has proposed them to be a hotspot to facilitate sex-determining loci (SD) (Abbott *et al.* 2017). The X chromosome on the other hand is carried by both males and females. Thus, if an allele of a gene expressed in both males and females is too beneficial for one sex but reduces the fitness in the other, it can cause intralocus sexual conflict (laSC). A shared gene pool hinders males and females from reaching their optimal fitness (Charlesworth 1996). Fitness can be described as an individual's ability to attract partners, produce many and healthy offspring, adapt to an environment and carry good genes. An individual with high fitness has higher odds of spreading their genes to the following generations (Arnqvist & Tuda 2010).

1.1 The study organism

The study organism *C. maculatus* is a legume pest. It lives in warm climates around the world and lives off of bean storages. In this species, the female is the larger sex with the larger body size and the male is the smaller sex (Berger *et al.* 2014). The large body size of the female is also correlated with higher fecundity (Fox 1993a). However, somewhat larger males can occur and previous work has shown that this is because of a specific Y chromosome haplotype. The reason why the large male Y haplotype occurs is unclear and this leads to the question of what its consequence on male fitness is. In this project, I test whether the males carrying a Y haplotype associated with smaller or larger size differ in different aspects of reproductive fitness. To study this, one needs to look into different traits of the two male haplotypes. One trait that may show effects of Y-linked variation between the two males is ejaculate weight (Savalli & Fox 1998). The ejaculate size is hypothesized to be proportionate to the body size of the males and would give an indication of the paternal investment. Both

sexes invest energy into producing the optimal offspring and the paternal investment includes the energy required to produce ejaculate.

The three lines of seed beetles used for this study are SL1b Y, SL3b Y and 2:11. The abbreviation "SL" stands for "sex-limited" which implies that these lines were artificially selected for smaller and larger body sizes of the males without considering the female size. Lines SL1b Y and SL3b Y have two different Y haplotypes, the former has a Y haplotype encoding for a small male (S) size and the latter has a Y haplotype encoding for large (L) males. Both of these two lines were subsequently introgressed into a common genetic background, line 2:11, and thus only differ for the Y chromosome. The lines do therefore have a common genetic background. The females in this study were used as a control group when measuring traits such as body size and development time which are life-history traits expressed by both sexes (Paukku & Kotiaho 2008), and which can show the line effect and thus the effect of the Y haplotype only in males.

The female decides which beans the eggs will be laid on (Paukku & Kotiaho 2008). Sometime later, the eggs hatch and the larvae crawl out of the egg and into the bean. An unhatched egg will be of transparent colour and a hatched egg will be observed as a white dot on the surface of the bean. The larvae will stay inside the bean for a time that is called the development time. Depending on the line of beetle or the species, the development time can differ but, in this case, the larvae stay inside the bean for approximately four weeks. There the larvae will eat from the bean and grow until it is ready to emerge as a full adult. Once the beetle has emerged, it will mate and live for approximately a week. Thereby the cycle repeats itself (Paukku & Kotiaho 2008).

A small body size for males could be beneficial since it is known that small males are more agile and active (Berger *et al.* 2014), which can be useful when trying to mate in pre-mating sexual competitions. An active male would have the opportunity to mate with more females faster and have a higher chance of spreading its genes. A larger male is not as active and would therefore not be able to mate with comparatively as many females. However, if the ejaculate weight is larger in larger males, they would have the opportunity to win in sperm competition. This study will look at which male has the larger ejaculate weight, mating success and lifetime reproductive success. Berger *et al.* (2016) has found earlier that smaller males do also have a shorter development time than larger males and females, which naturally have a larger body size. This means that the smaller males would be reproductively mature earlier and ready to mate as soon as the first females start maturing. Large males would be at a disadvantage at this point since they may need more time to develop and would therefore not have the chance to mate with the first emerging females, that may also be of higher genetic condition. On the other hand, large males may have greater nutritional resources gathered from larval stages inside the bean, which can ensure greater energy resources beneficial for mating activity and ejaculate transfer, which may ultimately benefit them in sperm competition.

Having this model organism instead of other model organisms such as *Drosophila* is of great importance. *C. maculatus* is currently the only model organism known to exist that has

evidence of sexually antagonistic polymorphism on a highly heteromorphic and degenerated Y chromosome that influences a sex homologous trait present in both sexes, the body size (Kaufmann *et al.* manuscript submitted), as opposed to only males like Y-linked traits in other systems are. Using this model organism does also contribute to a broader understanding of the Y chromosome since the data sampled is not solely restricted to one species such as *Drosophila*.

1.2 Aim of the study

The four hypotheses for this study are the following:

- L males will have higher pre-mating success, ejaculate weight and lifetime reproductive success. Thus, the ejaculate weight will be proportionate to the body size.
- A lager body size may also delay male development time, thereby causing a mating disadvantage in some contexts compared to the smaller male Y haplotype.
- S males will develop faster than L males.
- The body size of males will increase with a longer development time.

This research study aspires to test how S and L males, that are carrying two different Y chromosome haplotypes, affect male development time, ejaculate weight, body size and reproductive success under non-competitive conditions. To test for reproductive success, the lifetime offspring production will be measured. The pre-mating success will be tested and compared, as well as the pre-mating advantage and fertility for each haplotype. The question this study helps to unravel is how such polymorphism in the Y chromosome can be maintained. Is it possible that it is maintained under negative frequency-dependent selection whereby the rarer haplotype has a higher fitness? If this is the case, does it come to occur from how sexual selection acts on males or from viability selection? To begin to understand how this polymorphism and variation is maintained, more needs to be known about how each Y haplotype affects different male fitness components.

2. Materials and methods

The seed beetle populations used in this study were kept in laboratory climate cabinets at 29°C and 50% humidity, both during their development time and during their adult life. The climate cabinet has a 12:12 hour day/night cycle where it is installed to change light at eight twice a day. Line SL1b Y includes the beetles with a Y haplotype associated with a small male size and line SL3b Y includes the beetles with a Y haplotype associated with a large male body size. Originally, these lines come from a Lome population which refers to the city Lomé in Togo where they were collected. Numerous lines were created from this population, including the lines worked with in this study. The Y lines were created through introgression of the Y haplotypes to a common genetic background (2:11, also originally from a Lome population) by backcrossing for 15 generations. Therefore, these lines have a shared

background and the same mitochondrial DNA since they had the same mother. The lines were generated by Kaufmann *et al.* (manuscript submitted) at the Department of Ecology and Genetics. For this study, the grandparental and parental generation reproduced in a controlled manner at a young age and the oviposition was limited to 24 hours. This was performed to reduce any parental effects on the experimental generation of beetles. The F1 generation that this study started was created on the 15th of October. On that date, the eggs were laid on mung beans (*Vigna radiata*) and the females were again only allowed to lay their eggs over 24 hours for the development time to be scored precisely and to avoid multiple eggs being laid on the same bean. A week before the new beetles (F1) were expected to emerge, they were isolated into well plates hereafter called 'virgin chambers' (VC). Line SL1b Y was isolated into 13 VC, line SL3b Y into 20 VC and line 2:11 into 21 VC which sums up to a total of 54 VC. The initial plan was to fill up 70 VC but since the females from generation F0 had a limited amount of time to lay their eggs, there were not enough eggs on the beans to fill up that many virgin chambers. The beans that seemed to have more than one egg on them were also incubated into virgin chambers in case the study would need more individuals, which it in turn did. A total of nine virgin chambers were incubated for beans that seemed to have more than one hatched egg.

