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Density and Mass Effect on the Development of Phormia regina

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Density and Mass Effect on the Development of *Phormia regina*

By

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Forensic entomology is the application of the study of arthropods to the criminal justice system. This is primarily done through the development of a post mortem interval (PMI) based the insect evidence present. A practitioner must be able to determine the age of the insect through temperature data. One factor influencing the temperature dependent development is gregarious behavior. Current literature describes a faster development rate due to an increase in feeding efficiency and temperatures produced by this aggregate. However, there is very little literature defining a minimum number needed to induce this effect and little to none on it for *Phormia regina*.

Two experiments were done to explore the effect of aggregation on *P. regina* juveniles. Both experiments used growth chambers set to 25°C and egg masses from lab reared colonies. The first experiment used two chambers with differing densities of larvae at 25, 50, 100, and 200 in 490ml plastic containers with 2cm of pine shavings. Larvae were reared on 2g of liver in 29.5ml plastic cups in the containers and liver was added as needed. Each container was subsampled with replacement every day to check the development of the larvae (10-25%) until adult eclosion. Aggregate temperatures were checked with a digital heat thermometer gun (TES) and probe throughout the duration of the experiment. No significant difference in development was observed.

The mass temperatures in experiment one did not cause a decrease in development time as reported by the literature and the 100 counts had a higher average

than the 200s. Experiment two was designed to explore this by increasing the space provided from a 490 ml container to a 1.42L one and to increase the feeding cup size from 29.5ml to 88.7ml. 25 and 200 larvae were provided 5g of liver to feed on initially.. Development times were significant longer for the 200 counts in the third instar and caused a downstream effect.

Dedication

For Uncle Johnny, I said I'd get it done

I'm sorry I couldn't show it to you in time

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To say that my graduate career has been an easy one would be an incorrect statement. It has been a long journey that started five years and two schools ago. Without the help of friends, family, mentors, advisors, and colleagues I would not be here.

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CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

Introduction

Forensic entomology is the application of the study of insects and related arthropods to the criminal justice system. There are three ways in which this is done; urban entomology, stored-product (food storage) entomology, and medico-legal entomology (Amendt et al 2011, Haskell & Williams 2008). Of these three the latter is what most people are familiar with due to the popularity of shows like Bones and C.S.I.

An urban entomological assessment is typically required in situations where an infestation by a pest insect in a building or other economically important location that is not agriculturally related. These situations involve insects that are commonly regards as urban pests, such as termites and cockroaches. In food storage entomology, the practitioner is brought in to assess the presence of insects in food and to determine whether contamination happened pre or post production and if it goes over the government allowance of insect parts per weight. As it is feasibly impossible to prevent any contamination from insects occurring it is important for the entomologist to work with the regulatory agencies to determine when the contamination occurred and if it is a problem (Hall 2010).

Both differ from medico-legal entomology as it focuses on the interaction between insects and the human body in criminal and civil cases. The most common use for forensic entomology is in homicide cases where the practitioner is hired to provide the

prosecution or defense with a post mortem interval (PMI). However, forensic entomology can also be used in cases of abuse where insects have infested living tissue due to negligence. These cases typically involve persons who cannot take care of themselves hygienically and rely upon others to do so or are of low socioeconomic status. As such we mainly see human myiasis occur in the young, elderly, disabled, and homeless (Francesconi & Lupi 2012, Goff et al 1991). In either situation it is the job of the practitioner to analyze the insect evidence and determine the facts based upon the data.

Forensics

In 2009 the National Research Council (NRC) released a report calling for higher scientific standards in the forensic sciences. The report states how lack of standardization, accreditation, certification, training. education, and funding have harmed the efficacy of forensic science and its application. Counties, states, and the federal government have different levels of funding and regulation that they adhere to in an investigation. This disparity means that the resources for training, education, and oversight may differ from county to county, thereby increasing the likelihood that an improper application of forensic science may occur.

