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Cameron Wynn March, 10, 2020

The Costs of Being a Vector: Effects of *Serratia Marcescens* Infection and Environment on *Anasa Tristis* Performance

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2020

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Abstract

The costs of being a vector: Effects of *Serratia marcescens* infection and environment on *Anasa tristis* performance.

By Cameron Wynn

A vector is an organism that carries pathogens from one host to another. Certain vectors may also experience a disease state, or otherwise detrimental effects caused by the pathogens that they carry. The extent of these possible negative effects may be influenced by the environment that the vector lives in. Factors such as different stressors and types of nutrients available may alter the vector's possible immune response to a vectored pathogen. This thesis investigates these ideas using the relationship between the *Anasa tristis* insect vector and the phytopathogenic strain of *Serratia marcescens* that it carries. We performed experiments to test whether the presence of *S. marcescens* and the environmental rearing conditions of *A. tristis* affects the survival, development, and size of the insects. Though *Serratia* infection does appear to have an effect on the rate of development of insects in poorer environmental conditions, by most metrics the *S. marcescens* was not significantly detrimental to the insects' performance. This may show how pathogens must strike a balance so they can exploit the resources of the vector but not to such an extent that the vector is unable to spread the pathogen to other hosts.

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The costs of being a vector: Effects of *Serratia marcescens* infection and environment on *Anasa tristis* performance.

Cameron Wynn

1. INTRODUCTION

Vectors harbor pathogens that can have devastating consequences on humans, animals and plants. The evolutionary relationship of vectors and pathogens is of high interest. There is a potential that pathogens specifically work to not kill their vector, since this would ultimately harm the pathogen's ability to propagate. Alternatively, harboring the pathogen may have some unintended negative effects on the vector. From the perspective of the pathogen, an evolutionary balance must be struck in which the pathogen does not limit the survival of its vector to the point where it reduces pathogen transmission, but the pathogen can still use enough resources within the vector to multiply.

The consequences of pathogen vectoring on vector fitness has been studied in several systems, particularly in those of consequence for human health. For example, triatomine insects, commonly known as kissing bugs, serve as vectors of the trypanosome *Trypanosoma cruzi,* the causative agent of Chagas disease. Kissing bugs have an innate immune system that fights off potential pathogens and non-self microbes (Salcedo-Porras 2019). However, it may not be as robust as could be possible because it is dependent on a bacterial symbiont (Salcedo-Porras 2019), potentially allowing triatomines to be particularly adequate vectors. Kissing bugs do seem to have a differential immune response to the parasite that they vector, and the pathogen might be able to modulate the insect's immune response (Salcedo-Porras 2019). Interestingly, triatomine insects may, upon introduction of these parasites, support the growth of certain bacteria that secrete compounds to potentially harm or compete with parasites (Tobias 2016).

The availability of various bacteria to an insect in its environment could be crucial to its tolerance of, or defense against, the pathogen it vectors. One of these microbes that triatomines have been shown to harbor is *S. marcescens,* and it could theoretically have vectoring capabilities for this disease, possibly for human hosts (Vieira 2018). For the observed strains, *S. marcescens* does not increase the mortality of kissing bugs that contain it (Vieira 2018).

Insects are also important vectors in agricultural systems. Plant pathogenic bacteria, phytopathogens, vectored by insects can have commensalistic or parasitic relationships with their vectors (Nadarasah 2011). Generally, a less immunogenic pathogen-- a pathogen that does not elicit a strong immune response from its vector--that can be vectored more efficiently should be selected for, however, insect-phytopathogen relationships may start as parasitic relationships and progress to this benign state through evolution (Nadarasah 2011). Pathogens may still be detrimental to their vectors while undergoing this transition. Furthermore, phytopathogens may start as insect specific microorganisms and develop plant pathogenicity through continued contact with plants (Nadarasah 2011), which may explain why some vectored pathogens harm their insect hosts. Another possibility is that insect vectors may eventually become alternative hosts for vectored pathogens, resulting in negative fitness or performance effects on the vectors themselves (Nadarasah 2011).

Take for example the pea aphid. Aphids have been shown to act as primary hosts for phytopathogens (Nadarasah 2011), meaning that in addition to vectoring specific phytopathogens, the aphids themselves suffer a disease state. The pea aphid both vectors and hosts the phytopathogen, *Pseudomonas syringae* (Nadarasah 2011). Additionally, *Dickeya dadantii,* a phytopathogen causing rot in corn and potatoes, and several other phytopathogens, have been shown to cause mortality in pea aphids (Nadarasah 2011). This relationship may have evolved through aphids' repeated contact with infected plants (Nadarasah 2011).

Anasa tristis is a hemipteran insect that consumes plants of the squash family, *Cucurbitaceae,* primarily in the United States (Doughty 2015). Infestation with *A. tristis*, the common squash bug, can be particularly devastating to squash agriculture, since it is not only a damaging herbivore (Doughty 2015), but also a vector for *Serratia marcescens*, the causal agent of Cucurbit Yellow Vine Disease (CYVD) (Bextine 2001). CYVD is a phloem-limited disease that affects squash plants. CYVD-causing strains of *S. marcescens* are specifically adapted to live in phloem (Wayadande 2005). Phloem-blocking diseases like CYVD often have infection cycles involving an insect vector (Bendix 2018). In this case, *A. tristis* fills that role (Bruton 2001). Vectoring occurs when the insects insert their specialized mouthparts, stylets, into the plant in order to feed and in doing so directly interact with the plant's xylem, and, more importantly, phloem, introducing pathogens to it (Nadarasah 2011). It has been observed that *S. marcescens* can be transmitted by *A. tristis* erratically up to 21 days post inoculation, but is transmitted most frequently 1-3 days post infection (Bextine 2001). For phloem-limited diseases, pathogens are generally seen to cross through insect cell membranes and be carried internally by the insect, long feeding times are usually necessary for the insect to take up the microbe, and pathogens often replicate within the insect (Bendix 2018). Additionally, Hemipteran vectored phytopathogens usually have evolved highly specific relationships with their hosts; each phytopathogen strain is only associated with one specific vector, allowing for intricate evolutionary and ecological relationships to develop (Perilla-Henao 2016).

The relationship between *Serratia* and *A. tristis* is particularly interesting because *S. marcescens* is a known insect pathogen, and heteropterans are not usually vectors for phytopathogens (Wayadande 2005). Phloem diseases are generally understood to be circulative in their vector hosts, meaning that they enter the hemolymph and circulate through the insect. *S. marcescens* has been observed in the squash bug hemolymph (Bextine 2001), but sources are

conflicted as to whether it truly circulates within the insect (Nadarasah 2011). For phloem-limited diseases, intracellular colonization of the vector can and often does occur (Perilla-Henao 2016). This implies that since the pathogen multiplies in the tissue of the insect, it may be more able to cause a disease state in the vector by over exploiting it as a primary host. Such is the case with many leafhopper insects and the *Spiroplasma* phytopathogens that they vector (Perilla-Henao 2016). Intracellular colonization could be an important step in maximizing the vector's utility as a primary host of the pathogen, but it could also be harmful for the insect.

The CYVD pathogenic *Serratia* is phenotypically and genetically dissimilar from other strains that are associated with insect and human disease (Besler 2017). This might be due to evolutionary changes that have occurred based on its relationship with cucurbits and *A. tristis*. Based on *A. tristis'* fundamental herbivory on cucurbits and the evolution of many different *S. marcescens* strains, it is possible that *S. marcesens* did not initially have a relationship with *A. tristis* and that this vectoring relationship evolved over time (Bendix 2018). *A. tristis* and *Serratia* likely had their own separate relationships with squash plants before they came to associate with one another. This is to say that it is most likely that *Serratia* was initially a phytopathogen and evolved to associate with the squash bug (Bendix 2018).

The activation of an insect's immune system, such as the one that might be triggered when *A. tristis* is infected by *S. marcescens,* can have significant energetic costs to an organism (Ardia 2012). e Increased metabolic efforts have been measured in many insects undergoing an immune response (Ardia 2012). Infection by a pathogen can lead to many negative effects such as weight loss, for example, and these can be caused by the pathogen's direct action or by the animal's immune response (Zuk 2002). The activation of the immune system requires a reallocation of resources that could be important in growth, development or other key characteristics of an insect fitness. The level of immune response to be mounted is another

evolutionary balancing act. Though some immunity is necessary for protection, an immune response is costly and can even have effects on reproductive success (Zuk 2002). A strong immune system can help to increase fitness, but too much immune investment can result in negative effects to survival and reproduction (Zuk 2002). Stress can result in similar effects to an immune response; it can increase metabolic rate and reduce resources available for other activities (Ardia 2012). Under stressful conditions, an insect might also be at risk for having their immune system overperform, releasing chemicals that are possibly damaging (Zuk 2002). These fitness costs of having to mount an immune defense and being under stress could compound. In bumblebees, these negative effects of mounting an immune response to infection are highly exacerbated when the insects are starved (Zuk 2002). More generally, in terms of the consequences of diet on infection, in *Drosophila* undergoing infection, eating a high protein low carbohydrate diet has been shown to reduce survival rates (Ponton 2019), suggesting that the environment, in particular, available food, can influence immunity. Restricting diet in *Drosophila* has been shown to reduce the insects' ability to clear an infection quickly (Ponton 2019).

Here, we focus on the fitness consequences of *S. marcescens* vectoring for *A. tristis*. A previous experiment showed that *S. marcescens* infected squash bugs had higher rates of mortality than controls, suggesting negative effects of infection, however, these experiments used an injection technique as opposed to feeding the insects *Serratia,* which is how they would naturally acquire it (Wayadande 2005). Another study showed that a CYVD causing strain of *S. marcescens* had no negative effects on the survival rates of squash bug, however, other *S. marcescens* strains did appear to have negative effects on these insects, significantly reducing their life-spans (Heppler 2007). These experiments with *A. tristis* and *Serratia* have not considered how environment, and specifically diet, might influence the consequences of

infection. For *A. tristis*, diet has been shown to influence insect size, survival, and developmental times, especially the time to the fifth instar stage (Bonjour 1993). This may be because different varieties of plants have different defense mechanisms and susceptibility to damage by the squash bugs, and the more susceptible plants may generally produce more successful bugs (Novero 1962). This thesis utilizes two different diets, plants and fruits, both of which are fed upon in field insects and are biologically relevant. Both diets vary in nutritional content, and plants are generally more relied upon by *A. tristis* . Thus, in addition to considering the costs of pathogen infection for *A. tristis,* this research aims to understand if diet among other environmental factors, also influences *A. tristis*' ability to clear bacteria.