Testing for development time differences in the beetles of each line was conducted by observing the time it took for the larvae to develop into an adult and emerge from the bean from the day when parental egg-laying was initiated. The virgin chambers were monitored twice a day, morning and evening with a nine-hour interval, to clock their development time precisely. Once the beetles started coming out of the bean, their development time in days was noted. The beetles that emerged during the night and were counted in the morning at 9 o'clock were written as a natural number (e.g. 29) and beetles that had emerged during the day and were counted in the evening at 18 o'clock were written with a decimal (e.g. 29,5). If two males and two females from each line were available, a mating competition could take place. Since line 2:11 took longer to emerge from the beans while the other two lines were at their peak, the study was required to also use females from the two other lines, hence the females used came from three lines total. Otherwise, the males would have been too old once the females from line 2:11 emerged. As it showed later on, line 2:11 only produced a fourth of the number of females required for the mating assays in this study which made it a good decision to also use the females from the other two lines. The females from 2:11, SL1b Y and SL3b Y have essentially the same genetic background due to the backcrossing scheme and are therefore not expected to differ due to genetic reasons.

In preparation for the mating assays, the beetles were collected from the virgin chambers into Eppendorf tubes with lids punctured for air and got weighed. Weighing the beetles right before mating gives us the pre-mating weight (W1) and both males and females were weighted before mating. The scale used for this matter is Sartorius ME254S Genius Analytical Balance 250g x 0.1 mg. The majority of the individuals used for the matings were zero to one days old, but some individuals were up to four days old. For the mating competitions, one small male and one large male were placed in a Petri dish firstly and allowed to habituate for a minute before the female was added. The female was also placed in

the Petri dish with an equal distance away from both males. This technique was to assure both males had the same opportunity to mate with the female. It was recorded which male mated with the female first and succeeded to win the mating competition, which would be our mating success data. The male which did not succeed to mate first was placed with another virgin female to be able to measure the male lifetime reproductive success of both male lines in the end. The females used for the mating assays were also used an equal amount of times from both Y lines and it was ensured that one lines was not used more than the other. A total of 60 mating assays took eight days to complete.

After mating, the females were placed on Petri dishes containing a layer of mung beans to lay their eggs through their whole lifetime. At the same time, the males were weighted immediately after the mating to detect how much weight they had lost which would be an approximation of the ejaculate weight (Immonen *et al.* 2016). The faster the males were weighted after the mating, the more accurate the ejaculate weight was going to be. The sample size of the number of males from lines SL1b Y and SL3b Y that mated are 30 and 30 respectively. The number of females from lines 2:11, SL1b Y and SL3b Y that mated are 6, 27 and 27 respectively. Three females from 2:11 mated with males from SL1bY and the other three mated with the other line. 15 females from SL1b Y and SL3b Y respectively, mated with males within the same line. The rest of the 12 females from each line mated with the other line.

Once the mating assays were finished and the development time for these individuals involved was monitored, the statistical tests could be performed. All analyses were conducted using R in RStudio (RStudio Team 2016), with version 3.6.3. When the analysis of covariance (ANCOVA) test was performed to obtain the ANOVA tables, the package “car” was used in version three (John Fox and Stanford Weisberg 2019). The ANOVA type III tests had an F-test added in order to show the significance of different groups. The lifetime offspring production was measured to gather reproductive fitness data under non-competitive conditions. These are non-competitive conditions because there is no sperm competition involved. To simplify the measurement of the offspring (F2) from generation F1, the beetles were first put in a freezer after given enough time for all F1 adults to emerge. The beetles from line SL1b Y and SL3b Y were given nine and seven days respectively to emerge, in addition to their development time, before they were put in the freezer at -20°C. Once they had been in the freezer for at least 48 hours, the offspring were counted, the reproductive fitness data was gathered and was ready to be used for analyses.

3. Results

The average development time, body weight, ejaculate weight, growth rate and lifetime reproductive success for the different lines and sexes are shown in Table 1.

Table 1: Mean values and standard deviation (SD) of each response variable. *The mean and standard deviation are given for both sexes in both lines for each response variable for comparison.*

	Development time [days] (SD)	**Body size [mg] (SD)**	**Ejaculate weight [mg] (SD)**	**Growth rate [body size/ development time] (SD)**	**Lifetime reproductive success [Offspring] (SD)**
Male SL1b Y	28.531 (2.498)	3.471 (0.428)	0.186 (0.158)	0.122 (0.018)	32.333 (30.964)
Female SL1b Y	27.076 (1.481)	4.882 (0.718)		0.181 (0.030)	37.500 (35.240)
Male SL3b Y	28.343 (2.223)	4.277 (0.694)	0.263 (0.104)	0.153 (0.031)	54.500 (30.777)
Female SL3b Y	28.250 (2.287)	4.923 (1.017)		0.176 (0.043)	45.607 (30.249)

3.1 Body size

An ANCOVA was performed with body weight as the response variable and traits such as development time, sex, line and their interactions, as the explanatory variables. The significance of the effect was tested using ANOVA type III F-test. The effects of development time, line, sex, age, as well as the interactions between the development time and line, and between development time and sex, were all significant (Table 2). Presented in Table 2 below are the different variables with the significant results. The adult age affects how much the seed beetle will weigh every day and the sex of the individual does also affect its body weight. The ANCOVA test showed that there was an interaction between line and sex. To see which sex had an interaction with line, the data had to be split by sex.

The linear regression model with body weight as the response variable had a significant difference between the lines as well as an interaction effect between development time and line in males. Figure 1 shows how the males with a Y haplotype known to cause a larger body size (SL3b Y) are indeed larger and that their size also decreases with a longer development time. When performing the same test for the females, significant results are instead obtained for development time and age, not for line. This means that for females, the development time and age affect their body size, but the line does not. With females as the control group, these results point to the role of the Y chromosome and how it affects males differently regarding the effect of development time on body size. When splitting the model by sex, line has a significant effect in males but not in females. This means that the males from the two lines have different body sizes and that the females do not differ in body size between the lines. This explains the interaction between line and sex (Table 2). When performing a linear regression model with growth rate as the response variable (section 3.3), the same conclusions

hold which again point to the role of the Y chromosome affecting males differently regarding development time.