The problems also stem from the manner in which our judicial system operates. Daubert standards allow a judge to deem what forensic evidence is admissible (National Research Council 2009). However, the judge is not a scientist and typically lacks the necessary education to make these decisions beyond the criteria set forth by the courts.

Furthermore, the adversarial nature of our judicial system also does not allow a proper objective review of forensic evidence as the lawyers are lacking in the same education as the judge. Cross examination only allows for each lawyer to present the strengths and flaws of the evidence as it is presented to their knowledge, it does not allow for a thorough review of the science and whether it should have been admitted in the first place.

The report further details this point and how many of these fields lack empirical data to support their methodology and that much of their interpretation is subject and not objective. This is problematic in the face of newer fields, like DNA analysis, which have an academic counterpart that produces supportive research. New data driven forensic sciences have, in part, shown how improper application of other fields has been. DNA evidence has exonerated persons whom were previously found guilty with other forensic sciences (National Research Council 2009).

In response to these issues the National Science and Technology Council (NSTC) created a subcommittee under the Council on Science (CoS) to review the current state of forensic science and how to improve it at the federal level. The subcommittee began in July 2009 and completed its task on December 12th, 2012. Its subsequent report was published in 2014 and detailed the current status of certification and accreditation for various forensic personnel and the challenges for meeting the 2009 report's criteria. The last thing the report addresses is the necessity for a universal code of ethics for forensic practitioners that the 2009 report proposed. The subcommittee suggests the adoption of the code of ethics from the ASCLD/LAB *International* Supplemental as it addresses

many concerns and has a wide application. Furthermore, they suggest that adoption of this code, or any, as a universal code of ethics by a forensic organization can be used as a prerequisite for application for funding, certification, or accreditation (National Science and Technology Council 2014). If this tactic were implemented this means that the singular organization for forensic entomologists, the North American Forensic Entomology Association (NAFEA), would have the same code of ethics as others in the forensic community and be held to the same standards.

These standards, as proposed initially by the 2009 report, were a catalyst for the development of a new framework in forensic entomology referred to as the Period of Insect Activity (PIA) as proposed by Tomberlin et al (2011). The framework, in itself, proposes further areas where research is needed and an expansion of **Best practice in forensic entomology—standards and guidelines** (Amendt et al 2007). However, its proposal was the cause of some controversy amongst practitioners and led to multiple letters to the editor.

The initial letter states that the terminology proposed by Tomberlin et al has been improperly used and as such has not represented forensic entomology appropriately (Wells 2014). It goes on to state that many of the things suggested by the framework have not had an explanation as to how someone would accomplish them, thereby suggesting many of the concepts are esoteric in nature. Wells ends his letter by stating that no practitioner should use the terminology until “they really mean it.” In response three letters were published; Campobasso and Introna (2014), Michaud et al (2014), and Tarone et al (2014).

The letter by Campobasso and Introna (2014) states the misuse of terminology in common throughout multiple fields of science, that Amendt et al (2007) had already explained that PIA and PMI do not always correspond, and that Villet and Amendt (2011) had detailed how variable terminology adds to the confusion. They further emphasize that the need for standardization is essential for forensic entomology and all other fields. Michaud et al's (2014) letter states that the concerns brought up by Wells (2014) are semantic but indicate that some aspects of the framework are not possible without further research. This is often the case with frameworks/models as research typically confirms, modifies, or expands upon them. The framework provides a means for practitioners to research ecological principles that underly most of forensic entomology (Michaud et al 2014). The third letter involves authors from Tomberlin et al (2011) where they attempt to clarify their work in regard to Wells' comments and state that their framework will promote research in to the necessary topics.

Tomberlin et al's (2011) PIA framework was not the only publication to offer a critique of forensic entomology in response to the 2009 report. **Sampling Flies or Sampling Flaws? Experimental Design and Inference Strength in Forensic Entomology** by Michaud et al (2011) detailed how experimental design has been an issue in forensic entomology for quite some time. While it is true that most flaws in experiments are unintentional, flawed data when applied has consequences. The authors propose a variety of solutions that address replication, independence, and experimental/natural conditions in order to meet the criteria set forth by the 2009 NRC report.