A greater knowledge of the *A. tristis* system may further inform our understanding of the relationship between insect vectors and the pathogens that they carry. This thesis seeks to better understand what, if any, effects *S. marcescens* has upon its insect vector, *A. tristis* and what this implies for *A. tristis*' vectoring ability. Additionally, the interplay between *S. marcescens* infection and environment and its possible effects on development and fitness is also investigated. We hypothesized that harboring *S. marcescens* would have negative costs to performance on *A. tristis*, such as slower developmental times and smaller adult sizes, and that these harmful effects would be more apparent for those insects raised in an environment with poorer conditions.

2. METHODS

2.1 Squash Bug Rearing and *Burkholderia* Inoculation

Anasa tristis eggs were collected every two to three days off of yellow crookneck Squash plants where adults from the stock colony were left to breed. These eggs were surface sterilized in ethanol and bleach; they were then allowed to hatch and reach the second instar stage on a

fruit diet consisting of slices of organic zucchini fruit. All insects and plants were kept in an environmental growth chamber on a long day cycle (16:8, day:night) set to 27C and 60% humidity. At the second instar stage, insects were separated into the two environmental treatment groups and all insects were starved for an 8-16 hour period, after which they all received a liquid diet consisting of *Burkholderia* SQ4A, a squash bug derived symbiont strain known to be important for bug development and survival (Acevedo et al, in prep), at a concentration of 2 x 10^7 cells/mL. They were left to feed on this diet for 24 hours before being placed into their new environment conditions.

2.2 Treatment Groups, Diet, and *Serratia* Inoculation

Insects were separated into four major treatment groups at the second instar stage. One group received *Serratia* strain ZO1 at the third instar stage and was raised in the plant diet lower stress environment. Another group was also raised on a plant diet but was never fed *Serratia*. Yet another group received *Serratia* but was instead reared on a higher stress fruit diet condition. The last group did not receive *Serratia* and was raised on fruit (See Figure 2.1). Each treatment group had a little over ten replicates.

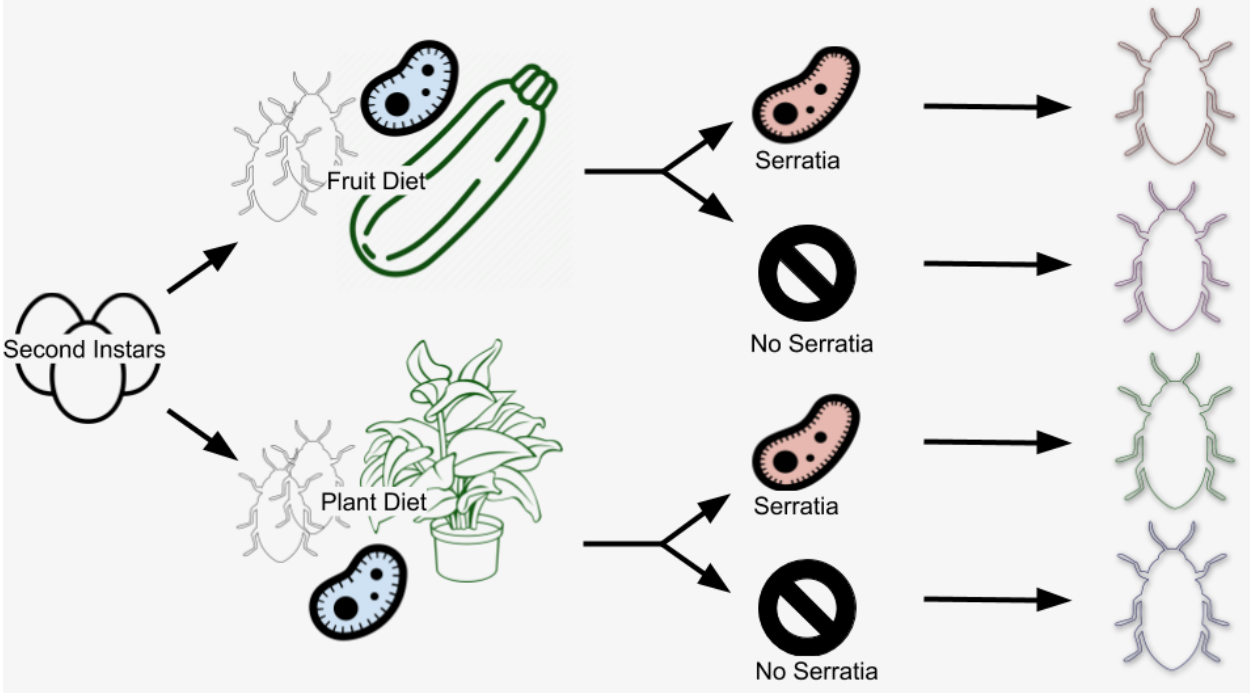


Figure 2.1: Separation of insects into 4 treatment groups. From top to bottom on the right the red insect represents fruit fed ZO1+ insects, purple is fruit fed ZO1-, green is plant fed ZO1+ insects, and blue is plant fed ZO1-.

The plant fed insects were placed in a 1 cubic foot netted cage with one squash plant in groups of 11 insects on average. Plants were replaced when noticeable browning or mold appeared or when the plant outgrew its enclosure. Plants were kept in a hydroponic system in which water and fertilizer were exchanged weekly. Fruit fed insects were placed in smaller (10cm x 10cm x 2.5cm) boxes in groups of approximately 15, with one parafilm wrapped piece of organic zucchini squash fruit which was exchanged for a new piece every two days. Insects in the ZO1+ treatment groups received *Serratia* at the third instar stage by giving them cubes of squash infused with a Red Fluorescent Protein (RFP) labeled strain of *Serratia* (strain Z01) at a concentration of 2×10^7 cells/mL and were left to feed for 24 hours.

2.3 *Serratia* Clearance Groups

Another group of second instars was collected from the same group of eggs and fed *Burkholderia* as previously described. Insects were then separated into two groups (n = 90 per group). One group was placed in the plant environment condition, and another was raised on fruit. Insects were raised to the 3rd instar stage before being fed *Serratia*. Twenty four hours after being fed *Serratia,* five insects from each group were sacrificed, crushed, and plated. Then, after allowing 24 hours for growth, the plates were examined for RFP labelling. Plates were considered to still have Serratia present if any RFP labeled bacteria was found. This was done every day for six days until there consistently was no RFP bacteria on any plates.

2.4 Data Collection and Analysis

Survival data was obtained by recording the number of deaths every two days and by comparing the number of second instars to the final number that reached adulthood. Insects were observed for sixty days past their collection as second instars or until all insects in a replicate had reached adulthood at which point they were removed from their environments to be measured. Adult insects were photographed and measured at the pronotum using ImageJ. Two measurements were taken and averaged for each insect. After being photographed, adults were dried in an incubator at 52C and then weighed. Dry weights were obtained by taking frozen dead adults and placing them in an incubator for two days until they no longer held water. Pronotum width and dry weight can both be used as an indicator of insect fitness. Stage development data were taken every two days based on morphology and the presence of molts. Data were analyzed using RStudio Version 1.0.153. Most data analyses use ANOVA models to consider the effects of the environment (fruit vs. plant rearing), *Serratia* presence and the interaction between the two. A cox proportional hazards model is used for the survival analysis (Figure 3.1) and the

Serratia clearance data (Figure 3.7). Sex is considered when analysing the size metrics. All time points use the collection as a second instar as the 0 time point.

3. RESULTS

The days until death for insects raised in different environments were significantly different ($Chi^2 = 211.74$, d.f. = 1, $p < 0.001$). *Serratia* presence ($Chi^2 = 0.003$, d.f. = 1, $p = 0.88$) and the interaction between environment and *Serratia* ($Chi^2 = 1.04$, d.f. = 1, $p = 0.31$) were not significant (Figure 3.1). We observed clearance of *Serratia* within six days of preliminary infection, so the effects of *Serratia* presumably would be most strongly seen in the first six days.

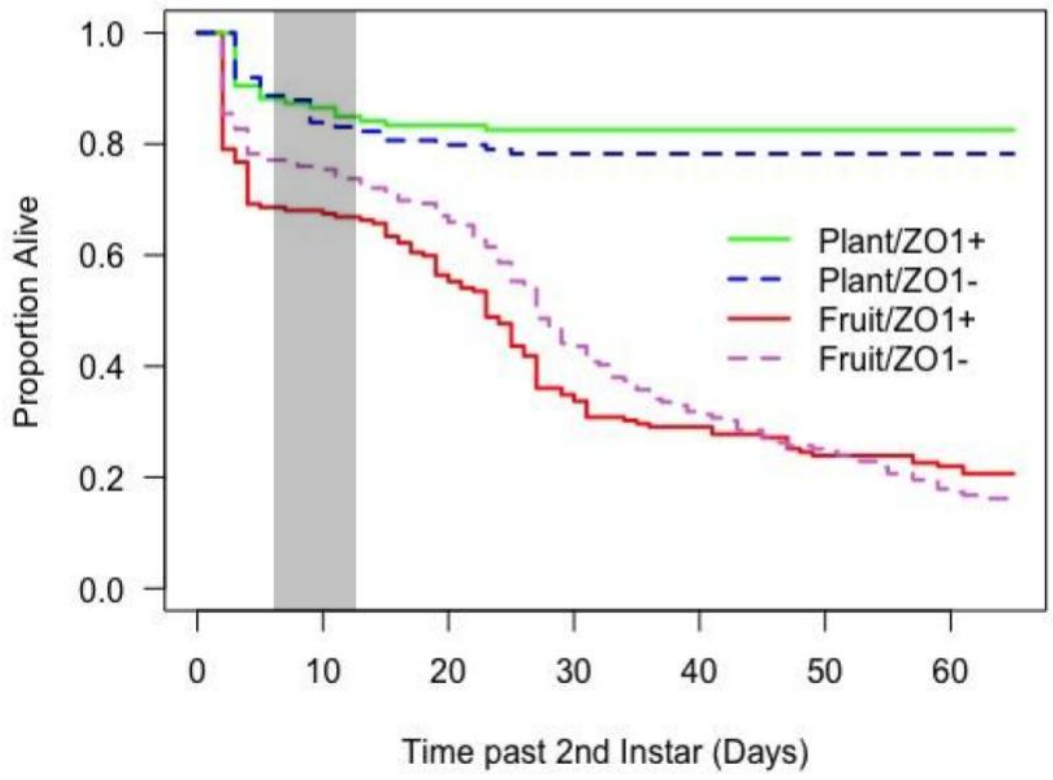


Figure 3.1 Plant fed insects have enhanced survival compared to fruit-fed insects

This graph shows the average proportion of insects per replicate within each treatment that survived over 2 months. ZO1+ refers to *Serratia* fed groups; ZO1- refers to non-infected controls. The gray bar shows where we might expect to see the influence of *Serratia* on survival. *Serratia* feeding occurred 6-11 days after day 0, the 2nd instar starve date. Z01+ refers to those bugs that received *Serratia.* Data was collected every two days. The last insect to die or molt to adulthood did so at 60 days past the second instar time point and as such 60 days is the end point for survival data collection.