Table 2: ANOVA table (Type III test) with added f-test*. This test is performed with body size as the response variable.*

	Df	**Sum Sq**	**F-Value**	**P-Value**
Development time	1	2.930	7.618	0.006772 **
Line	1	1.990	5.172	0.024888 *
Sex	1	4.014	10.435	0.001631 **
Age	1	8.919	23.185	4.727e-06 ***
Development time:Line	1	1.632	4.243	0.041775 *
Development time:Sex	1	1.975	5.133	0.025430 *
Line:Sex	1	2.248	5.843	0.017283 *

Included below is Figure 1 where the respective plots are illustrating body weight as the function of development time. The three plots show the pattern of males and females from line SL1b Y and SL3b Y. The plots were performed to see how the body size of each male type is affected by their development time in the bean, compared to the females from the same lines but without the possibility of being affected by the Y chromosome. From these obtained results one can see how the males grow at different rates (Figure 1c). L males grow faster than S males, when developing for a shorter time, but if they have a longer development time, their body size will actually become relatively smaller approaching the S males. The L males thus have a negative trend in the slope whereas the S males have a positive trend. Females do however grow at very similar rates (Figure 1b), as expected since they are the control group and have not had the body size selected on. To know if the L males who develop longer and get smaller body sizes will become sick and defect or fit like the small males from SL1b Y, one needs to look at the reproductive fitness data presented in 3.6.

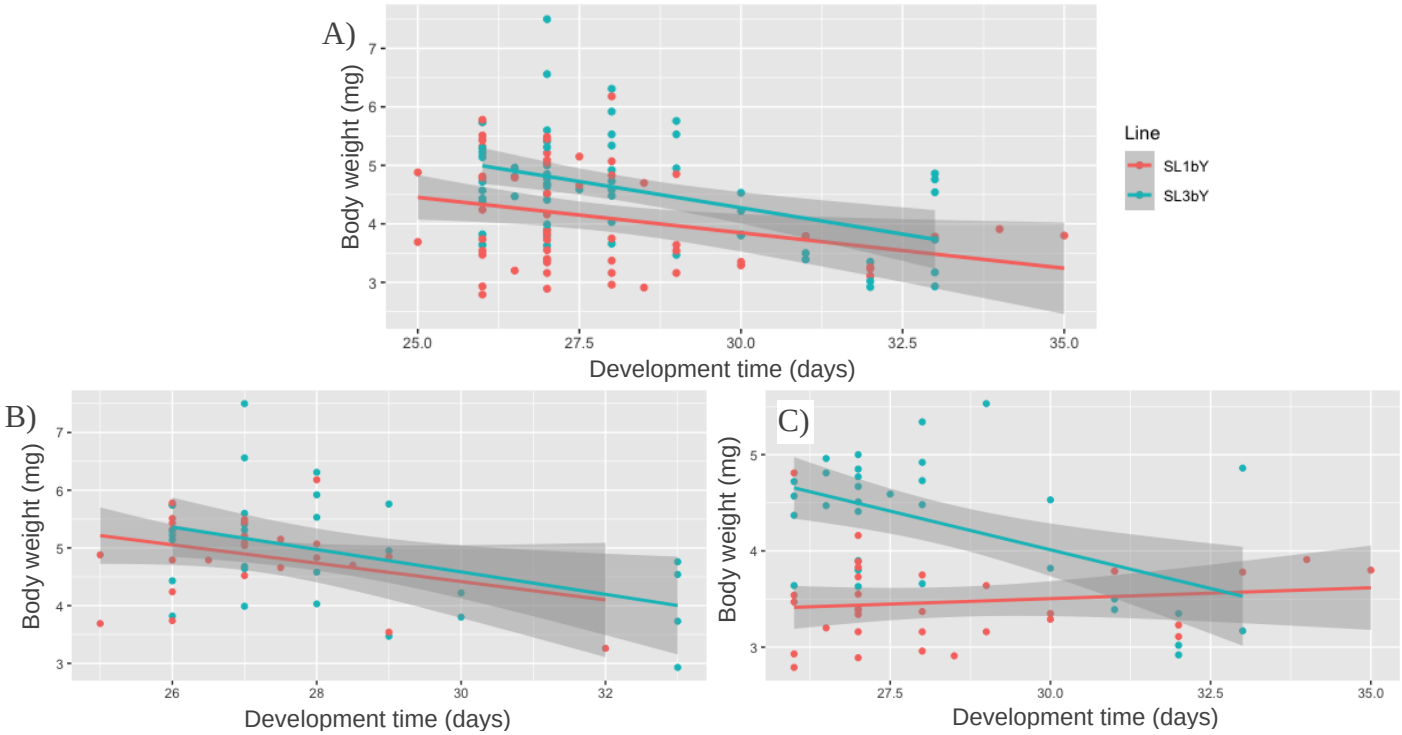

Figure 1: Plots with body size as a function of development time. *A) Shows both sexes together in one regression line and how the body size generally decreases with a longer development time in both beetle lines. Line SL3b Y (coloured blue) has a higher intercept. The sample size of SL1b Y and SL3b Y are 58 and 60 respectively. B) Shows females from both lines. The body size of females from both lines is affected similarly by their development time. The sample size of SL1b Y and SL3b Y are 26 and 28 respectively. C) Illustrates the male distribution. Males from line SL3b Y are larger when they have a shorter development time (~26,5 days) and get a smaller body size the longer their development time lasts. Small males seem to get a larger body size the longer they develop. The sample size is 32 for SL1b Y and SL3b Y respectively.*

3.2 Development time

The average development time per line and sex and their differences can be observed in Figure 2 and Table 1. Both show how males took slightly longer to develop than females, which was not expected because of their larger body size. It is also illustrated how the development time is very similar for the males. Males from line SL1b Y do also seem to show more variation than the large males, especially at the late emerged beetles. Some outliers do also occur among the females and the small males.

