Of maze and men: Effects of structured laboratory environments on female longevity in the seed beetle *Callosobruchus maculatus*

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Acknowledgments

Abstract

The seed beetle *Callosobruchus maculatus* is an established model organism used to study resource competition and reproductive investment. The beetles have short generation time, and populations are easy to maintain in the lab. The environment in which the lab populations are kept may however deviate from natural conditions, where the varying crops of beans and vegetation or vast storage rooms are swapped with a plain petri dish. Being inspired by previous studies on fruit fly *Drosophila melanogaster,* this study looked at how accommodating a more structured environment would affect mating interactions, the reasoning being that structures would allow for females to hide from prospecting males and avoid unwanted attention. From previous studies we know that a skewed sex ratio in favour of males leads to decreased female longevity, as copulation physically damages females. The experiment was done by preparing two different environments, one open, which was a plain lidded petri dish and is what the beetles are normally subjected to, and one structured, containing a 3-D printed maze within a petri dish, and having the sex-ratio be 16:2 male:female in each. The results did not show any difference in female longevity and overall behaviour of the beetles, thus showing that a structured environment in a laboratory setting does not impact female longevity nor beetle behaviour.

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Introduction

In 1976, Scottish hard-rock band Nazareth released their hit song "Love Hurts", with the famous opening lines:

"Love hurts, love scars
Love wounds and marks"

Though meant as a metaphor, the lyrics come hauntingly close to the real proverbial love life of many insect species. An example is the fruit fly, *Drosophila melanogaster*, where the male seminal fluid is toxic to the female, hence reducing her life expectancy after continuing copulation, basically poisoning her (Lung et al., 2002). In dung fly, *Sepsis cynipsea,* the barbed male genitalia causes wounds and harm to the female's reproductive tract physically harming the female (Blanckenhorn et al., 2002; Edvardsson & Tregenza, 2005). Sexual conflict is widespread (McNamara et al., 2020), and sexual antagonism is ubiquitous throughout the animal kingdom (Adler, 2010). A plethora of diverse weapons and tactics have been developed in the struggle between the sexes. An example is the water strider (*Gerridae sp.)* who forces himself on females by pouncing on- and then grasping on to her, thereby securing himself copulation (Arnqvist & Arnqvist, 1989; Davies et al., 1981 p. 210). Another example are the convoluted genital organs of ducks, with both the penis and vagina made in a corkscrew fashion, the vagina to make unwanted intrusion harder, the penis to counter the shape of the vagina (Brennan et al., 2007; Davies et al., 1981, p. 219)

The male seed beetle, *Callosobruchus maculatus*, acts much like the dung fly, physically hurting the female with each copulation, where the male punctures the walls of the female´s reproductive tract with its spined genitalia (Arnqvist et al., 2005; Gay et al., 2011). Studies on the beetles behaviour seem to indicate that the harm the male causes the female is not in itself an advantage to the male, as the spined penis of the male may serve more as an anchor point to secure prolonged copulation, the harm being a side-effect (McNamara et al., 2020). Beneficial to the males or not, the damage the females receive during mating leads to a decrease in their longevity. Females are not totally defenceless, as they can kick off males who have outstayed their welcome (Edvardsson & Tregenza, 2005).

Having been used as a subject of research since at least the 1970's (Janzen et al., 1977; Utida, 1972), *C. maculatus* has become a very popular model organism (Beck & Blumer, 2006). There are many reasons for this; a fast generation time, no food requirement, and though they are capable of flight, beetles seldom use this ability. All of these traits make the beetles easy to handle and keep (Beck & Blumer, 2006).

The lab environment in which the beetles are kept when studied, have little resemblance to environments in which they are found in the wild, or in storages of legumes. Blumer et al (2006) argue that storage facilities and lab environments are similar, but a varied 3-D environment filled with hiding spots and topological variations as in storage facilities are not found in small petri-dishes in the laboratory. In agricultural fields, the differences are even bigger, as beans are spaced further apart, there are fluctuating temperatures and light conditions, and beetles are exposed to predators at all stages of their life (Beck & Blumer, 2011). These differences may also impact the rate at which encounters between mates occur within a population.

