Density Effects on Reproduction in *Callosobruchus maculatus*

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Abstract. A reduction in population viability due to excessively high densities is a fundamental principle of population dynamics. Resource overuse is a constant factor in this regard, but density-induced stress manifests itself uniquely among different species due to distinct life-history strategies. Bruchid beetles present one such distinct life-history, as they must lay their eggs on individual beans, many of which contain only enough resources for a single offspring. Thus, it is necessary for female Bruchid beetles to distribute their eggs evenly in order to maximize fitness. In this study, we used Messina & Mitchell's U-score to investigate the effects of increasing density on the fecundity and egg dispersion of *Callosobruchus maculatus*, a representative member of the Bruchidae family. Treatment conditions of 1, 2, 5, and 10 females were each placed with 40 mung beans into a plastic petri dish, and were allowed to distribute eggs until death. We found that female beetles laid significantly less eggs on beans, and distributed their eggs less evenly at higher densities ($p < 0.01$, df = 15). We hypothesize that the observed behavior exhibits a protective mechanism against bean overloading, thus promoting greater offspring survival.

INTRODUCTION

It has been observed in a broad range of biological systems that high population density negatively impacts population viability, both through density-induced stress and resource scarcity (Sibly and Hone, 2002; Peters and Barbosa, 1977). The more commonly discussed of these mechanisms is intraspecific competition for limited resources. Smallegange and Tregenza (2008) concluded that the presence of competition dictates population dynamics because of individual differences in evolved behaviour when exploiting said resources. These individual differences involve factors such as relatedness, age, density and dispersal distances. It is through the competition for limiting resources that the natural variance within organisms is magnified and specific fitness is selected. Although less commonly discussed, the impact of competition on population growth is further accentuated by density-induced stress because excessive densities introduce maladaptive behaviors. For example, white-footed mice are more likely to engage in fights in response to higher densities (Tarsi and Tuff, 2012). The Bruchid family of beetles allows for a unique case study when considering these density effects, as their larvae must develop on individual seeds. This presents a novel system in which the mechanisms of competition and density stress may be explored. The ideal Bruchid for laboratory investigation is *Callosobruchus maculatus* due to their short generation time, low maintenance and ease of condition manipulation.

C. maculatus are known agricultural pests that feed on the internal endosperm and embryo of various legumes (Beck and Blumer, 2014). They can be found around the world but are known to be associated with stored grain infestations in Africa and Asia (Messina, 1993). The species has a one to two week lifespan which involves mating and the distribution of eggs on beans by females. *C. maculatus* requires no food or water supplementation as an adult and instead depends on the oviposition and environmental conditions that its mother chooses (Beck and Blumer, 2014). Since *C. maculatus* larvae cannot move from their chosen bean, the bean acts as a closed system, where resources are highly limited. This means that if multiple eggs are confined to a single bean, larvae must compete

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to ensure survival. As a result, larval survivalship decreases as the larval density and egg/bean ratio increases (Smallegange and Tregenza, 2008). It then can be stated that the degree of parental care directly controls the emerging offspring's chance of survival (Messina, 1989). As discussed by Mitchell (1975), this reproductive life-history strategy introduces a strong evolutionary pressure for the female to make sophisticated oviposition decisions. Indeed, Messina (1993) and Mitchell (1990) found that females do have a discrimination mechanism for egg distribution by choosing beans that possess little to no eggs. Due to this fact, the limited resources in each bean cause increased intraspecific competition among larvae at increased density (Smallegange and Tregenza, 2008; Vamosi and Lesack, 2007; Messina, 1991). In order to mitigate this competition, female beetles attempt to distribute their eggs evenly among the available beans (Messina, 1990).

In terms of maximizing fitness, the most advantageous strategy for egg distribution is variable, depending on the situation. While it is possible that beetles will try to maximize their own individual fitness by laying as many eggs on beans as they are able, it is also possible that beetles will restrict the number of eggs they lay in order to maximize the number of emerging beetles. This latter possibility has been investigated by recent studies between kin and non-kin competition. Researchers hypothesized that the species fitness was dependent on the survival of the family's genes, rather than solely on the individual's genes (Smallegange and Tregenza, 2008). It is therefore conceivable that Bruchids could be capable of altruism. It is therefore possible that kin selection will affect egg dispersion. Overall, this study aims to assess the effect of female density on egg dispersion in relation to oviposition competition.