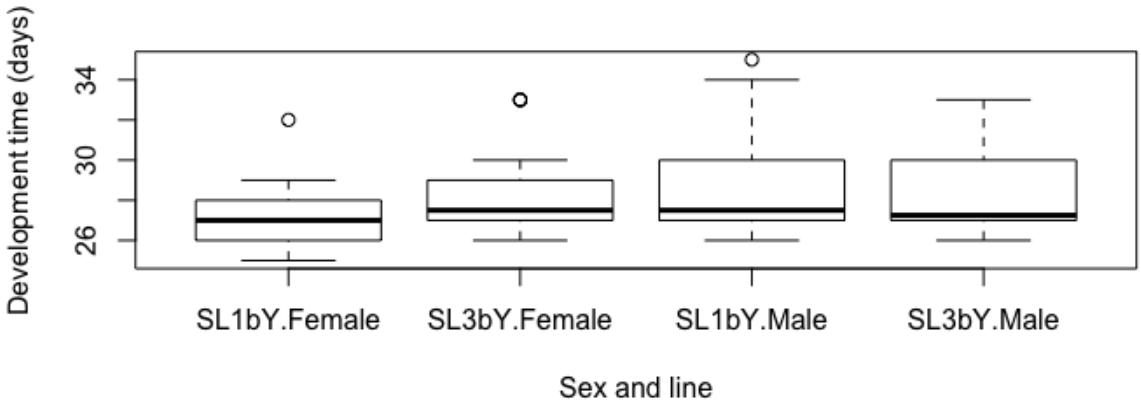

Figure 2: Average development time per sex and line. *This boxplot presents the time it took for the beetles from each line and sex to mature inside the bean and come out as adults.*

A generalized linear model (GLM) was fitted with development time as the response variable and with both sexes included in the data, where body size, line and sex, as well as their interactions, were fitted as explanatory variables. Since the data had Poisson distribution, which is common for count data, a Poisson regression model was added to the GLM test. The significance of the effects was tested using ANOVA type III F-test. To further see how each sex may differ, the data was split by sex. When splitting the data by sex and performing an ANOVA type III F-test, males got significant values for line and the interaction between line and body size (Table 3). However, the effects of body size alone were not significant. This means that the development time in males differs between the lines but also as a function of their body size, as already indicated in the model using body size as the response variable. A larger body weight does however not mean that the male beetle will develop for a longer time. To get the full picture, this data needs to be compared to the female data which is the control group in this case. Additionally, Table 1 shows a very slim difference between the average development time of the males and it is odd that the test picks this up as significant. However, it is likely that this slight difference between the male types is that distinct that it is showed as significant.

Table 3: ANOVA table (Type III F-test) for male data. *This test was performed with development time as the response variable. The table presents the result when the data was split by sex.*

	Df	**Sum Sq**	**F-Value**	**P-Value**
Line	1	24.16	4.847	0.03154 *
Body size	1	2.77	0.556	0.45868
Line:Body size	1	21.57	4.3279	0.04177 *

Females got significant values for line and body weight as presented in Table 4. That means that female development time is affected by the line and by the body size of the female. If the female has a larger body size, she will have a longer development time and if the female has a smaller body size, she will have a shorter development time. Since the line is significant in females as well and not only in males, it means that it is not the Y chromosome alone that affects the development time. Given that the two lines share autosomal and X chromosome variation, the line effect on development time is likely caused by environmental factors.

Table 4: ANOVA table (Type III F-test) for female data.

	Df	Sum Sq	F-Value	P-Value
Line	1	17.28	5.296	0.025490 *
Body size	1	32.23	9.8791	0.002785 *

The data was also split by line to see how the sexes may differ in development time within each line. Sex and body weight, as well as their interactions, were used as explanatory variables. For line SL1b Y, sex was significant (F_1= 6.842, p-value= 0.01142), meaning that males and females within line SL1b Y have different development times. Hence, one of the sexes take longer to develop than the other. Table 1 shows that the females have a shorter mean development time than the males and this information is now also statistically supported. For line SL3b Y, body size became significant (F_1= 13.959, p-value= 0.0004). This implies that the body weight of the individuals from line SL3bY affects their development time. Additionally, it is reasonable that line SL3b Y did not get a significant result for sex when looking at Table 1 since their mean development time is very similar.

3.3 Growth rate

Growth rate was also calculated from the data (as body weight per development time) and was used as a response variable, with line, sex, development time and their interactions as explanatory variables. Their significance was tested with an ANCOVA type III F-test and Table 5 shows the results. Sex affects the growth rate of the individual and the interaction between sex and line does as well. When the data is split by sex, males show significant results for development time, line and the interaction between the two. Females show significant results for development time and age but not line. This shows how the growth rate differs between male lines, whereby the males from line SL3b Y have a faster growth rate. Such an effect is not found in females meaning that the growth rate does not differ between females from the two lines since line was not shown significant. Their development time and age do however affect their growth rate. These patterns are captured by the significant interaction effect between line and sex, shown in Table 5 below. Moreover, the females have a higher growth rate than the males. The growth rate detected in males is due to the Y chromosome and this can be determined because no such growth rate was detected in females.

Table 5: ANCOVA table (Type III) for growth rate.

	Df	Sum Sq	F-Value	P-Value
Development time	1	0.012	23.713	3.770e-06 ***
Line	1	0.003	5.4216	0.0217167 *
Sex	1	0.006	12.549	0.0005826 ***
Age	1	0.009	19.718	2.145e-05 ***
Development time:Line	1	0.002	4.514	0.0358479 *
Development time:Sex	1	0.003	6.603	0.0115186 *
Line:Sex	1	0.003	6.352	0.0131631 *

3.4 Mating success

In the mating competition assays, I tested whether the mating success differs between the males with different Y chromosome haplotypes. To get an idea of what the data looked like, it was first checked how often each male line won the mating competitions by calculating the proportion of wins out of all trials. Large males won 20 out of 32 times (62.5%) and small males lost 20 out of 32 times. An exact binomial test was performed since it is also appropriate for smaller data sample sizes. The p-value was not significant (p= 0.11) even though the proportion of winners is 0.625 and thus higher among the males with a large haplotype. With a larger sample size, this effect could likely become significant.

It was also tested if the winners, in general, had a significantly larger body size than the losers. For instance, the 12 winners from line SL1b Y may have been larger than expected and this might not have been caused by their Y-lineage alone but instead due to environmental or genetic variation in autosomal or X chromosomal loci. From the performed paired t-test, the p-value was close to one (p= 0.95). That almost completely rejects the alternative hypothesis and accepts the null hypothesis that there is no difference between the groups. A larger body size does not result in a higher pre-mating success, neither does a small body size and there is no selective advantage. Figure 3 below displays the average body size of those who won and those who lost the mating competition assays. Beetles from both lines are amongst winners and losers. The winners seem to have a larger body size than the losers, but the difference is not statistically significant.