The proportion of insects that survived over sixty days was significantly different between rearing conditions (ANOVA, $F(1, 41)=250.60$, $p < 0.001$), with a higher proportion of insects reared on plants surviving (Figures 3.1 and 3.2). *Serratia* inoculation ($F(1, 41)=250.60$, $p = 0.29$) and the interaction between environment and *Serratia* ($F(1, 41)=250.60$, $p = 0.77$) were not significant.

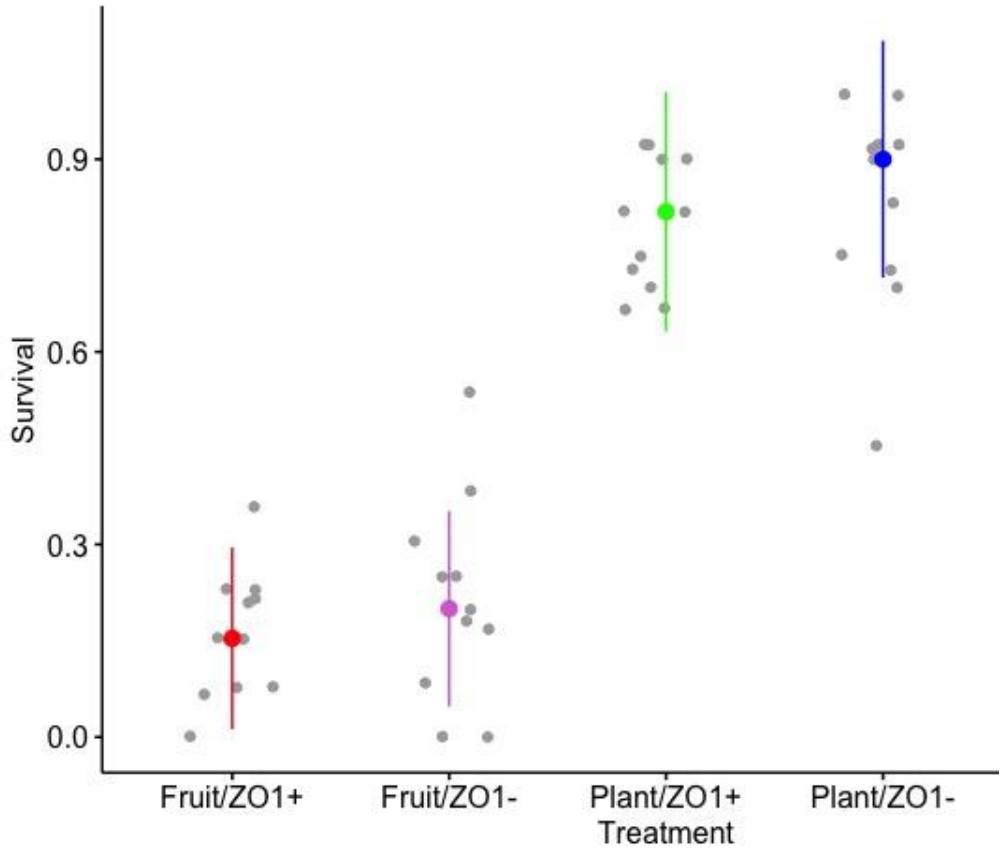
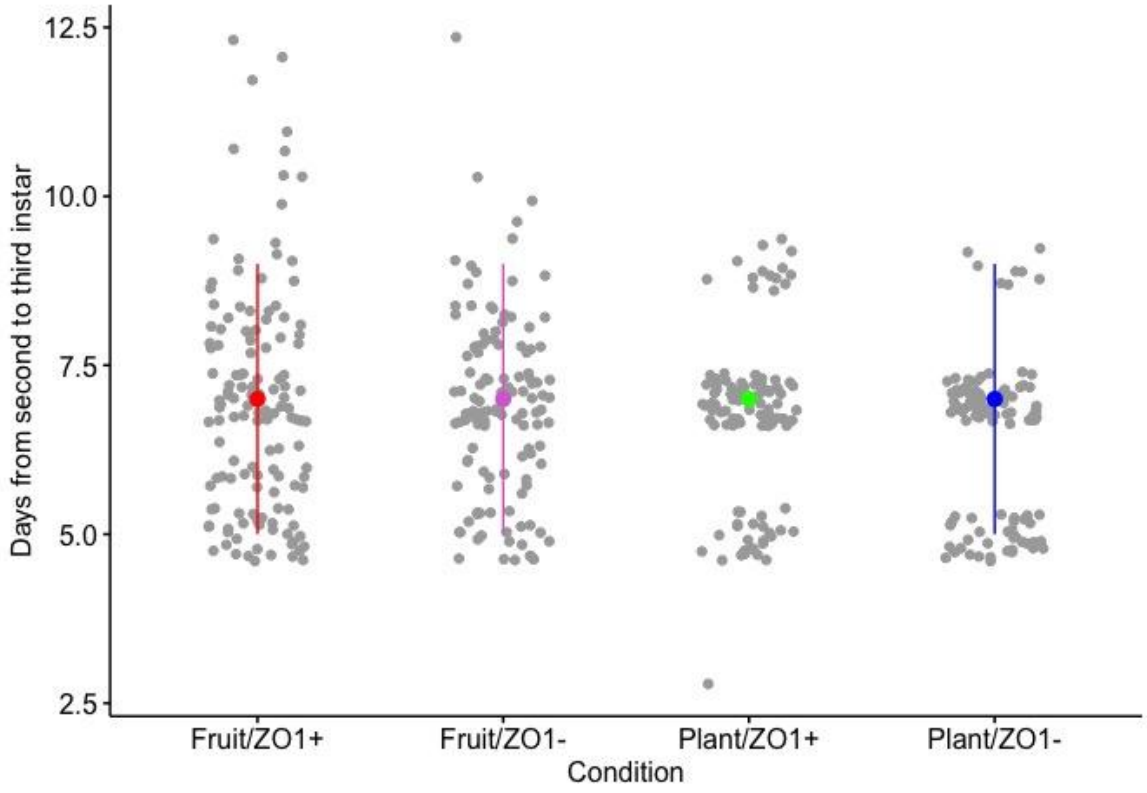
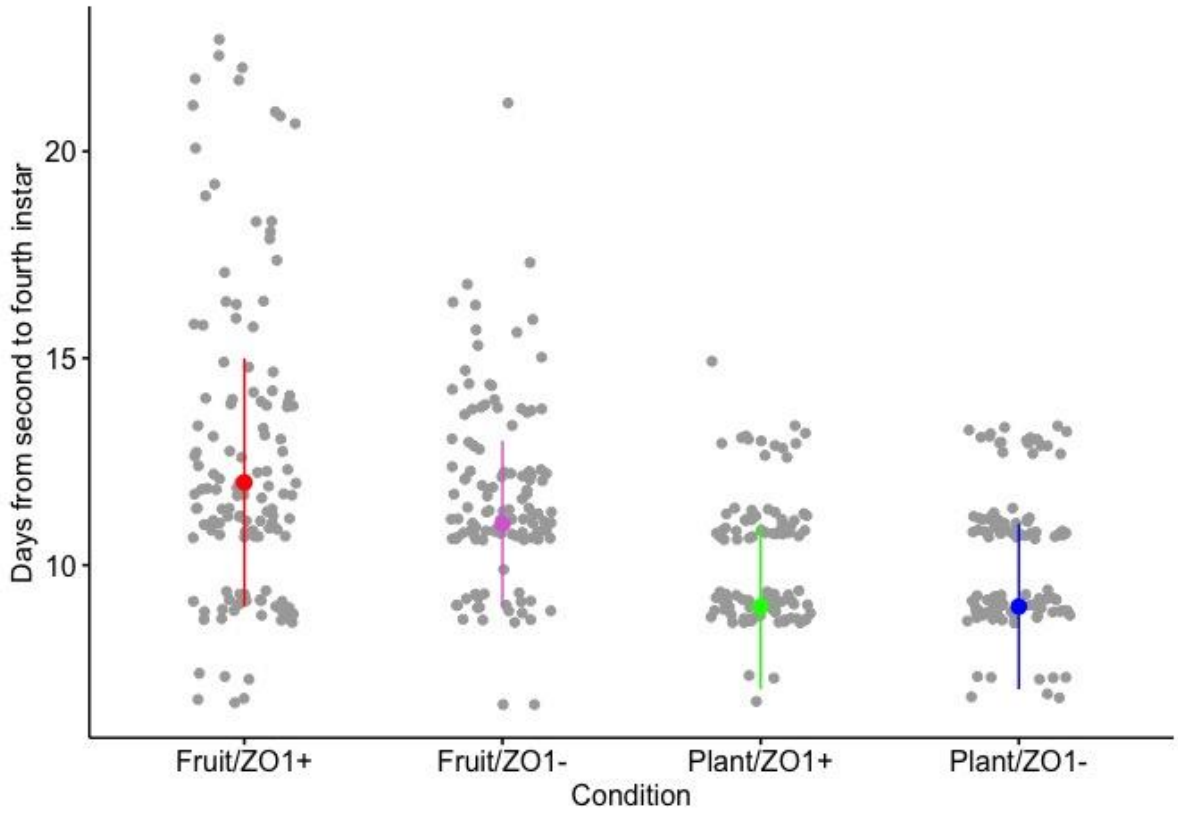


Figure 3.2: Higher proportions of plant-fed insects survive for 60 days compared to fruit-fed insects. This graph shows the median proportion of insects in each replicate, a replicate being either a box or plant cage, that survived to the adult stage. Bars show interquartile range. Each point represents a singular replicate.