In this study, we subjected *C. maculatus* females to various density conditions, and allowed them to lay eggs until death. We then quantified egg dispersion as well as the total number of eggs laid in order to investigate how oviposition behavior is influenced by increasing density. We hypothesize that as female density increases, females will lay less eggs on beans due to altruism. We also expect that increasing density will not influence egg dispersion, as a uniform distribution of eggs is beneficial at all densities.

MATERIALS & METHODS

Procedure. For this study, Callosobruchus maculatus beetles were obtained from the University of Alberta (strain unknown). To prepare the colony, the bean beetle stock was placed in a 32 oz. Pro-Kal Polypropylene clear container with $\frac{1}{4}$ of the container filled with green mung beans. The colony was maintained in a dark incubator at $\sim 28°C$ and new beans were added after every new generation. To conduct the study, 4 experimental replications were prepared: A, B, C and D. Each replication had 4 different density conditions: 1 female, 2 females, 5 females, or 10 females. 40 green mung beans were distributed into each petri dish. Bean beetles from the colony were sexed and the number of females for each density condition were obtained using a scoopula and placed into each designated petri dish. (Ex. 10 females from the colony were each placed into A10, B10, C10, and D10.) Subsequently, an equal number of males were placed into each petri dish to ensure there were no unmated females. Allowing the mating to occur in the petri dish during the experiment was preferred, as it simplified the laboratory procedures. It is also important to note that male exposure has shown no effect on the fecundity and fitness of the females (Jigisha et al., 2020). All the petri dishes containing the green mung beans, female beetles, and males beetles were placed in a controlled environment at a temperature of $\sim 28°C$. Once the females and males had mated, the females were allowed to lay eggs until all individuals had died. At this point, all the dead beetles were discarded in a waste container and the number of eggs on each bean were counted for each replication and density. In order to dispose of the unused beetles from the colony, the petri dishes and the colony were placed in a freezer at a temperature of $-20°C$ for 2 weeks allowing all beetles to die. After all the beetles were dead the petri dishes and the colony were discarded in accordance with laboratory regulations.

U-score. In order to quantify egg dispersion, we used Messina & Mitchell's U-score. U-score is an index of dispersion of some resource among a discrete number of suitable sites. This index was devised in the context of bean beetles, so its use is justified in this study. A good outline of U-score and how it is calculated can be found in the appendix of Messina and Mitchell (1989). In
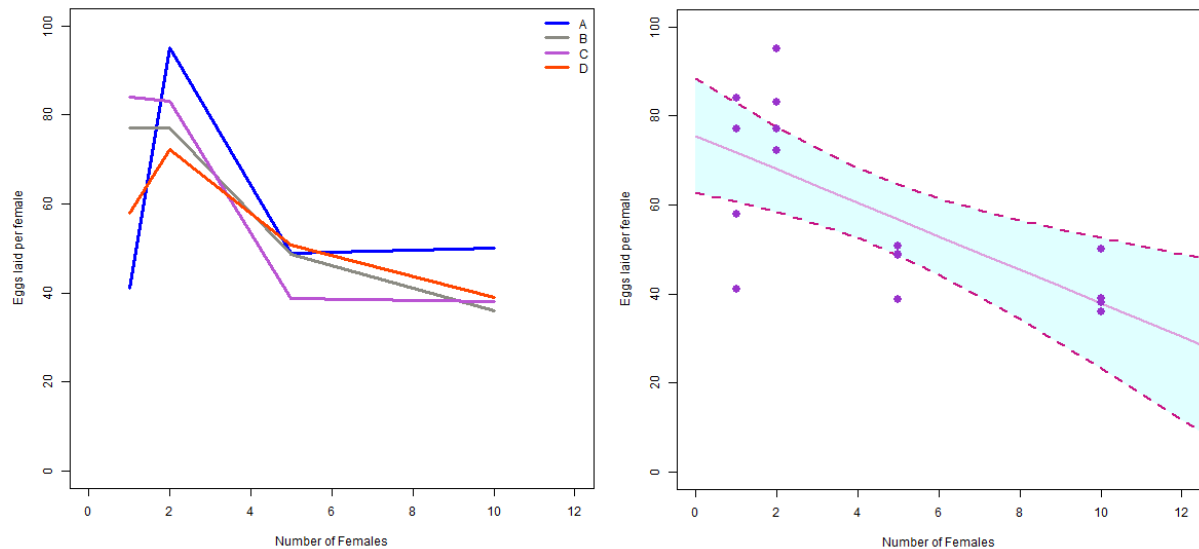
Figure 1: A scatter plot showing the eggs laid per female on beans (individual fecundity) as a function of female density. Left: eggs laid per female on beans by each repetition (A through D). Right: the data from left plotted with the regression line ($y = -3.8x + 75.5$). Dashed lines indicated the 95% confidence interval on the slope. The relationship between the eggs laid per female on beans and female density was found to be statistically significant (p = 0.00289, df = 15, $R^2 = 0.444$). Regression is justified as the residuals were found to be normally-distributed via the Shapiro-Wilk test (p = 0.9975).