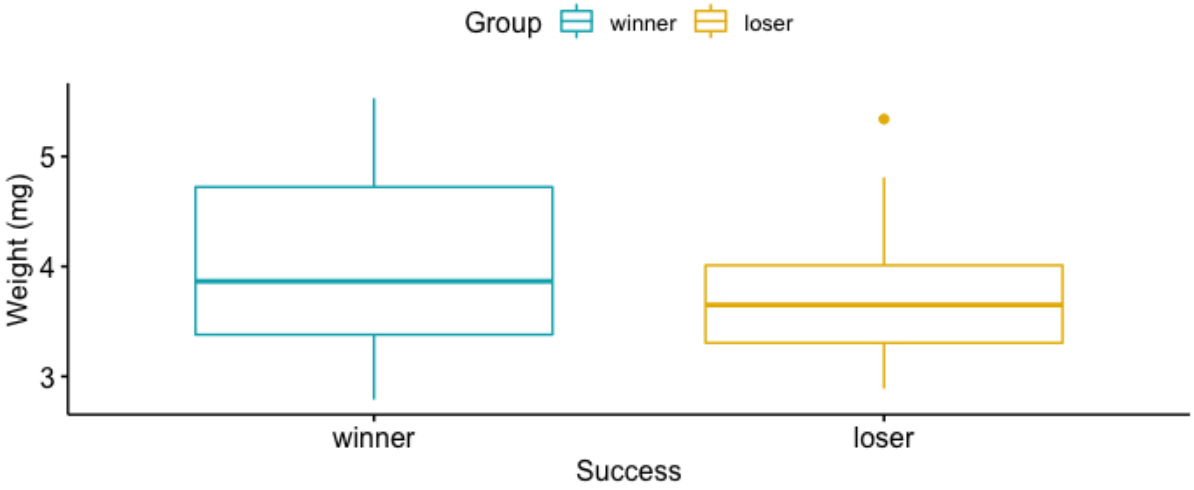

Figure 3: Boxplot showing the body weight of winners and losers in the mating competitions. *In the group of winners there are 20 individuals from line SL3b Y and 12 individuals from line SL1b Y. In the group of losers there are 12 individuals from line SL3b Y and 20 individuals from line SL1b Y. The winners seem to have a larger variation and the losers have one outlier with a large body weight.*

3.5 Ejaculate weight

The ejaculate weight of males from the two different lines does indeed differ. First, a linear regression model was conducted and then an ANCOVA with line and body weight as the explanatory variables. Line was shown as a significant variable as seen in Table 6 and body

weight was approaching the threshold for statistical significance. The interaction effect was not significant and that means that the body size has a similar relationship with ejaculate weight in both lines. It was also examined if body size became significant when line was not fit into the model and the result came out significant as seen in Table 6 as well. Body size affects the ejaculate weight indirectly since the size of the male is defined by the line and the ejaculate weight is proportionate to the body size of the male. This suggests that the Y chromosome affects the ejaculate weight directly and thus there is a difference in the ejaculate weight of S and L males, as illustrated in Figure 4.

Table 6: ANCOVA table of ejaculate weight. **Body weight only showed as a significant variable when line was not fit into the model. This table has therefore incorporated two tests and tables into one.*

	Df	**Sum Sq**	**Mean Sq**	**F-Value**	**P-Value**
Line	1	0.089	0.089	5.078	0.028 **
Body size *	1	0.137	0.137	8.078	0.006 **
Line:Body size	1	0.002	0.002	0.109	0.743

The S males have a larger variation in ejaculate weight and hence a larger standard deviation compared to L males which also have seemingly five outliers (Figure 4). However, a visual inspection of the models did not reveal issues of outliers or heteroscedasticity and therefore no data was removed.

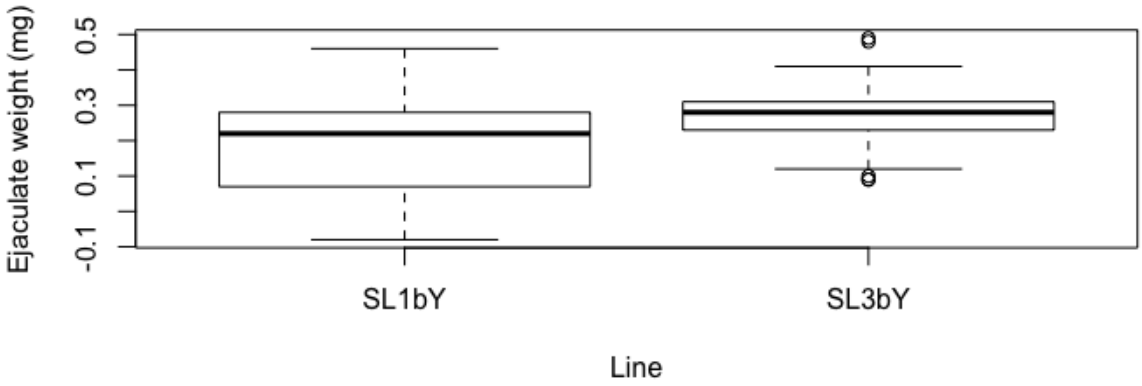

Figure 4: Ejaculate weight of males from the two Y lines. *The boxplot is illustrating their mean, standard variation and standard error. Large males (SL3b Y) have a heavier average ejaculate weight.*

The effect of body size on ejaculate weight is illustrated in Figure 5, with separate regression lines for each beetle line. This gives a clearer idea of how the beetle lines differentiate from one another. Figure 5 reveals how body weight has a positive effect on ejaculate weight. Due to the fact that line SL3b Y has heavier individuals on average, their ejaculate weight is also heavier on average (Figure 5c). Based on these results, line SL3b Y has a higher intercept

since the regression line starts at ejaculate weight 0.2 mg instead of at approx. 0.15 mg as it does for SL1b Y (Figure 5b). The two lines have a similar slope although the slope of line SL1b Y is slightly shaper since the regression line for beetle line SL1b Y goes from approx. 0.14 to 0.28 mg and the regression line for beetle line SL3b Y goes from approx. 0.2 to 0.325 mg. Given that some SL1b Y males were also large and hence have a larger ejaculate, the difference is subtle between the lines.