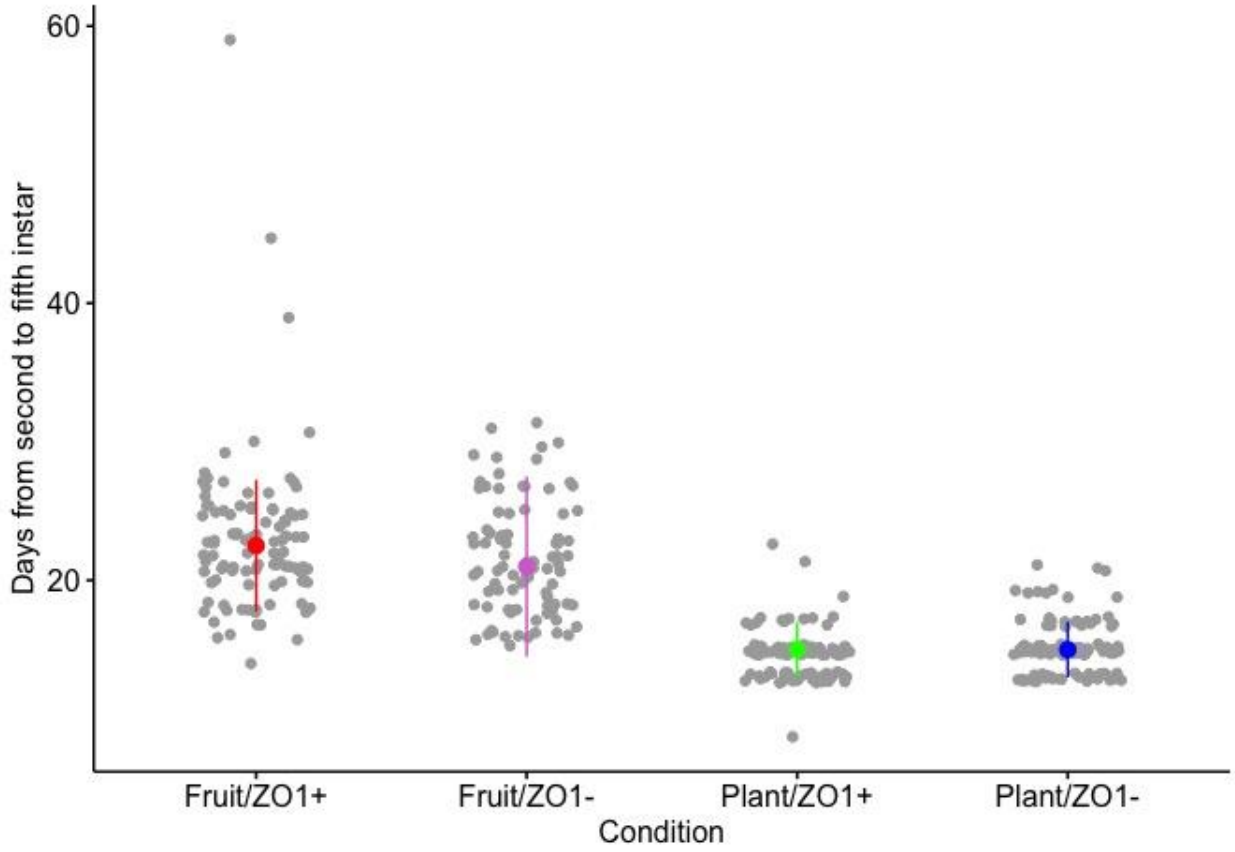
The days to each instar stage were significantly different between the fruit-reared and plant-reared groups (Figure 3.3). There was a significant effect of *Serratia* presence for fruit reared groups at later developmental stages. The days between the second instar and third instar stage are significantly different between the two environment conditions ($F(1, 475) = 5.82$, $p = 0.02$), with fruit-fed insects taking more time to reach the third instar stage. Insects were fed *Serratia* after the third instar stage so it would not have an effect on the developmental speed at this point. The same results hold for the time until the fourth instar stage: environment was significant with fruit fed insects again taking longer to develop ($F(1, 460) = 76.62$, $p < 0.0001$). Neither *Serratia* inoculation nor the interaction between environment and *Serratia* infection were significant (*Serratia*: $F(1, 459) = 3.43$, $p = 0.06$; interaction: $F(1, 458) = 2.45$, $p = 0.12$). he confidence interval for *Serratia* infection, however, does not overlap with zero, suggesting a marginal effect of infection on development. The environment was again significant in determining time until the fifth instar stage ($F(1, 397) = 461.63$, $p < 0.0001$). *Serratia* presence also had a marginal effect on development to the fifth instar stage ($F(1, 396) = 0.94$, $p = 0.33$). The interaction between *Serratia* and environment was significant ($F(1, 395) = 6.84$, $p < 0.001$). This is seen in the widening gap between the average development time for the fruit fed insects based on the presence of *Serratia* (Figure 3.3). For the total time between second instar and adult, environment ($F(1, 278) = 695.40$, $p < 0.0001$), *Serratia* inoculation (($F(1, 277) = 9.70$, $p < 0.01$) and the interaction (($F(1, 276) = 13.57$, $p < 0.001$) were all significant. Overall, fruit fed insects developed slower than the plant fed insects in every stage, and *Serratia* presence does seem to slow development in the fruit reared insects, at least at later stages. This suggests that *Serratia* is potentially detrimental to insect performance for this specific, less optimal environment.


12.5
10.0
7.5
5.0
2.5
Days from second to third instar
Fruit/ZO1+
Fruit/ZO1-
Plant/ZO1+
Plant/ZO1-
Condition

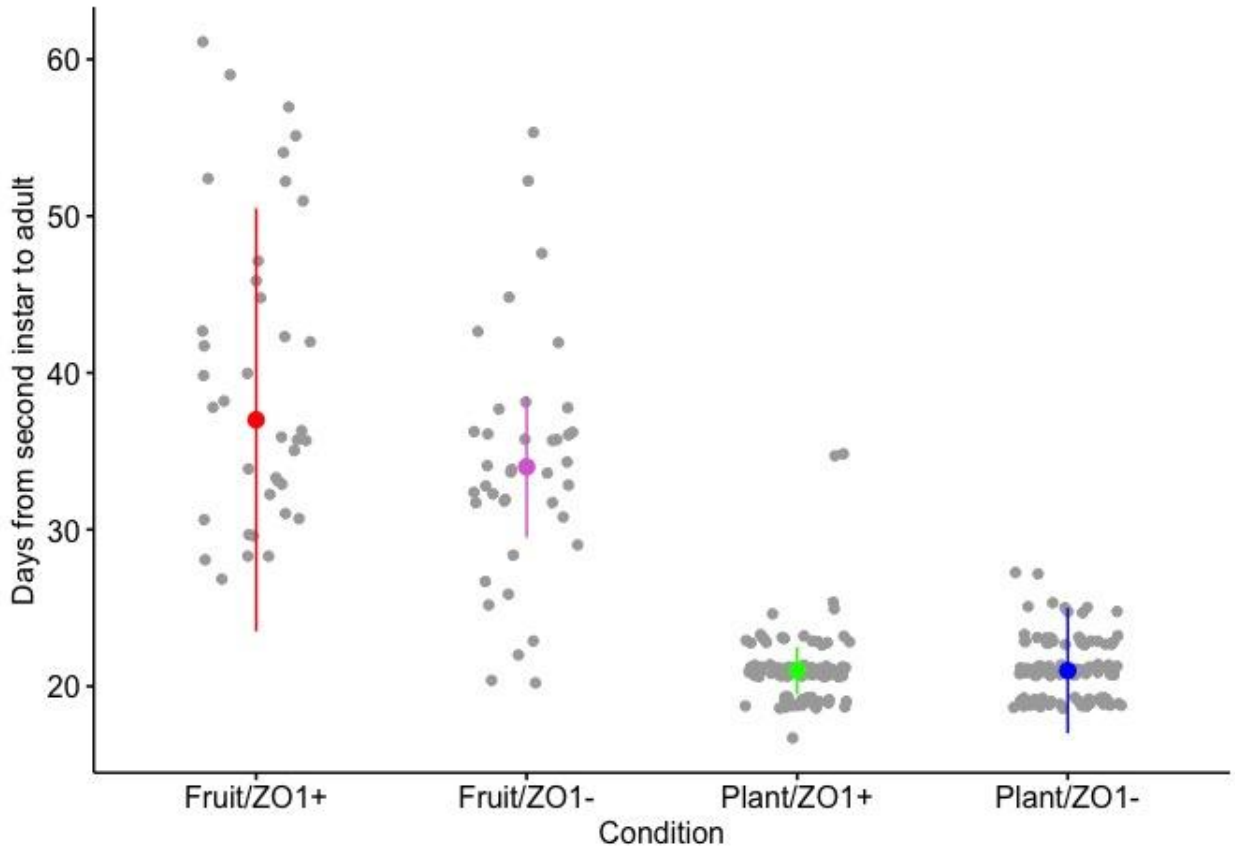
(A)


20
15
10
Days from second to fourth instar
Fruit/ZO1+
Fruit/ZO1-
Plant/ZO1+
Plant/ZO1-
Condition

(B)


60
40
20
Days from second to fifth instar
Fruit/ZO1+
Fruit/ZO1-
Plant/ZO1+
Plant/ZO1-
Condition

(C)