Methods of Use

There are two primary ways in which a forensic entomologist produces a post mortem interval (PMI) estimate for investigators. The first is referred to as Accumulated Degree Days/Hours (ADD/H) and it relies upon the physiology of insects. Insects are poikilotherms that develop at variable rates depending on the temperatures they experience during their juvenile stages. Individuals of a given species that experience higher temperatures, excluding extremes, will therefore develop faster than those at lower ones. The development rate is calculated through a heat unit called Degree Days/Hours (DD/DH). This unit considers the amount of time the insect stays in a given stage and the average temperatures it experienced during that time. Insects must then experience a certain numbers of degree days to progress in development. Each degree day is then added cumulatively in a total called Accumulated Degree Days/Hours (ADD/H). The formula for which is:

$$DD = (\text{average daily temperature} - \text{developmental threshold}) * \text{days}$$

$$ADD = DD1 + DD2 + DD3 \ldots$$

The ADD/H calculation accounts for fluctuations in ambient air temperature that the insect experiences, the amount time spent in a stage, and the minimum temperatures needed for development. The fluctuations are important to account for because a stable

constant temperature is rarely seen in nature. The minimum development temperature, also called base temperature, is either acquired from reference materials that have already researched it for a given species or it has yet to be produced. For some practitioners a base of 10 ºC is typical whereas others use 0 ºC, this is usually dependent on the species they are looking at and the region in which they were found (Higley & Haskell 2001).

An alternate to degree days is degree hours, where the practitioner calculates the time spent at given temperatures per hour. This can be converted to ADD by multiplying by 24 for ease of use but ADD cannot be reversed to ADH as it gives an improper hourly temperature (Higley & Haskell 2001). This type of analysis is typically more thorough but requires hourly temperature data and can be time consuming.

The second method that a practitioner uses is an ecological principle called succession. The principle is that over a period of time the species present in an ecosystem will change. For forensic entomology this concept is important as the corpse is a new environment lacking in established populations (Anderson 2010, Perez et al 2014). As such we can predict insects are expected to appear over a given time and at what intervals in order based upon the area that the corpse was found.

This concept of succession on a corpse/carrion and its application to forensic entomology is typically to Megnín 1894. This publication, and others from that period, primarily looked at the necrophagous insect community, their successional patterns, and then organized them in to eight waves. Subsequent research in 20th century later expanded upon Megín's hypothesis through similar experiments (Catts & Goff 1992, Lefebvre & Gaudry 2009).

An alternate to the eight waves concept was proposed by Lefebrve and Gaudry (2009). Their research details distinct groups, units, periods, steps and phases in necrophagous insect succession. Where the presence and overlap, or lack thereof, of a species unique to a labeled group determines the period of time that corpse is in. Period is then paired with phase of development for the observed population, thereby telling the practitioner how long that given successional community has been present.

Calliphoridae

Calliphoridae is a family of Diptera commonly called blow flies. They are found throughout the world and are attracted to carrion and excrement. However, some species will lay their eggs on living tissue, which causes a medical condition known as myiasis. This process can happen to humans and animals, which is why some species are considered agricultural pests. Typically, the flies use the carrion or excrement as a resource for reproduction. Adults will consume either as a protein meal to facilitate egg and sperm maturation, and then lay their eggs on a similar substrate some time later.

The larvae are usually a shade of white throughout their development. They go through a number of juvenile stages, called instars, where they feed on the carrion/excrement. They will then enter a wandering/meandering phase for some time until they pupate. After a given amount of time, they will then eclose as adults (Byrd & Castner 2010).

Of interest to forensic entomologists are blow flies that are frequently found at crime scenes and their larvae. One species of interest is the black blowfly (*Phormia regina)*. This species is Holarctic and is ubiquitous throughout the United States (Byrd & Castner 2010, Hall 1948). Adults are more commonly found in the spring and fall but can be seen during the summer months (personal obs). The adult fly can be distinguished from other callophorids by its orange anterior spiracle and the lack of setae on the stem vein (Whitworth 2017). Instars are cream color and may wander away from food. They will also exhibit a massing behavior throughout their development. The development of the larvae is dependent on the ambient temperature of their surroundings.