summary, U-score compares the number of "mistakes" made by female beetles (number of eggs that would have to be moved in order to achieve a perfectly even distribution) to the number of mistakes expected (based on a Poisson distribution). Values vary between one (a perfectly even distribution), zero (a random distribution, synonymous with Poisson) and negative one (all eggs on one bean, "clumped").

Data Analysis. All statistical analyses were performed in R version 4.0.2. Code was built (in base R) to calculate the observed number of mistakes for each petri dish, as well as the expected number of mistakes for each dish based on a Poisson distribution generated from the number of beans and total number of eggs laid. R was also used to fit linear models to U-score, total fecundity, and average female fecundity. In each case, Shapiro-Wilk tests were done to confirm normally-distributed residuals, and a t-test was done to determine if the slope differed significantly from zero (ie. if the observed relationship was statistically significant). It should also be noted that greater or lesser fecundity of individual beetles will not affect their ability to distribute eggs evenly (Messina, 1993; Cope and Fox, 2003). Therefore, fecundity per female will not have to be held as a covariate in the regression involving U-score.

RESULTS

After the adult beetles died, we counted the number of eggs deposited on each bean. In order to show the relationship between female density and reproductive behaviour, we fit linear regressions between female density, fecundity per female (Figure 1) and U-score (Figure 2). We calculated fecundity per female by taking the total number of eggs laid in each dish and dividing it by the number of females in that dish. U-score was calculated as previously described (Messina and Mitchell, 1989). The dataset used in this analysis can be found in the Supplementary Information document.

Fecundity per female. For all replications, the number of eggs laid per female on beans was found to decrease as female density increased (Figure 1). This relationship was found to be statistically significant (df = 15, p = 0.00289). Interestingly, in the higher two density conditions, a plateau was observed in fecundity at 40 eggs laid per female. This is the same as the number of beans per dish. For the lower two density conditions, the 'A' replication represents a potential outlier.