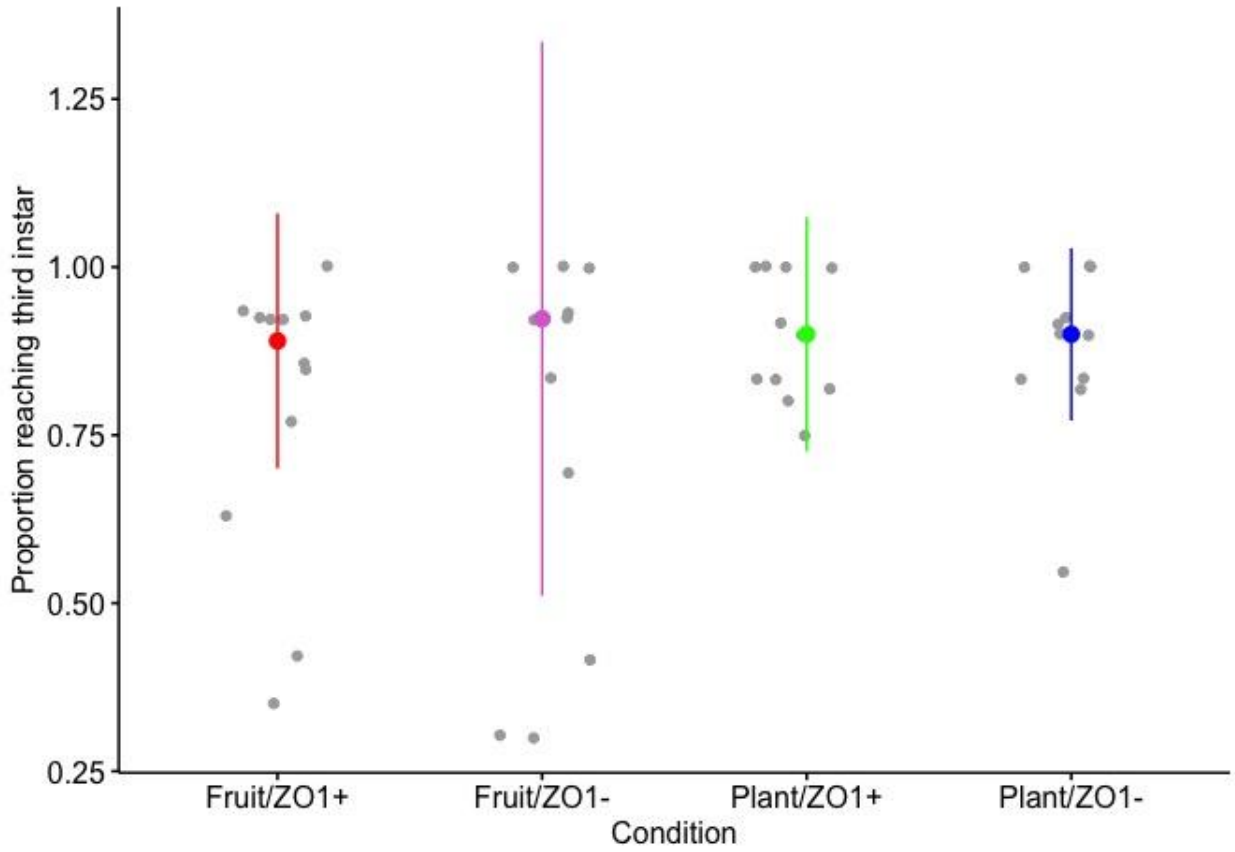


(D)

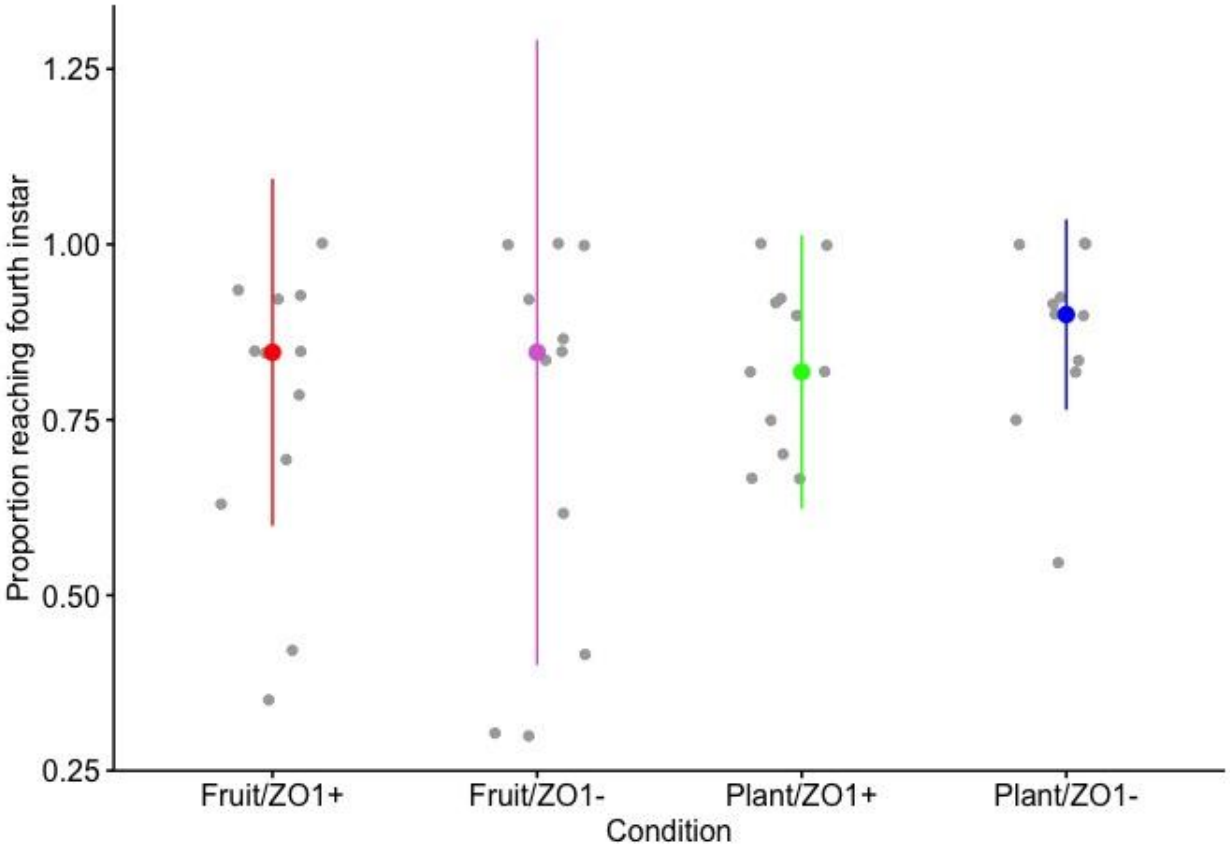
Figure 3.3 *Serratia* positive insects take longer to reach developmental milestones in the fruit-fed condition. These graphs show the median time taken to reach a developmental stage for each of the four treatment groups. Bars represent the interquartile range. Graph A shows the time between second instar and the third instar stage, B shows the time between second and fourth instar, C shows the time until the fifth stage, and D shows the time until adulthood. and Each dot corresponds with an individual insect. All measurements are the time between collection after the second instar stage and the molt to the marked stage. *Serratia* feeding occurred after the third instar stage and before the molt to fourth.

The environmental conditions also had an effect on the proportion of insects reaching the different larval stages. For the 3rd instar stage, (Figure 3.4); the environmental condition was significant ($F(1, 43) = 6.99$, $p = 0.01$). Insects were fed *Serratia* after the third instar stage so the presence of Serratia would not have an effect on the proportion of insects reaching the third instar stage. For the proportion of insects reaching the fourth instar stage similar results hold. The environmental condition was significant ($F(1, 43) = 5.45$, $p = 0.02$). *Serratia* presence was

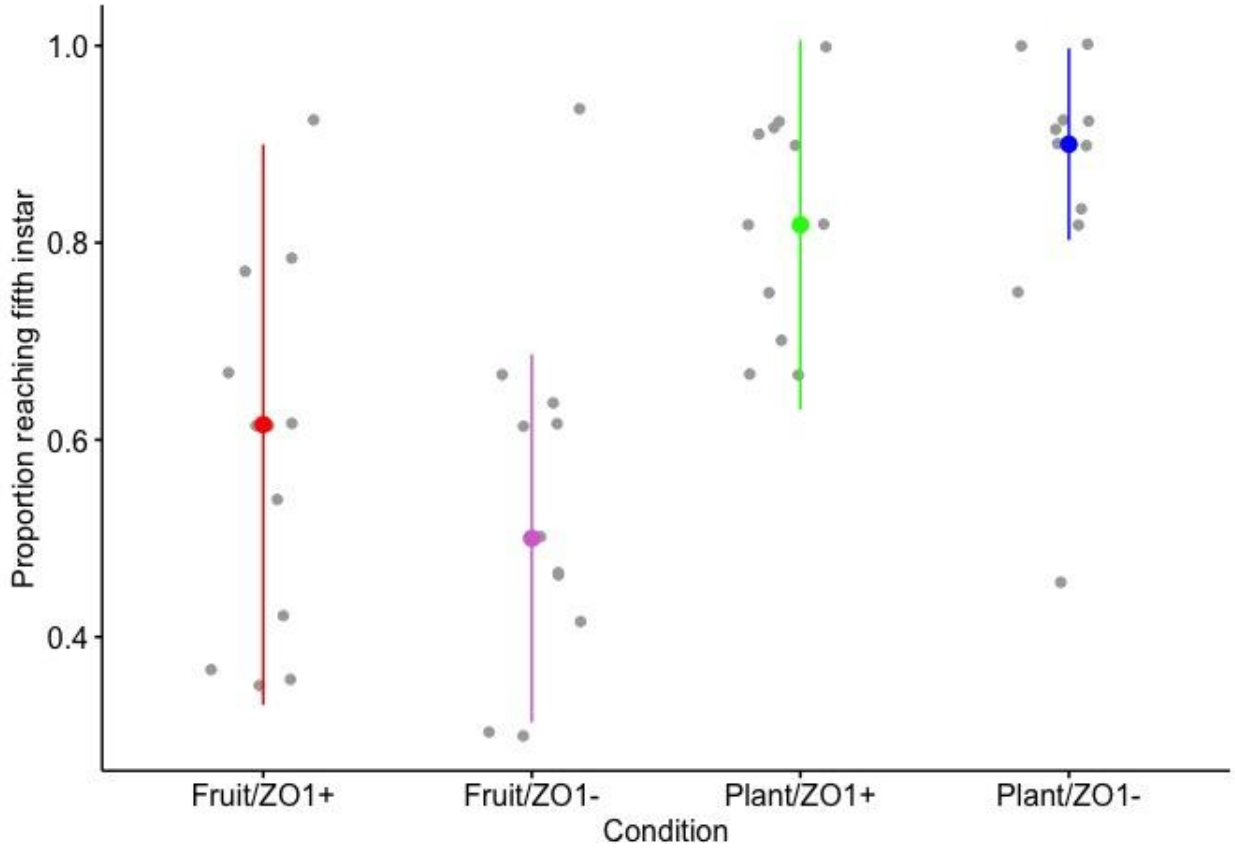
not significant ($F(1, 42) = 0.09$, $p = 0.76$); the interaction between environment and *Serratia* was also not significant ($F(1, 41) = 0.68$, $p = 0.41$). For the proportion reaching fifth instar the same results are also seen; environment is once again significant ($F(1, 43) = 33.04$, $p < 0.0001$). *Serratia* presence is not significant ($F(1, 42) = 0.10$, $p = 0.75$) and neither is the interaction ($F(1, 41) = 0.70$, $p = 0.41$). The results are the same for the proportion reaching the adult stage. Environment was significant ($F(1, 43) = 132.99$, $p < 0.0001$), presence of *Serratia* ($F(1, 42) = 0.39$, $p = 0.54$) and the interaction between the two variables ($F(1, 41) = 0.14$, $p = 0.71$) was not. From these results, we can see that the only significant variable having an effect on the proportion reaching each life stage is the environment. A greater proportion of plant-fed insects generally survive to each stage. It can also be seen that the odds ratio for the effect of environment on the proportion of insects reaching each life stage is increasing with each analyzed life stage. This might simply be due to the cumulative nature of the measurements.