Vyalkova (2019) observed that different sex ratios of seed beetles in a dish affected longevity of the females, the most long-lived females were found in the 1:1 sex ration group, while those who lived shortest had a male skewed sex ratio of 4:1 (Vyalkova, 2019). These results, along with Hartåker (2019) and Tveitnes (2019) confirmed the negative impact of male presence on female adult life expectancy in the laboratory.

The fruit fly, much like the seed beetle, is a model organism, and is thus kept with a minimum of effort and expense during normal laboratory conditions (Tolwinski, 2017). In this species, a complex spatial environment has been shown to reduce male-to-female harm (Malek & Long, 2019). In their 2018 experiment, fruit flies were either placed in vials fitted with acetone strip in a zig-zag pattern or in open vials. The strip provided a physical hindrance to the males seeking females, thereby reducing mating induced harm and increasing female longevity (Malek & Long, 2019). The point to be made is that, even though both beetles and fruit flies seem to be thriving under the recommended conditions, this does not necessarily give

genuine insight into how a population of seed beetles behaves in the wild (Hoffmann & Ross, 2018).

The aim of this study was to investigate whether the traditional way in which seed beetles are kept in the laboratory influences cost of multiple mating and male harassments (Hjartåker, 2019; Vyalkova, 2019). In line with Malek & Long (2019), two environments were provided for the beetles: one structured, containing a maze in a petri dish, and one open unstructured environment. The open environment represents normal rearing conditions in the laboratory. I used a highly skewed male to female sex ratio since the difference in life expectancy is expected to be more pronounced here. Two distinct hypotheses were formulated and tested in this study:

1. Adult life expectancy is lower in female beetles in open compared to structured environments.
2. Females experience more encounters and more mating attempts with male beetles when in an open compared to a structured environment.

Materials and methods

Study organism

The seed beetle is a common model organism and has been utilised in a variety of ecological studies. It is also a serious pest, feasting on cowpeas, lentils and green gram, among others. The beetle originated in continental Africa, and has since spread to much of the tropic- and subtropic world, and are currently found on all continents except Antarctica (CABI, 2019; Gevina et al., 2016).

After mating, the females lay their grey, inconspicuous eggs on legumes, the larva hatches within 5-6 days, and starts burrowing into the seed. As it feeds, the egg is filled with faecal matter, turning it white and highly conspicuous against the skin of most bean types. After a varying amount of time, the minimum being three weeks under optimal conditions, the adult seed beetle emerges from the bean, and is ready to mate after 24-36 hours (CABI, 2019).

The adult seed beetle lives one average 10-14 days after they emerge from the bean (Beck & Blumer, 2011; Moreno et al., 2000). During this time, the females are exposed to mating attempts by males, where each copulation can be harmful to the females (Edvardsson & Tregenza, 2005). Females may also benefit from multiple matings as spermatophores transferred during copulation contain nutrients and water that may increase their lifespan (den Hollander & Gwynne, 2009; Fox, 1993), making it a nuptial gift (Gwynne, 2008).

The beetles are easy to maintain, and they only require beans of a compatible variety and suitable temperature to reproduce. Adult beetles do not require sustenance of any kind, supplying an everlasting colony of seed beetles is only a question of allocating them fresh beans as the generations come and go (Beck & Blumer, 2011).

Rearing

The beetles used in this experiment are descended from seed beetles imported from Carolina Biological Supply in USA in 2016, and the strain has since been raised on *Vigna* legumes (Vyalkova, 2019). The particular strain used in this study has exclusively been raised on mung beans since they arrived (Tveitnes, 2019).

Rearing operations, from setting up new cultures to keeping the beetles and disposal of undesired populations, follow "A Handbook on Bean Beetles, *Challosobruchus* maculatus" (Beck & Blumer, 2011). The beetles used in these studies are all kept in lidded petri dishes, variables being types of beans, amount of beans, sex ratio, number of beetles, and the size of the dishes. A start culture of beetles was prepared from UiB's supply of beetles in 15 cm diameter petri dishes. Starting with a population of around 200 beetles, these were placed in batches of 50 per dish, with the dish being filled with mung beans. The beetles were then given time to mate and oviposit. When the parent generation had died out, beans with eggs were carefully transferred to "incubation chambers", a sealable plastic container with 24 individual "cells", one for each bean. As the new generation started to emerge from their host bean, their sex was identified, as well as their date of emergence. The adult beetles were kept in separate containers to ensure they were all virgins.