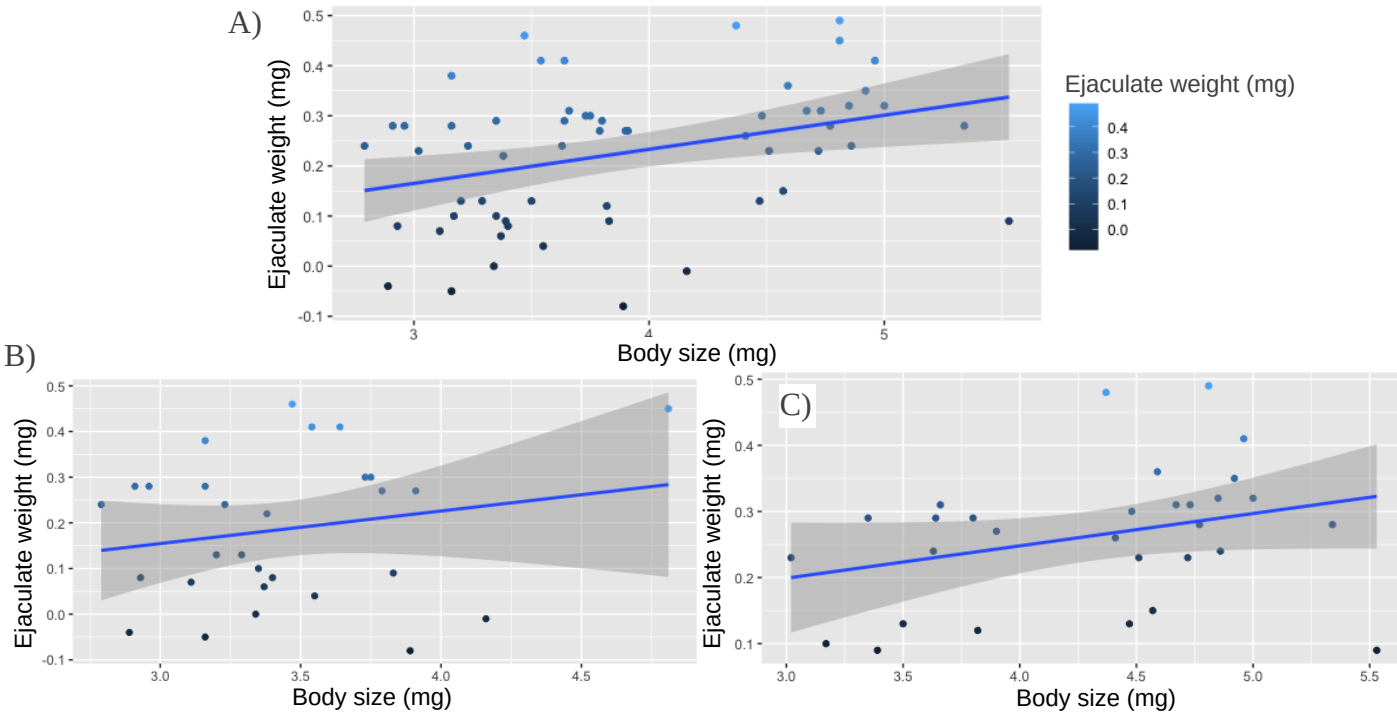

Figure 5: Illustration of scatterplots with body weight as a function of ejaculate weight. *A) Shows the ejaculate weight of males in general from both lines in the same regression line. The ejaculate weight increases on average along with a larger body size. B) Shows the ejaculate weight of small males (line SL1b Y). This slope seems slightly steeper, however, the two lines do not have significantly different slopes. C) Shows the ejaculate weight of large males (line SL3b Y) which has a higher intercept.*

3.6 Lifetime reproductive success

A zero-inflated Poisson regression model was used to test the lifetime reproductive success data, which had an excess of cases with zero offspring. Table 1 shows how line SL3b Y has a higher number of offspring and can give us an idea of what the data looks like. The zero-inflated Poisson regression model tests the Poisson model coefficients of the data separately from the zero-inflated model coefficients. The male line is significant in the first part of the test as seen in Table 7a. This means that the two male Y haplotypes differ in the number of offspring they produce. Moreover, the L males produce more offspring than the S males. However, the zero-inflated part of the test is not significant for the line effect, as seen in Table 7b. This means that the two male Y haplotypes do not differ in how likely they are to produce zero offspring and they are both just as likely to produce zero offspring. The female mating partner's line was also included into the model and there is no significant difference between

the two Y lines of interest, SL1b Y and SL3b Y, in female fecundity. However, females from the original genetic background, line 2:11, differ in fecundity which is likely due to the line being inbred.

Table 7a: Summary table on the zero-inflated Poisson regression coefficients of lifetime reproductive success. *This is the first part of the data including the Poisson model coefficients. The estimate shows the difference to line SL1b Y, in the case of the male line effect and the effect of the line of female mating partner.*

	Estimate	**Std. Error**	**Z-Value**	**P-Value**
Male SL3b Y	0.306	0.053	5.727	1.02e-08 ***
Female 2:11	0.233	0.063	3.695	0.00022 ***
Female SL3b Y	0.075	0.044	1.702	0.08880 .

Table 8b: Zero-inflated model coefficients. This is the second part of the data which showed no significance.

	Estimate	**Std. Error**	**Z-Value**	**P-Value**
Line SL3b Y	-0.762	0.631	-1.207	0.2274

A box plot was also created to illustrate the average number of offspring produced by each male Y haplotype (Figure 6). L males produce more offspring on average compared to the S males.

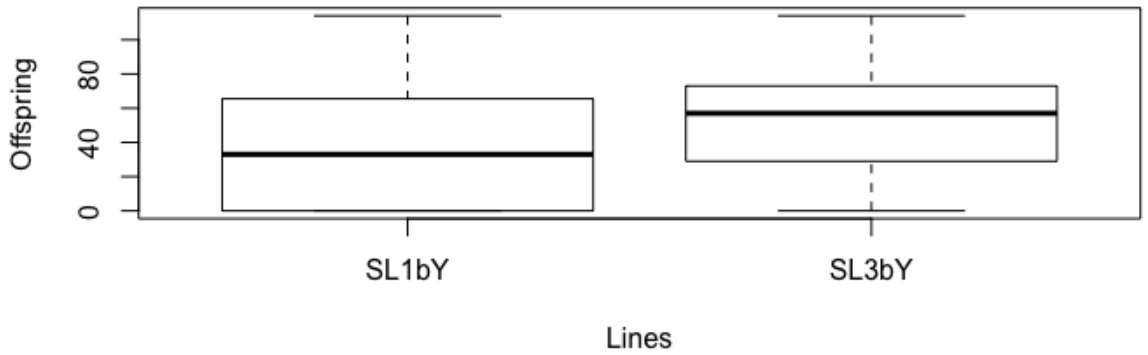

Figure 6: Illustration of the number of offspring per male Y haplotype. *It is visually shown how line SL3b Y has a higher mean and how line SL1b Y has a larger variation of the number of offspring.*

Figure 7 below helps to visually illustrate how much offspring the fathers produced depending on how long they had developed inside the bean themselves. This is to see if those L males that developed especially long (and hence emerged with a smaller relative size) were of lower condition, and unable to produce as many offspring as those with a shorter development time and hence larger size, compared to the S males whose body size increased with development time. Looking at Figure 7a, one can see how L males produced a similar number of offspring on average when they had developed for 33 days as when they had developed for 26 days. This shows that the L males who have a long development time are similarly fit as the S males and not sick or defect. The late emerged fathers produce approximately the same amount of offspring as the early emerged fathers. Figure 7 gives the impression that the L males which developed between 29 to 31 days did not produce many offspring. However, there were only three individuals who emerged on these days and therefore the result is the following. Additionally, these results have shown that L males are

able to produce more offspring. There were four L males that developed after 26 days and this group had a surprisingly low number of offspring because three out of these four produced zero offspring. Both mothers and fathers were one day old when they mated, they had large body sizes and the males had lost weight after mating which indicate on their ejaculate weight. These are all optimal conditions and qualities and cannot be the reason that zero offspring was produced. However, there is one interesting factor which is that two out of these four males lost in the mating competition assays and did also not produce any offspring. The third male lost in the mating competition but produced offspring and the fourth won the competition and did produce offspring. Perhaps some of these males were defect and did therefore lose the mating competitions and did not manage to produce offspring. However, all the L males that developed after 26.5 days did produce offspring and won in the mating competition assays. Hence, the reason zero offspring was produced from the L males that developed after 26 days, cannot be because all L males that develop earlier are defect, but simply because a fluctuating fraction can be.