Proportion reaching third instar
1.25
1.00
0.75
0.50
0.25
Fruit/ZO1+
Fruit/ZO1-
Plant/ZO1+
Plant/ZO1-
Condition

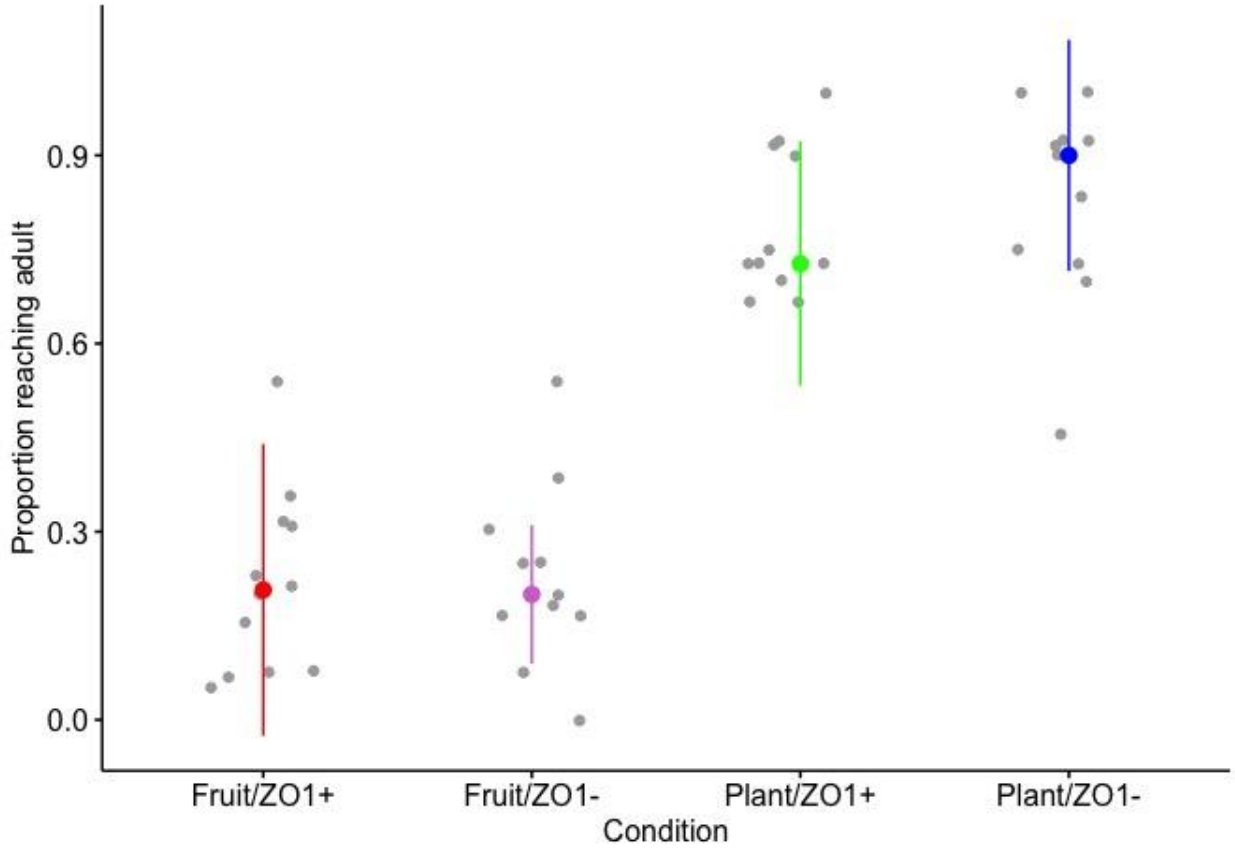
(A)


1.25
1.00
0.75
0.50
0.25
Proportion reaching fourth instar
Fruit/ZO1+
Fruit/ZO1-
Plant/ZO1+
Plant/ZO1-
Condition

(B)


1.0
0.8
0.6
0.4
Proportion reaching fifth instar
Fruit/ZO1+
Fruit/ZO1-
Plant/ZO1+
Plant/ZO1-
Condition

(C)

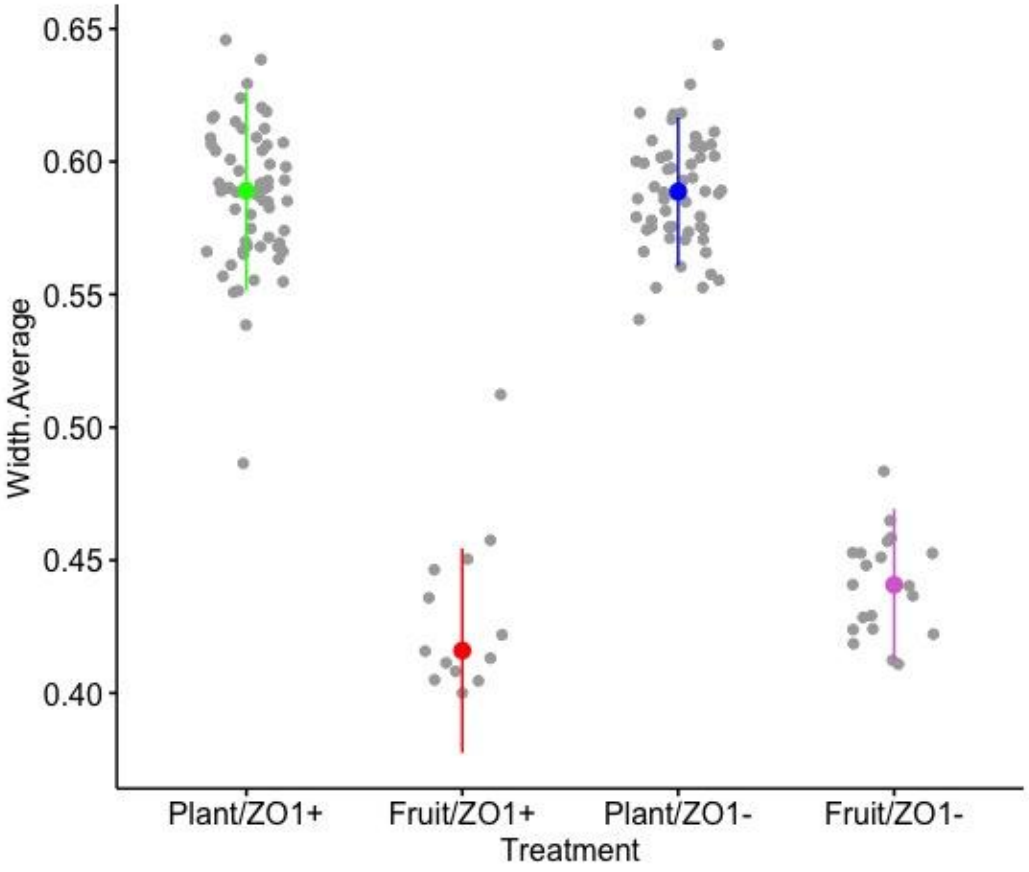


(D)