Experiments

I exposed the beetles to two "environments": The "Open" environment was a traditional 15 cm diameter petri dish with a single layer of mung beans (200/dish). The "Structured" environment was an identical petri dish as used in the open environment, fitted with a 3-D printed maze and 200 mung beans (Figure 1). In both environments, the beans were counted meticulously to be exactly 200. Two females and 16 males were added to each dish. Only females that emerged from beans within 24 hours before the experiment were used to guarantee similar age. I performed 15 trials for each environment (open and structured). All beetles, both the starting population as well as the test population, were kept at 28°C under constant light in a specialised cabinet, only taken out briefly when needed for counting and observations.

Structured environments – the maze: 3-D print designs were made with the program TinkerCad®, a web-based free-to-use service which allows you to make various 3-D structures for printing (Backman, 2020). I tested multiple alternative labyrinths, before settling on the one used in the experiment (Figure 1). Although it is not a natural habitat, the structure contains nooks and crannies that may be used as hiding spots for females. The mazes were made in plastic with a FlashForge® 3-D printer. A limiting factor was the opaque plastic, which

prevented me from making overhangs or even multiple layers of the labyrinth, as the experimenter needed to observe the beetles.
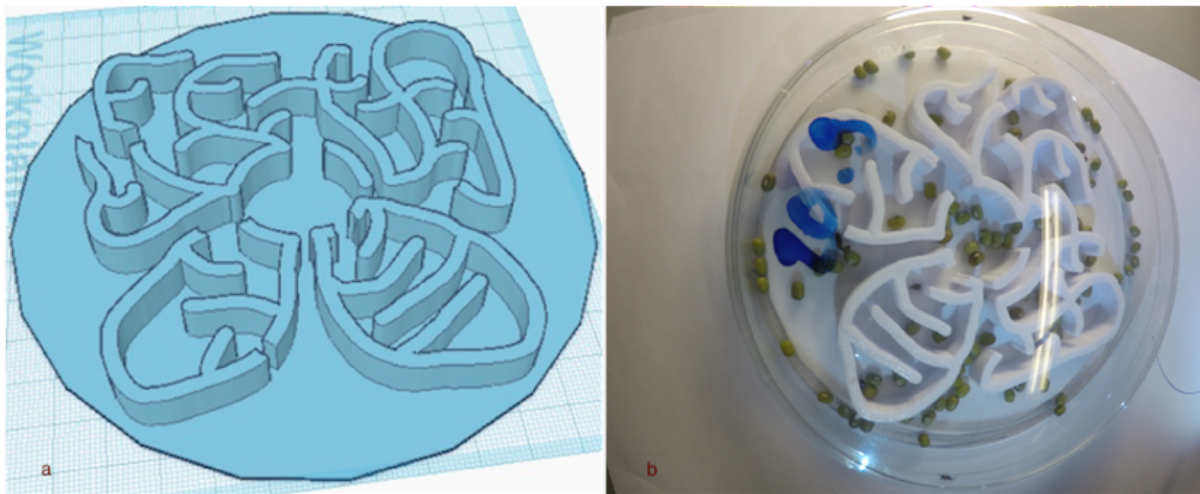

Figure 1: Maze structure a) TinkerCad® model of the maze with four different regions, all protruding from a central hub. The underlying plate which binds it all together has a diameter of 15 cm, while the walls of the maze have a height of 1 cm. When in use the plate fills the bottom of a petri dish. Maze structure b) Photo of the maze in use in a petri dish, with mung beans and beetles.

Longevity and behavioural observations: The number of surviving females were recorded in all dishes each day, until all females had died. The beetles sometimes stand perfectly still, even when alive. In these cases, the beetle in question was touched gently with a brush, thus triggering movement if alive. In addition, interactions between females and males were observed over a ten-minute period per day for the duration of the females' lifespan in 5 dishes of each environment. I recorded "close contact", which was defined as a male and female beetle touching each other, typically antennae contact or grazing past each other with no pursuit by either party. I also recorded "attempted mating" categorized as a male climbing on to the back of a female trying to mate, a mating was carried out, or a male interrupted or interfered with an already ongoing mating.