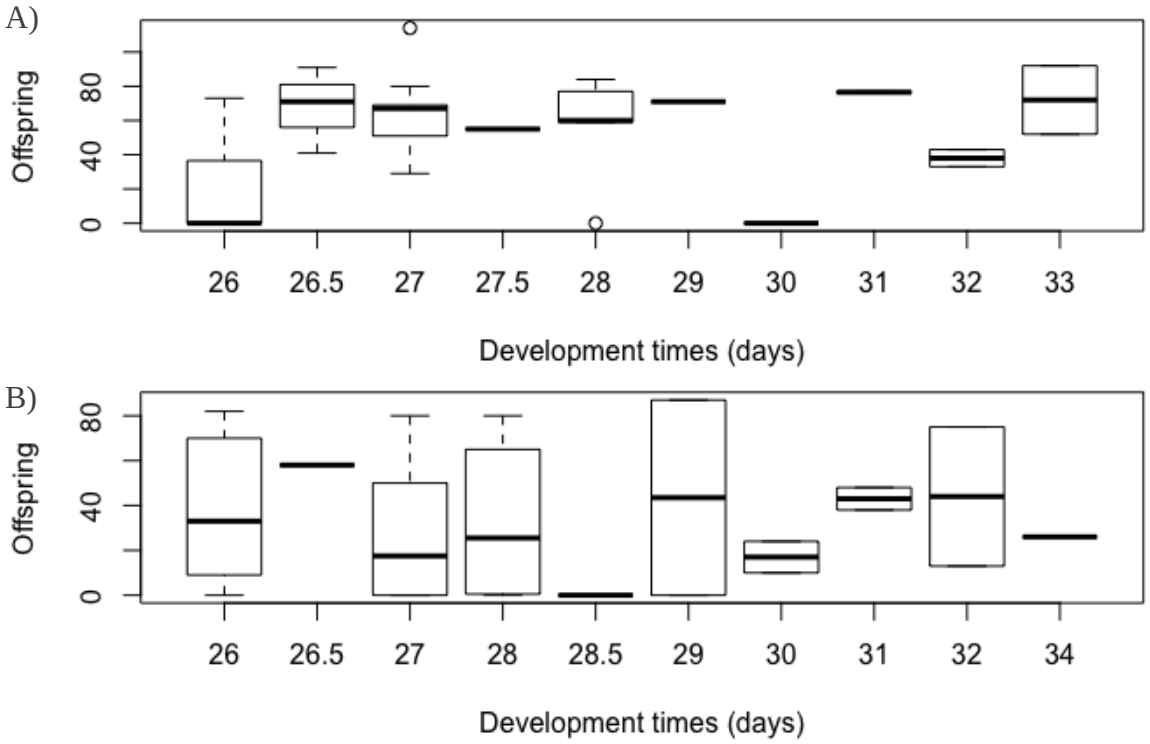

Figure 7: Illustrates the offspring produced by males with different development times. *A) Shows L males which mostly produced a large amount of offspring when they had developed for 26 or 33 days. B) Shows the S males which produced a large amount of offspring when they had developed for a short and medium amount of time, but not when they had developed for long.*

4. Discussion

The aim of this study was to test how the Y chromosome polymorphism, found between S and L males (Kaufmann *et al.* manuscript submitted), affects life-history traits, mating success, as well as reproductive success under non-competitive conditions. The question this study helps to answer is how the polymorphism in the Y chromosome can be maintained. The

hypothesis was that Y chromosome polymorphism could potentially be maintained under negative frequency-dependent selection, if each haplotype has a higher relative fitness when rare in the population. Although this study was not aimed to directly test how rarity of each Y line in a population affects their fitness, it can provide insights into what types of traits the males can show differences in, and subsequently differ in advantages and disadvantages of their respective Y haplotypes depending on the competitive context. A bit more is now known about how these two Y haplotypes affect different male fitness components. This helps to further understand how this polymorphism affects the male fitness and how it may be maintained in a population. The key findings include that the L males (line SL3b Y) have a higher lifetime reproductive success and ejaculate weight, suggesting that they may even have a higher overall fitness. However, the S and L males do not differ in mating success which means that L males do not have a higher pre-mating success and this finding is consistent with the study performed by Savalli & Fox (1999). The males from line SL3b Y does also show a higher growth rate. Although not tested in this study, a higher growth rate may require more larval resources. This could constrain optimal growth for L males when food is scarce, e.g. when population densities are high. It is therefore possible that under some conditions, the lower growth rate and hence smaller size of the S males is more beneficial.

A general pattern in *C. maculatus* is that the body size increases with longer development time in the bean seed (Berger *et al.* 2016). The prediction for the S and L males, as well as for females was therefore that both males would have a larger body size the longer their development time was. The results do not support this, since L males had a smaller body size when they had a longer development time. It was also hypothesised that S males would develop faster than L males because of their lighter body weight but the results do not support this. For a given development time, the L males emerge from the bean with a larger body size, and hence have a faster growth rate than the S males. However, with increasing development time, the size difference between the male types decreases as the growth rates become more similar. This is because the L males show a negative and the S males a positive relationship between body size and development time. For the mating success, it was hypothesised that L males would win more often at the mating competition assays and have a greater pre-mating success, if a larger body size benefit in a male-male competition. Although it was factual that the L males won the mating competition assays more often, the results do not support this hypothesis since neither male Y haplotype has a significantly greater pre-mating success. However, the results on the ejaculate weight and lifetime reproductive success were higher for L males, supporting the hypothesis that a larger body mass encoded by the L haplotype provides higher resources for reproductive investment.