Mass Effect & Density

Aggregation of the larvae can influence the development of the juveniles. This aggregate is commonly called a maggot mass and can produce temperatures up to 40°C and differentiate from the ambient temperature by 25°C (Charabidze, 2011, Deonier 1940, Gruner et al 2007). There have been previous attempts to characterize masses according to their size and density (Charabidze 2011, Goodbrod, & Goff 1990, Gruner et al 2007).

There is, to this author's knowledge, no published study that investigates the minimum number of individuals to cause a significant temperature effect due to aggregation. Experiments that use low counts are often geared towards base line temperature research than they are for mass effect whereas the papers that investigate mass effect use counts in excess of several hundred. It is understandable that this has

been the design as in the majority of situations the practitioner approaches a corpse with several thousand maggots upon it. However, even with knowing the upper range of the mass effect and what temperatures it can produce it is still essential to understand the minimum amount needed to induce a change.

It is thought that accessibility of food may be one reason as to why the larvae aggregate in mass. The large number of larvae present on a corpse help break down tissues thereby making it easier for the individual and others to feed. Typically it is unnecessary for them to seek out additional food beyond looking for a new place to feed on the substrate. However, Charabidze (2011) mentions that the size and surface area of food source has an effect on the aggregation and displacement of the maggots during feeding, and Roe (2014) noted that *Phormia regina* would exhibit wandering behavior throughout their development. If the substrate is large enough then why is this wander/displacement behavior being exhibited? The larvae tend to mass when feeding, so why are individuals wandering away? There is some thought that the aggregates themselves become too hot for the larvae to endure and individuals will rotate to the outside of the mass. If this is the case then why do we see individuals leaving the mass?

Research Objectives

Our initial goal was to answer one question during this experiment; what is the minimum number of larvae needed to cause a significant change in mass temperature? However, during the first experiment it was observed that *P. regina* larvae would wander away from the provided food even though there was a sufficient amount to support all

individuals. Upon review it was noted that the cups in which the liver was placed may not have been large enough to accommodate a high number of individuals. While masses were observed in the cups the containers with high counts would often have two masses that formed; one inside and one outside. As such this led to a second experiment where we questioned if there was an effect between larval density and physical space that had an influence on development time.

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CHAPTER 2. MINIMUM POPULATION SIZE TO OBERSVE MASS EFFECT IN PHORMIA REGINA

Introduction

Phormia regina (Meigen) are necrophagous flies, commonly called the black blowfly, that belong to the family Calliphoridae (Diptera). They are a Holarctic species found in the United States, sans southern Florida, that has importance in agriculture, medicine, and forensics. The black blowfly is a common cause of myiasis in animal operations (Byrd & Allen 2001) and its larvae have been used to clean wounds in maggot therapy (Byrd & Castner 2010). In forensics the black blowfly can be one of the first insects to colonize a corpse (Joy et al 2002) and/or arrive within minutes (Byrd & Castner 2010, Marhenko 2001, Shean et al 1993).

Females use the corpse as a source of protein and for oviposition upon arrival. Depending on the temperature eggs will typically hatch between 6-40 hours and larvae will then begin feeding. The juveniles will then progress through three stages, called instars, that are separated by a molt and increase in size. The third instar then enters a wander/meandering phase where they move away from the corpse in order to pupate and develop in to adults (Byrd & Castner 2010, Heaton et al 2014, Roe 2014).

As stated in Chapter 1, insects are poikilothermic and *P. regina* are no exception to this rule. During the juvenile stages their development rate is dependent on the ambient temperature. However, it is during these stages that we observe gregarious behavior commonly called a maggot mass. This mass can create a microclimate that is higher than

the ambient temperature. As such the development rate of the maggots can then be affected by the higher temperatures they experience in the mass than that of the ambient area. This may influence the development of a post mortem interval as the forensic practitioner may mistake the individuals in the mass for being older than those outside of it (Heaton et al 2014).