Statistical Analysis

Statistical analysis was carried out in R Version 4.0.5 (R Core Team, 2021). A Kaplan-Meier survival plot and models was made using the "survival" package (Terneau & Grambsc, 2021)

and "survminer" package (Kassambara et al., 2021) and the appropriate predictor model "extreme" was found through a one-way ANOVA test of a multitude of different predictors, checking for the lowest unexplained variability (-2*LL). The behavioural tests were made with the package "nlme"(Pinheiro et al., 2021), which produced two two-way repeated measures ANOVA tests, one for contact ratio and one for mating attempt ratio. Both models included a random effect variable; the individual dishes. In the mating ratio model, an extra consideration was made to accommodate surviving females in each dish. From these models two behavioural plots were made.

Results

Female longevity

Female longevity was recorded each by identifying surviving females in each dish. Female longevity did not differ significantly between females in structured environment with a predicted age of death at 7.3 days, compared to 7.2 days in an open environment (Chisq = 0.71, df = 1, p = 0.4, see Figure 2.
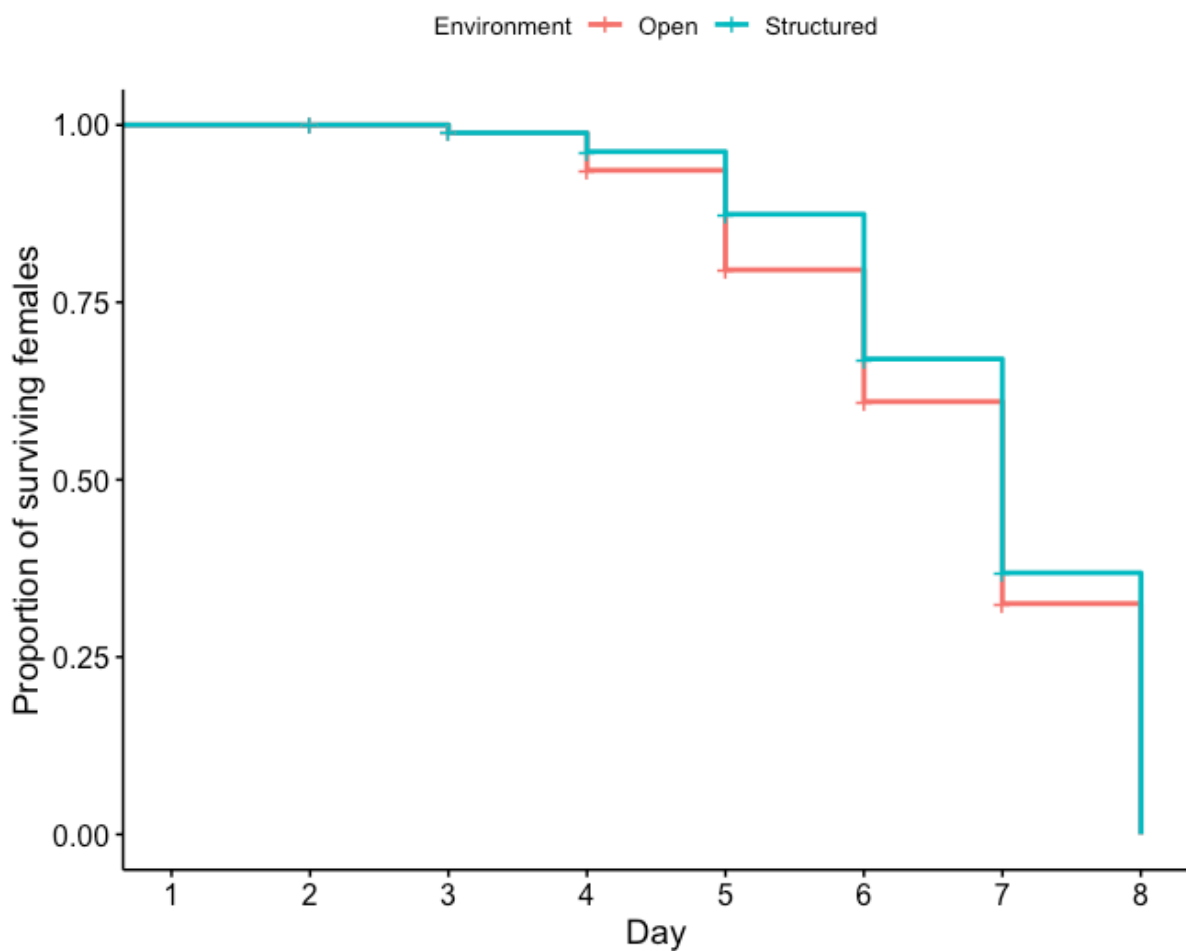

Figure 2: Adult female survival as a function of days after emergence from the bean. The red line represents female in the open environment, whereas the blue line represents the group in structured mazes. Mortality rate/day is more or less constant over time. The lifespans of 60 individual females make up the data for this graph.