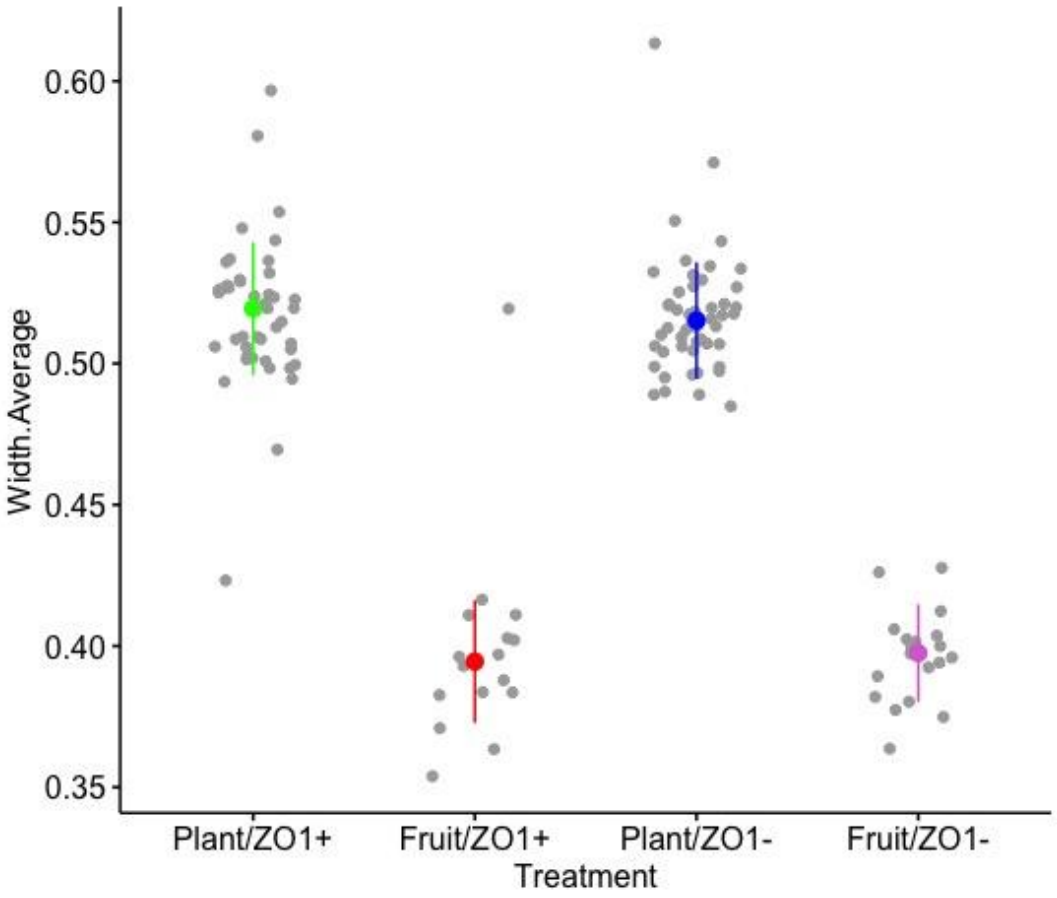
Figure 3.4: Proportion of insects reaching each individual developmental stage is higher for plant fed insects. Each individual group of points shows the median proportion of insects that reached a particular stage for each experimental group. Bars show interquartile range. Individual points represent the replicate from which the proportion was attained, either the box or the plant cage. Graph A shows the proportion reaching the third instar stage, graph B shows the proportion reaching fourth, C shows fifth instars, and D shows adults. *Serratia* feeding occurred after the third instar stage and before the molt to fourth.

The pronotum widths analysed here (Figure 3.4) can be used as a proxy for the fitness of the insects. Plant fed insects were generally larger than fruit fed insects, and *Serratia* presence did not appear to affect the insects width measurement. The environment had a significant effect on pronotum width ($F(1, 277) = 0.44$, $p < 0.0001$). As expected, females were significantly larger than males ($F(1, 276) = 0.18$, $p < 0.0001$). The interaction between sex and environment was also significant ($F(1, 276) = 0.18$, $p < 0.0001$). Those in the plant fed condition developed a more

apparent difference in size between males and females than those in the fruit condition. Presence of *Serratia* ($F(1, 275) = 0.18$, $p = 0.76$), environment *Serratia* interaction ($F(1, 273) = 0.16$, $p = 0.64$), sex *Serratia* interaction ($F(1, 272) = 0.16$, $P=0.4110$), and the interaction between all the variables ($F(1, 271) = 0.16$, $p = 0.43$) were all insignificant. Overall, *Serratia* has no noticeable effect on pronotum width.



(A)

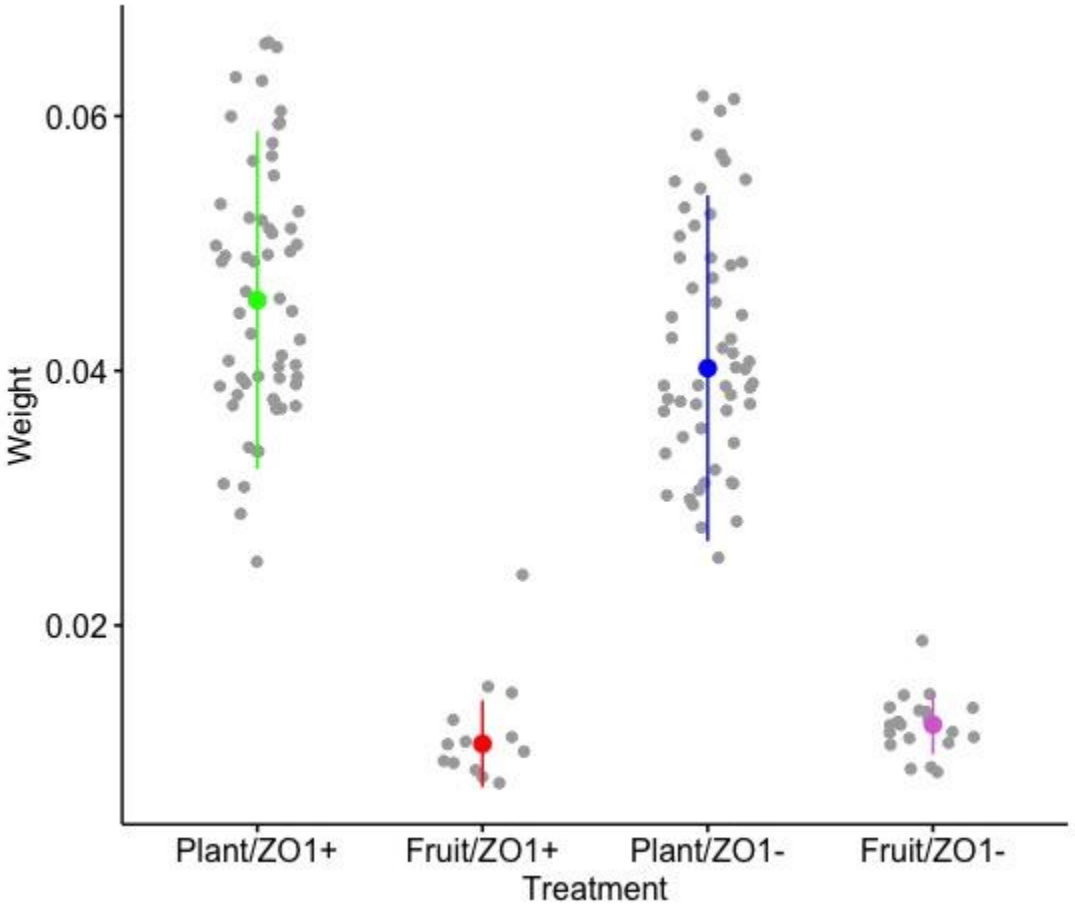


(B)

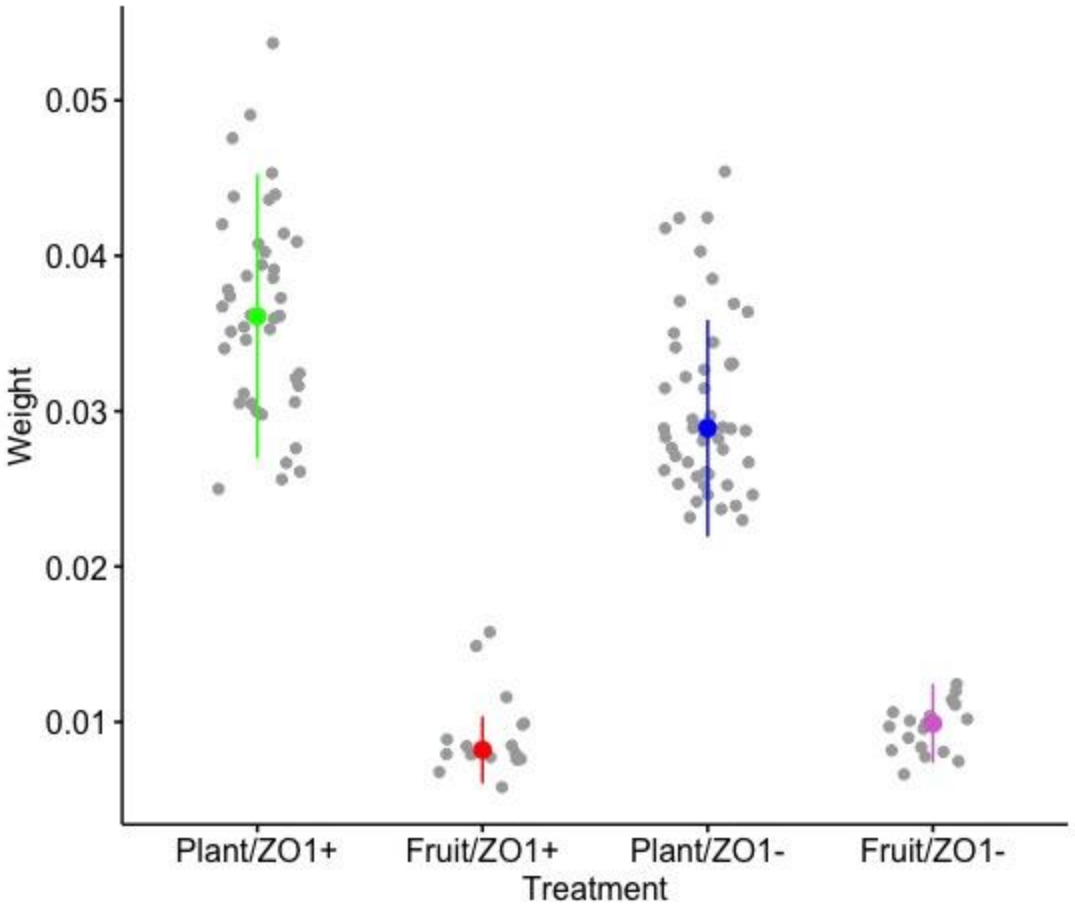
Figure 3.5: Environment affects the pronotum widths of insects. This graph shows the median pronotum widths. Error bars show the interquartile range Single points correspond to the measurement of one adult insect. Width is measured in cm. Graph A shows the female widths and Graph B, below shows the male widths

Similar to the pronotum widths, the environment significantly influence adult dry weight ((F(1, 275) = 0.06, $p < 0.0001$), as was sex (F(1, 274) = 0.02, $p < 0.0001$), and the interaction between sex and environment (F(1, 272) = 0.02, $p< 0.0001$) (Figure 3.6). Surprisingly, *Serratia* (F(1, 273) = 0.02, $p < 0.0001$) had a significant effect on the weight. The interaction between environment and *Serratia* infection also had a significant effect on weight (F(1, 271) = 0.01, p = 0.0094). The interaction between sex and infection status was not significant (F(1, 270) = 0.01, p = 0.42) and the interaction between all the variables was also not significant (F(1, 269) = 0.01, p

= 0.64). It can be seen that insects that were infected with *Serratia* and raised on plants had higher weights than non-infected insects that were also plant reared. Specifically, the plant fed *Serratia* negative males had a surprisingly low weight. Potentially, insects benefited from the short amount of time feeding on fruit where they contacted *Serratia* because of the variation in nutrition source. The interaction between *Serratia* infection status and environment is of interest. *Serratia* fed insects that feed on fruit appear to perform the worst in most of the metrics. The effect that *Serratia* has on the insects is changed based on the environmental condition, with it negatively impacting fruit fed insects and possibly positively influencing plant fed ones.