U-score. For all replications, U-score was found to decrease as female density increased (Figure 2). This relationship was found to be statistically significant (df = 15, p = 0.00284).
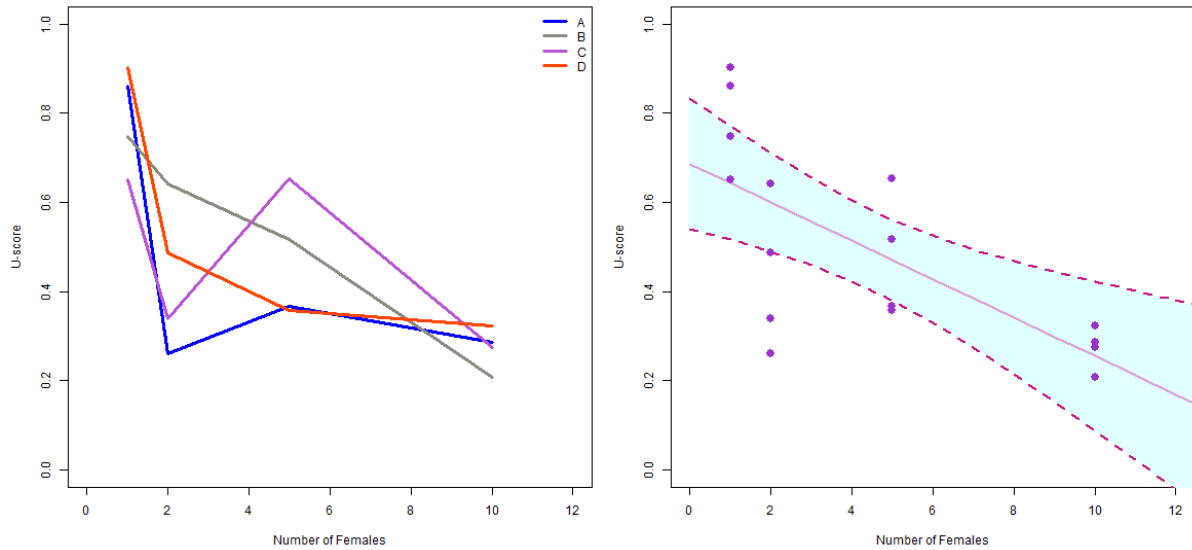
Figure 2: A scatter plot showing U-score as a function of female density. Left: U-scores of each repetition (A through D). Right: the data from the left plotted with the regression line ($y = -0.043x + 0.69$). Dashed lines indicated the 95% confidence interval on the slope. The relationship between U-score and female density was found to be statistically significant ($p = 0.00284$, df = 15, $R^2 = 0.4451$). Regression is justified as the residuals were found to be normally-distributed via the Shapiro-Wilk test ($p = 0.7276$).

DISCUSSION

Our results confirm that egg fecundity on beans is indeed impacted by increased density. Figure 1 demonstrates that female fecundity decreases as female density increases. It is important to acknowledge the nuance behind this statement; when counting eggs, we observed that eggs had been laid on the side and bottom of the petri dish. However, we did not include these eggs in our data analysis. Thus, it should be stated that Figure 1 demonstrates that the number of eggs laid per female decreases **on beans** as female density increases.Therefore, it can be hypothesized that females may not necessarily be laying less eggs, but are simply laying less eggs on beans. Note that at the highest density conditions, some beans had more than 15 eggs deposited on them, and so it is a possibility that this aversion from laying eggs on beans is a protective mechanism that prevents bean overloading (Messina et al., 1987). This behaviour would increase beetle fitness by improving larval survival, but it is unclear whether or not it suggests kin selection.

We also observed a plateau in fecundity at y = 40 in the two higher density conditions. This may point to the possibility that at higher densities, female beetles are only willing to lay one egg per bean, and the remaining are deposited elsewhere, such as on the side of the container (Wilson and

Hill, 1989). The ability of beetles to distribute their eggs in this manner is likely mediated by some sort of oviposition marker, such as a chemical or physical signal (Messina et al., 1987). However, if individual fitness is considered, laying eggs on the container may not be an optimal strategy. Seeing as there are many eggs on each bean, many of which do not belong to any one individual female, a female could increase the odds that the one emerging beetle is her own by laying many eggs on each bean. As such, this female would outcompete the other females based on sheer number. The fact that females only lay one egg per bean at higher densities raises the question as to why they do not adopt a strategy of laying multiple eggs per bean. One possibility is that it could cause instability within the population. If all female beetles utilized this strategy, it may overextend the limited resources of the bean so that no beetle emerges (Messina and Renwick, 1985; Patel, 2012). Another possibility may be that the females are unable to detect other female's eggs, and thus each female is simply trying to distribute their own eggs evenly. This is unlikely, however, as previous research has not observed a selective detection mechanism between self/kin eggs and eggs from unrelated females (Messina et al., 1987; Smallegange and Tregenza, 2008; Messina and Tinney, 1991).