Contact

The frequency of close contacts between females and males did not differ significantly between those placed in an open environment and those placed in the structured environment (Linear Mixed Effects Model, F= 0.13, df = 10, N = 59, p = 0.73.) see Figure 3. A significant decline in number of contacts was found in the open environment as the beetles grew older (p = 0.007), while in the structured environment I found no difference in contact rate over time (p =0.24).

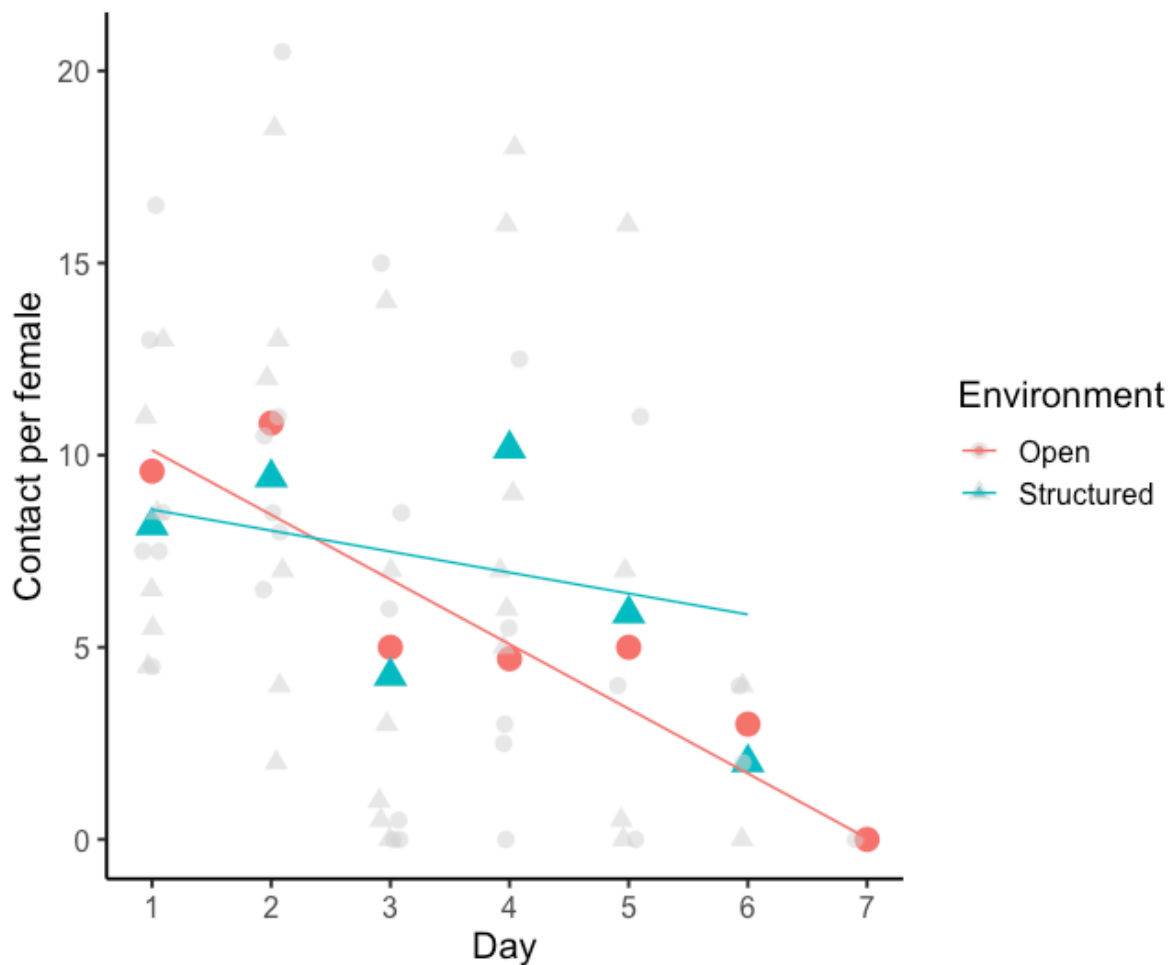Figure 3: Number of contacts per female between males and females as a function of day after emergence from the bean. Each dish, with both open- and structured environments, was observed for ten minutes per day. The coloured dots are the average for each day and group with individual dish data in grey. The coloured line shows the estimated slope for the open (red) and structured (green) environment.