(A)



(B)

Figure 3.6: *Serratia* may have alternate effects on the dry weights of insects based on environmental condition. This figure shows the dry weights of the insects. Each point represents the weight of one adult insect. These graphs show the median for each treatment group and the interquartile range. Weight is measured in grams. Graph A shows results for females and B shows the weights for males.

For the data on the speed at which the insects in the two environments cleared the *Serratia* infection, visualized in figure 3.7, the fruit reared insects were estimated to clear the infection on average in 2.22 (95% confidence interval 1.42 to 3.59)The plant fed insects were estimated to clear the infection in 1.87 days (CI: 1.20, 2.99). Since these confidence intervals overlap, the treatments do not appear to have an effect on the ability of the insects to clear the

infection. These results were calculated using a maximum likelihood estimator to parameterize a survival curve with an exponential hazard function and are not statistically significant.

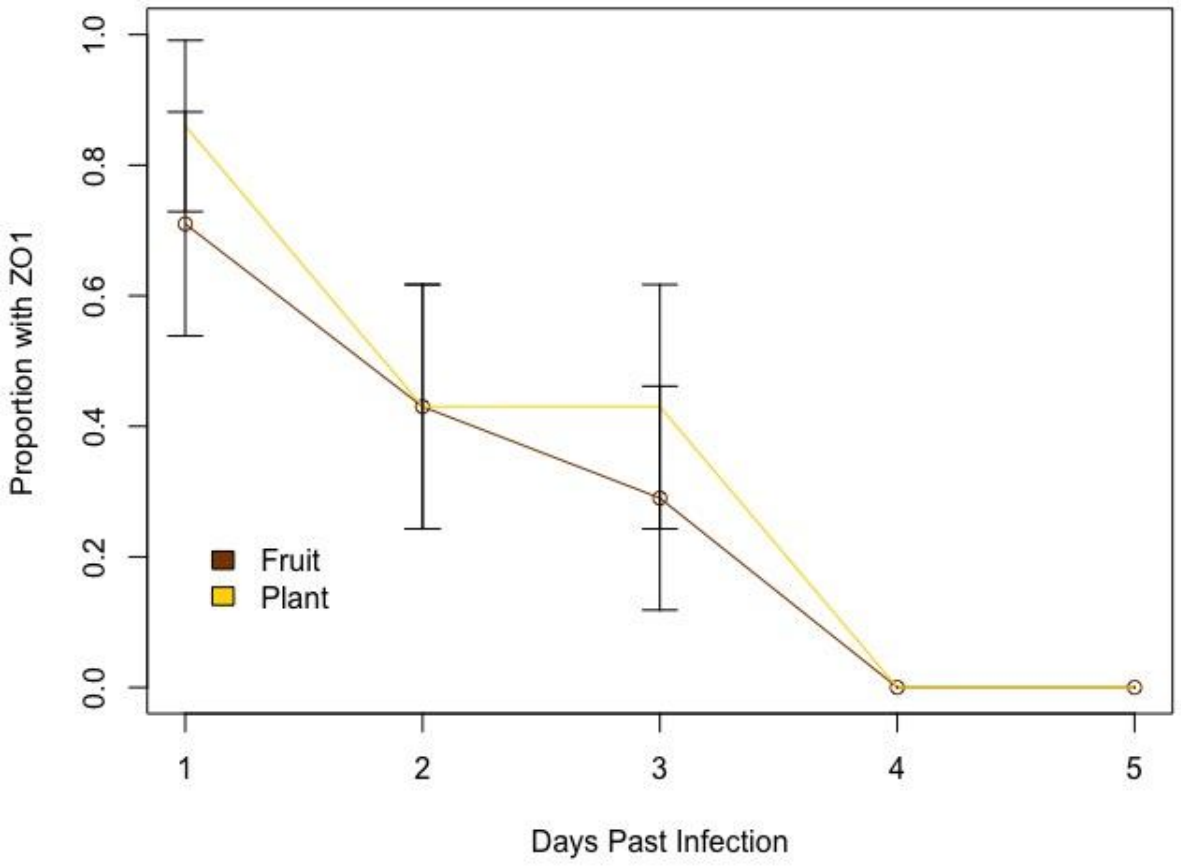


Figure 3.7: Environment does not appear to affect the ability of *A. tristis* to clear the *Serratia* infection. Bars show the standard error of the proportion.

4. DISCUSSION

Based on the results as a whole, the presence of the ZO1 *Serratia* strain does not appear to have a significant negative effect on the performance of the squash bug. This is of interest because it suggests that either the bacteria or the insect have evolved so as to ensure that infection does not negatively influence the vector. We do see some interesting effects where the

fruit fed ZO1+ insects fare the worst in survival, developmental time, and size measurements. These phytopathogens must tread the fine line between using the insect as a primary host and not affecting the insect too heavily so it can continue to properly serve its role as a vector, thereby exploiting the insect host to its fullest extent. This results in a somewhat gray outcome. The results seen with *Serratia* in the fruit fed insects are not seen in the plant diet insects so potentially the environment is playing some role in the insects' response to *Serratia*. Notably, *Serratia* infected insects had higher dry weights than non-infected individuals in the plant condition. It does appear that the environment made a difference in squash bug performance, with the plant fed insects performing better in most metrics. The similar patterns of *Serratia* clearance by both diet groups would suggest that there is not an interaction between the environment and the *Serratia* status. It's difficult to ascertain what exact factor in each of the two different environments might be causing the difference in development, survival, and size, as well as the lack of difference with *Serratia* clearance time. It could be due to space constraints, amount of food, quality of food, or available bacteria in the surroundings. Additionally, *Anasa tristis*' symbiont *Burkholderia* might behave differently in the two environments which could have an effect on the insect's performance and it's tolerance of infection. It might be of interest to separate out these variables and see what is the biggest factor influencing performance in these insects.

Most data collection focused on survival and size. To draw strong conclusions about fitness and to hypothesize on the evolutionary consequences of these results, it would be beneficial to collect fecundity and fertility data. However, in the current study size may serve as a proxy for fitness. Males prefer to mate with *A. tristis* females with a larger pronotum width (Hamel 2015). This suggests that the pronotum width measurement may have bearing on the fitness levels of the insects. This allows us to draw conclusions about the effects of our tested

variables on fitness. However, pronotum width in *A. tristis* has not been shown to affect the number of eggs produced overall (Hamel 2018). This result comes from a paper that did include interspecies pairings which may have interfered with these findings. It has been shown with insects of many different families that there is a consistent positive relationship between female body size taken as a dry weight and eventual fecundity (Honek 1993). This allows the dry weight measurement to not only provide information about performance but also about evolutionary fitness. This link is important because it allows for this data to provide insight into the evolutionary history of the organisms in this system. Additionally, regarding survival, it could be important to analyze the specific effects of the introduction of *Serratia* by focusing on the first few days after inoculation with the bacteria. Based on the current results, this analysis was deemed unnecessary at this point in time.

The results presented here are the third iteration of this experiment. The first two experiments failed due to a mislabeling of *Serratia* as *Burkholderia*. This resulted in insects not receiving their symbiont and having extremely poor survival rates. This experiment utilizes the correct bacteria for *Burkholderia* and *Serratia*; all results presented here are valid and use the correct bacterial strains. Additionally, there was some difficulty in collecting accurate developmental stage data based on the similarity of the second and third instar stages as well as the difficulty observing insects in the plant environment. It could be beneficial to take data every day instead of every two days for improved accuracy. Additionally, the time for which insects are starved is crucial to keeping results standard. Some replicates were excluded because a long starvation time was affecting survival. This still might be altering results and should be carefully monitored in future experiments. Ideally insects will be fed on the exact same time frame for every replicate.

Future experiments and research into the possible genetic basis for traits beneficial in the possible coevolution between *S. marcescens* and *A. tristis* could be of interest. For example, it could be useful to have a greater understanding of what makes phytopathogenic *S. marcescens* different from its insect pathogenic strains. Experiments could also be designed that consider *Anasa tristis*' symbiont *Burkholderia* or attempt to break down the environmental factors to understand what exactly drives these differences. More experiments that explore the cost of vectoring and its relationship to diet and stress could be entertained using other ecological systems. Another experiment could be included that considers intermittent transmission up to 30 days. A different design than that used here with multiple sacrifice treatments would be needed. Based on the variables studied, such as environment or symbiont presence, this could shed light on how intermittent transmission might be occurring. Alternatively, it could be beneficial to have a larger sample size of insects and to only look at insects for the week where they seem to be strongly influenced by *Serratia*. This study would likely drop the environmental variable in order to focus on the costs of the infection. With the results of this experiment and this suggested further investigation the relationship between *S. marcescens* and *A. tristis* can be better understood. The work done with this system can inform an understanding of other pathogens and help aid in the fight against all kinds of insect vectored disease.

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