The most likely hypothesis is that females are

able to detect the presence of eggs, but are not able to distinguish the eggs of other females from their own. In this scenario, it is difficult to speculate how an individual female would be able to increase her fitness. Perhaps, a female may choose to lay all her viable eggs in an effort to increase the chance of the emerging beetle being her own offspring and thus, passing on her genes. However, "overloading" beans with eggs has been shown to decrease the size of emerging beetles as well as lower the chance of a beetle emerging at all (Messina and Renwick, 1985). Alternatively, it is possible that the number of eggs a female can lay is highly limited, and thus females do not have enough resources (eggs) to "play the odds." They must instead focus on increasing their own fitness by evenly distributing their eggs rather than allocating a large number of eggs on any one particular bean in order to outcompete other females. This, however, is unlikely, as demonstrated by the large number of eggs laid on the container. Thus, our results suggest that female bean beetles have evolved an egg-laying strategy that is beneficial to the population rather than individuals. Female beetles are willing to sacrifice viable eggs on the sides of the dish in order to ensure that **a** beetle emerges (even if it is not theirs) rather than risking the survival of the emerging beetle just to ensure that it is theirs. Interestingly, this result has been previously predicted by theoretical models developed by Smith and C.M. (1985) and Ives (1989).

While it is clear that female beetles lay less eggs on beans in high densities, altered egg distribution was also observed. As demonstrated by Figure 2, female beetles distribute their eggs less evenly as the density of beetles increases. This suggests two possible hypotheses. The first suggests that the lack of uniform distribution is intentional and somehow provides an evolutionarily beneficial effect. The second hypothesis suggests the decrease of uniform ovipositions represents an error in egg distribution behaviour.

In the first hypothesis, it could be speculated that by overloading some beans with eggs, other beans would be left "under-loaded." This would correspond with increased fitness if certain beans maintained an egg per bean ratio lower than some threshold value. However, if this limit was exceeded, the resources provided by the bean would be spread too thin, and the bean would then bear no beetles at all (Messina and Renwick, 1985). Thus,

clumping of eggs would keep some under-loaded beans below this threshold, ensuring some beans will still bear offspring. This hypothesis could be further proven by additional experiments. If, at extremely high densities, the U-score value decreases and remains at zero, it could be concluded that a decreasing U-score value is simply a mistake. However, if the U-score values continue to decrease, surpassing zero into the negative numbers, then it is possible that a decreased U-score value at very high densities may indicate a fitness advantage. In the second hypothesis, the beetles' ability to evenly distribute their eggs is inhibited by density-induced stress. However, the specifics of this mechanism remain unclear (Wilson and Hill, 1989). There are several possibilities by which this inhibition could occur. One possibility may be that the beetles have adequate memory of the eggs oviposited on the previous bean, but have an insufficient ability to detect eggs, especially at high densities. This could be the case if egg detection by females occurred via some form of pheromone mechanism. Then, when many beetles are placed in a small area, the released air-bourne chemical signals begin to intermingle to the point that detection becomes difficult. This is unlikely, however, as ablation experiments performed by Messina et al. (1987) showed that the recognition of conspecific eggs by members of *C. maculatus* is likely a physical mechanism, not a chemical one. A second possibility is that beetles have sufficient egg detection abilities but inadequate memory. In this case, the beetles would only be able to "remember" a certain number of eggs per bean (ex. 5), and when this is exceeded, they would then tend to lose track of how many eggs are on any one bean (Mitchell, 1975). Therefore, when evaluating where to oviposit at higher densities, the beetles would no longer be making well-informed decisions, resulting in more mistakes and a lower U-score. This would be detrimental to the female's fitness, as overloaded beans bear fewer offspring of smaller size (Messina and Renwick, 1985).

By averaging the U-score values from the lowest density condition (Figure 2), we obtained a value of 0.79. This U-score value is of particular interest because this density condition matches the experimental conditions of a study by Messina & Mitchell (1 female with 40 beans), in which they calculated U-score for 7 different geographical strains. Our results are consistent with

Messina and Mitchell's F strain (Florida, USA), for which they found a U-score of 0.81 (Messina and Mitchell, 1989). No other U-score values reported by Messina and Mitchell (1989) are consistent with our observed value. Unfortunately, the beetles used in this study were obtained from the University of Alberta, and the geographic strain type is unknown. This makes it impossible to confirm this connection. However, it is worth noting that the values obtained in this report are similar to those in a study with the same experimental conditions.

Overall, our results suggest that density does indeed have an impact on reproduction behavior in *C. maculatus*. We observed that female beetles laid less eggs on beans at higher densities, and distributed these eggs less evenly. This suggests a protective behaviour which prevents bean over-exploitation.

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SUPPORTING INFORMATION

Additional supporting information and data used may be found in the file named "group5_CUREsupp.pdf."

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