Matings

The frequency of male mating attempts did not differ significantly between open environments and structured environments (Linear mixed effects model, F = 3.1, N = 59, df = 10, p = 0.11), see Figure 4.
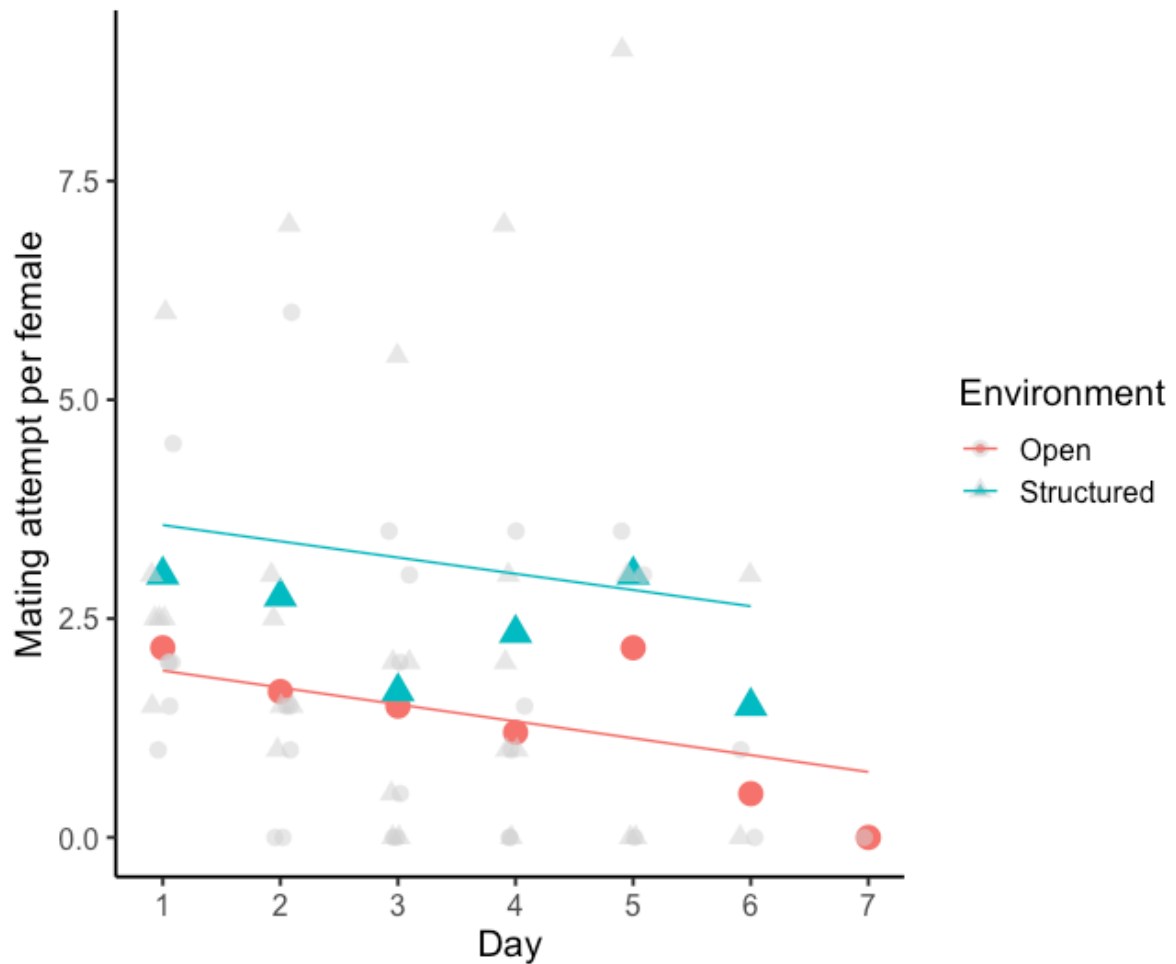

Figure 4: Male mating attempt per female as a function of days since emerging from the bean. Each dish was observed for 10 minutes per day. The coloured dots are the averages for each day and individual dish data are given in grey. The coloured line shows the average estimated slope for the open (red) and structured (green) environment.