An additional remark about the results on body size is that the interaction between sex and line presented in Table 2 was confirmed in this study, as expected based on the study done by Kaufmann *et al.* (manuscript submitted), which discovered how the Y chromosome affects body size in males. I also discovered that, although overall body size increased with a longer development for the S males, there were some that developed longer but had a smaller body size because they were most likely sick. These individuals also had trouble mating, even after repeated tries. Their development time must be this extensively long since it is harder for

these individuals to survive, grow, and mature enough to emerge from the bean. However, the body size can also be affected by environmental reasons and not only by the beetle line. It can be affected by parental effects, e.g. if the female is young when she is mating, she will have more energy to invest on the egg (Fox 1993b). If she is older, there will be less energy invested on the eggs and in turn to the larvae, which can cause the individual to be smaller as an adult. The bean that the egg is laid on does also have an effect on the beetle's body size. A healthier bean means that there is more nutrition for the larvae to feed on, which does also result in a larger body size (Małek *et al.* 2019). It is known that the female chooses a bean that seems fit (Paukku & Kotiaho 2008) but beans can still vary in their health.

The development time of S males was relatively longer per body weight than it was for L males (section 3.2). This was surprising since previous work (Berger *et al.* 2016) has shown how body weight should increase with development time, leading to the expectation that the L males should have a considerably longer development time to achieve their greater size. Instead, they show much faster growth rate. For future studies, it is worth having more lines that carry the same L and S haplotypes but on different genetic backgrounds to be able to compare them and have a broader idea of whether the effects can be detected independently from genetic variation in the autosomes and the X chromosome, as well as to separate environmental effects better which can obscure the Y line effects. There are other limitations in the study regarding how similar the mating scenarios are to the ones occurring in nature, as it is for most laboratory researches. In nature, there can occur sneaky mating where the beetles are in crowded places and can go from one female to the other very fast and have higher activity (Nakayama & Miyatake 2010a, b). In this case, the small males would benefit as mentioned. In a laboratory environment, this activity cannot be easily tested since it would be challenging to monitor several beetles simultaneously. Hence what we see here might not explain the behaviours of *C. maculatus* in nature. In addition, it is noteworthy that the females from both lines develop faster than the males from each line. The difference is rather small but enough for it to be picked up as a significant value. This is unexpected because females have a larger size and are hence expected to develop for longer.

The females have the highest average growth rate (Table 1), thereafter comes the L males and last comes the S males with the slowest growth rate. It seems that a larger body size results in a higher growth rate, perhaps to assure that bigger individuals do not to take extensively long to develop and can emerge around the same time as the lighter individuals for the possibility of mating. The females that have the fastest growth rate, do in fact also take shortest to develop (Table 1 and section 3.2). The L males with the intermediate growth rate do also emerge after the females and before the S males. Lastly, the S males which have the slowest growth rate, have the longest development time. I see this as a pattern, and this could be the explanation to why S males took longer to develop compared to L males, despite the smaller body size. Additionally, the development time in this study can have been affected by proceedings acted upon the previous generations of the beetle lines. The development time in these lines has in a way become selected on since the beetles chosen for the previous experiments had emerged between 23 and 25 days. The genes from these beetles are therefore the ones that were passed on to the next generations, thus, as a result, the development time

might have become analogous in the two lines which could explain the observations in Table 1 and in section 3.2.

Regarding the results about the pre-mating success, in most cases during the mating competitions, line SL3b Y had heavier males. However, there were five cases where the males from line SL1b Y were heavier and larger than the males from SL3b Y. These cases where the males from line SL1b Y were larger did however occur at the very end of the mating competition study. Additionally, those males were one of the last individuals within their line to emerge from their bean. Their large body size does explain the extensive time it took for them to develop inside the bean, as it is also presented in Figure 1. Amongst these five cases where the males from SL1b Y were larger, three of them failed to mate during the mating competitions. Since the larger body size did not guarantee a success in mating, it gives further evidence that the results from the mating assays presented in 3.4 are accurate. Sometimes during the mating assays, the females were not interested and would shake off both the L and the S males. There was therefore no male-male competition and the larger body size did not come as an advantage. The S males did many times show to be more patient than the L males and could sometimes mate with the female later after she came around. A larger body size is mainly more beneficial when there is a male-male competition and they try to push each other away. However, it is not always a competition when it comes to mating. There were also cases where the males were more interested in each other than in the female. Furthermore, when one male was not interested in mating and the other one was, there would be no need for a competition between the males to be able to mate. One observation I made during the mating assays was that old individuals were not interested in mating, even though they were virgins. Individuals that had emerged on the same day or a day before were more interested.

Regarding the lifetime reproductive success results in section 3.6, if large males who develop earlier have more offspring than the large males that develop longer, then the former is more fit than the latter (Figure 7). I would say that the late emerged L males are still prominently fit since they are able to invest such energy into their ejaculate and produce that much offspring. Moreover, the lifetime fitness data under competitive conditions and with access to several females (i.e. with multiple males and females in an assay), is not obtained, which would have provided more information about the fitness of the L male haplotype. If large males would have had a higher mating success along with more offspring, then this would have suggested that under these scenarios, males would benefit from being bigger. However, large males do not have a higher mating success rate and therefore they do not benefit from being bigger in all scenarios. This is possibly the reason why different haplotypes exist in nature, because the male body size affects fitness differently depending on the context. A large male body size is beneficial in sperm competitions and could be of big interest in populations with a shortage of females or with a high rate of female polyandry. A small male body size is beneficial since these individuals are expected to be more active and can thus potentially mate with more females (Nakayama & Miyatake 2010a, b). This body size would be beneficial in most beetle populations where the sex ratio is more even, where population densities are high and where larval food resources can be scarce. When the environment changes, it can be helpful to have

different chromosomal haplotypes to aid adaptation. Especially if the different Y haplotypes affect selection on the autosomal and the X-linked genetic variation, through epistasis (Kaufmann *et al.* manuscript submitted), they can participate in maintenance of variation in the loci interaction with the Y chromosome.

Furthermore, it is worth investing more time studying a variety of male Y haplotypes to fully be able to understand how the polymorphism in the Y chromosome is maintained. More knowledge can be gained from these Y haplotypes by studying them in a wider range of contexts. More lines would also give a fuller and clearer picture of how the Y chromosome acts on traits subsequently male fitness, helping to answer how genetic variation can be maintained by selection in the face of degenerative forces acting on a chromosome in the absence of recombination (Charlesworth *et al.* 2009).

5. Conclusions

The results from this study indicate that L males have a higher growth rate than small males. The growth rate is affected by the Y chromosome which can be concluded because no such growth rate difference was detected in females. The body size difference that is observed in the adult males, but not females, of the two Y lines is due to the growth rate and the development time differences. The interaction between development time and sex, as well as the interaction between line and sex, does also affect the body size of the beetle. With every day that the adult beetle lives, the less it will also weigh, revealing an age effect. Lastly, there is a difference in ejaculate weight between the males from the two Y lines. The ejaculate weight is directly affected by the Y chromosome which is indirectly affecting the body weight and in turn the ejaculate weight. As a net effect of these, the Y chromosome also influences male fertility and hence the reproductive success from a single mating.

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