Discussion

Females in structured environments did not live longer than females in open environments. There was no significant difference in number of mating attempts or contact frequency between males and females in the two environments. This suggests that the maze structure did not alter female-male contact and male mating attempts and hance had no effect on longevity of females. There may, however, be more to this than this study managed to unfold.

Malek & Long (2019) elaborate on the sexually antagonistic relationship between male and female fruit flies, and how "complex spatial structures" in some cases mitigate harm to females done by males (Malek & Long, 2019). The study documented an increase in female longevity in more structured habitats (acetone strips in vials). This was not found in the similarly structured environment in seed beetles in this experiment, and there may be several reasons why: No female beetle in this study survived past day 7, while the normal survival length of *C. maculatus* is somewhere between 10-14 days (Beck & Blumer, 2011), with females usually outliving males by a few days (Fox et al., 2004). This indicates that the experiment provided a highly harmful environment for females, regardless of being open or structured. Earlier studies have never tested sex ratios higher than 8:2 male:female (Hjartåker, 2019; Tveitnes, 2019; Vyalkova, 2019), I used a sex ratio of 16:2 in all dishes. This was done in order to ensure that we had an impact on life expectancy in the open environment, as we already knew that more males lead to decreased female longevity in this environment (Hjartåker, 2019; Tveitnes, 2019; Vyalkova, 2019). The extreme sex ratio may have led to overcrowding; what would otherwise have served as hiding spots, became places where females were trapped in instead. In retrospect, it might have been better to cap the sex ratio at 8:2.

The plastic mazes used in the experiment were course and rough, making them easy to climb by the beetles. Had the walls of the maze been slick and smooth, the males would probably not have been able to follow the females as thoroughly as they did. Also, because the beetles needed to be observed each day, the mazes needed to be made in a way that allowed for this. This limited the design and excluded features such as overhangs or multiple floors which

somewhat could have mitigated the beetles climbing and would have increased the amount of hiding spaces available to females.

The transfer of spermatophores in *C. maculatus* could be considered a nuptial gift, as it is a seminal gift from a male to his mate, containing nutrients (Gwynne, 2008). This is also costly for the male, which in some cases loses nearly 8 % of his body weight to copulation (Arnqvist et al., 2005). During the course of the experiment, the beetles did not have any available water, which is normal procedure when experimenting on *C. maculatus* (Beck & Blumer, 2011). However, this may have lead females to allow more matings than they otherwise would (Edvardsson, 2007; Vyalkova, 2019). Males provide females with sustenance with each copulation (Arnqvist et al., 2005), and if a male invests heavily in providing water to his mate, he may extend the time until she chooses to mate again, thereby increasing his own fitness (Edvardsson, 2007). For females in a dry environment, multiple matings are a double-edged sword: on one hand they receive water as well as increased fitness for their young by increasing genetic variety (Arnqvist & Nilsson, 2000; den Hollander & Gwynne, 2009; Fox, 1993; Fricke & Arnqvist, 2007; Petrie et al., 1998), but on the other hand, they are physically wounded from copulations (Edvardsson & Tregenza, 2005). In an environment where there is water available, the females still have the added benefit of increased offspring fitness, but the benefit of water reception is lost (Edvardsson, 2007). This means that females have reasons both to avoid and to seek out extra copulations, but in a parched environment, such as the one in this experiment, the incentives are stronger than when in a water-accommodating environment.

The strain of *C. maculatus* used in this study has been raised solely on mung beans since they arrived in 2016 (Tveitnes, 2019). This may have impacted their evolution during their stay, as a study on fruit flies proved that males raised on different food sources eventually gave rise to novel traits in their offspring (Arbuthnott et al., 2014). In another study on seed beetles, males raised under sexually competitive conditions gave rise to increased male-to-female harm, but female resistance to harm did not co-develop as a result of this (McNamara et al., 2020). This would imply that "domesticated" beetles do not necessarily behave the way their wild counterparts would in similar circumstances, as "domesticated" beetles have evolved separately from the wild for a long time. Because they arrived in Norway five years ago, the

beetles have been in captivity for at least 25-35 generations, given a generation time of six generations per year (Moreno et al., 2000). This has given them ample time to adapt to their new environment, as there are bound to be differences in selection pressures between wild and "domesticated" populations of beetles. Different organisms, especially insects, have been proven to evolve rapidly in laboratory settings, adapting quickly to their new environments (Fricke & Arnqvist, 2007; Hoffmann & Ross, 2018). A recent study has shown that sexual conflict in a population is prone to change along with the environment (Plesnar-Bielak & Łukasiewicz, 2021), in addition different strains of *C. maculatus* from different regions have been shown to inhabit a varied set of behavioural and selective characteristics (Mitchell, 1990). This blends well with the fact that different studies of the beetles sometimes produce conflicting results (Arnqvist et al., 2005).

This study does not indicate a difference in seed beetle longevity and behaviour between a structured environment and an open environment, but I think there is more to be discovered here. I therefore suggest repeating the experiment with a lower sex ratio, preferably with a fresh strain of seed beetles, newly harvested from wild populations and with available water for the subjects. In addition, a highly transparent material for the mazes would be beneficial, thus opening up for more complex structures to be built while still allowing for observations to be made.

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Appendix

A copy of the R-script used to make survival- and behavioural models and run tests:

#Model: Survival

```
fit1.surv <- survreg(Surv(Day,Status)~Bowl, dist="weibull", data=Deaths.df)
fit2.surv <- survreg(Surv(Day,Status)~Bowl, dist="extreme", data=Deaths.df)
fit3.surv <- survreg(Surv(Day,Status)~Bowl, dist="exponential", data=Deaths.df)
fit4.surv <- survreg(Surv(Day,Status)~Bowl, dist="gaussian", data=Deaths.df)
fit5.surv <- survreg(Surv(Day,Status)~Bowl, dist="logistic", data=Deaths.df)
fit6.surv <- survreg(Surv(Day,Status)~Bowl, dist="loglogistic", data=Deaths.df)
fit7.surv <- survreg(Surv(Day,Status)~Bowl, dist="lognormal", data=Deaths.df)


anova(fit1.surv, fit2.surv, fit3.surv, fit4.surv,fit5.surv,fit6.surv, fit7.surv)
```

#fit2.surv has the lowest unexplained variability, and is therefore the best response variable distribution.

```
fit2.surv <- survreg(Surv(Day, Status)~+1,
            dist="extreme",data=Deaths.df)
fit3.surv <- survreg(Surv(Day,Status)~Bowl,
            dist="extreme", data=Deaths.df)


anova(fit2.surv, fit3.surv, test = "Chi")
summary(fit3.surv)
anova(fit3.surv)


predict(fit3.surv, list(Bowl="Maze"), type="response")
predict(fit3.surv, list(Bowl="Control", type="response"))
```

```
#Model: Contact ratio

fit1.lme <- lme(Contact_ratio~Observation*Group,
        random=~+1|Bowl,
         data=Matetacts.df)


fit3.lme <- lme(Contact_ratio~Observation*Group,
        random=~+1|Bowl/Surviving_females,
        data=Matetacts.df)


anova(fit1.lme, fit3.lme)
# There is no significant difference between the two models, we therefore choose the
simplest one.
anova(fit1.lme)
summary(fit1.lme)


#Model: Mating Ratio


fit2.lme <- lme(Mating_ratio~Observation*Group,
        random=~+1|Bowl,
        data=Matetacts.df)
fit4.lme <- lme(Mating_ratio~Observation*Group,
        random=~+1|Bowl/Surviving_females,
        data=Matetacts.df)


#fit4.lme has lower AIC and is therefore the best model.


anova(fit2.lme, fit4.lme)


anova(fit4.lme)


summary(fit4.lme)
```

R being an open-source program means that many different people have made the varying packages that were utilised in the making of the plots and analysis in this study. These are "tidyverse" (Wickham et al., 2019), "scales" (Wickham & Seidel, 2020), "ggplot2"(Wickham, 2016), "naniar" (Tierney et al., 2020), and "plyr" (Wickham, 2011).