To address this problem, it is important to understand the relationship between the number of maggots present and the temperature of a mass. An aggregate can only develop significant temperatures once the numbers exceed a given minimum. One reason for this mass is thought to be that it optimizes feeding behavior for the larvae. By aggregating the larvae produce enough proteolytic enzyme for tissue breakdown and digestion. If this behavior is beneficial for development, then why are there individuals outside of the mass? The temperatures of the mass may exceed the higher threshold limits for the larvae and actually have a negative impact on the development. Gruner and Slone (2007) detail that masses are capable of producing temperatures up to 50 °C, which is above the lethal limit for blowfly larvae.

The objective of this study was to determine the minimum number of larvae needed in an aggregate to effect a significant change in temperature. The current literature has primarily focused on larval counts of several hundred or higher and not a minimum amount needed. In addressing the minimum, it may be possible that a formula between the number of maggots in an aggregate and temperature could be produced. Thereby allowing the practitioner to accommodate for the maggot mass effect when making their PMI estimates.

Materials and Methods

Adult *Phormia regina* were provided by Dr. Amanda Roe from the College of Saint Mary in Omaha, Nebraska. Flies were then placed in a mesh cage (46 cm x 46 cm x 46 cm) (Bioquip Products, California) and kept at 22°C with a 12:12 photoperiod. Adults were given water through a flask filled with water that had a paper towel in it secured with a rubber band. Sugar cubes were also provided in petri dishes for the flies to feed upon. Sugar and water were replaced ad libidum.

A week prior to egg collection the flies were provided beef liver for 24 hours in 59.15ml plastic cups for ovarian development. After a week flies were then provided another set of cups with beef liver in them to oviposit on for 24 hours. The cups were then removed from the cages and the eggs present on the liver were transferred to a glass petri dish. Distilled water was then added to the dish to help aid in breaking up the egg clusters with the use of a fine paint brush. Eggs were counted in sets of 25, 50, 100, and 200 and then placed on 2g (± 0.2) of beef liver in a plastic 29.5ml cup. Counts of 25 and 50 were repeated eight times, counts of 100 were repeated four times, and counts of 200 were repeated five times (N = 25). Counts were limited by the number of eggs produced by the flies.

Cups containing liver and eggs were then transferred to 7 x 7 x 10cm plastic containers with approximately 2cm of pine shavings. The pine shavings were to act as a pupation substrate for the larvae. The plastic containers were then randomly assigned a letter and placed in a growth chamber (*DigiTherm*® 38-liter Heating/Cooling Incubators).

Chambers were kept at 25.0°C (± 0.1). Each chamber contained four of each 25 and 50 count, and two of each 100 and 200 count with one chamber having three 200 count containers.

Liver was added as needed in 2g pieces to each cup as the experiment progressed. Containers were checked each day to ensure that there was adequate liver, the larvae were developing, and that the larvae were alive. If an aggregate was observed during this then the mass temperature was taken with an infrared heat gun (TES) and probe. The probe was inserted in to the mass and the temperature recorded, it was then inserted in to the liver and its temperature was recorded as well. The probe was cleaned and then this process was repeated for each container. Between 10 – 25% of each container was subsampled with replacement for larval stage. Development rates were then compared to the rates from Roe 2014. Behavior was also noted during each check. Containers were monitored and maintained in the growth chambers until adult eclosion.

Analysis

Data were statistically analysed with SAS University Edition Software. Where data sets were unbalanced (missing) data, a mixed methods analysis (Proc Mixed in SAS) was used.

Results

200 Count		100 Count		50 Count		25 Count	
Stage	Temp	Stage	Temp	Stage	Temp	Stage	Temp
1	24.1	1	23.74	1	23.21667	1	23.6
2	25.02	2	25.41429	2	25.05625	2	24.15
3	26	3	26.5	3	25.37692	3	24.84286

Table 1. Average mass temperature in °C observed at different instars.

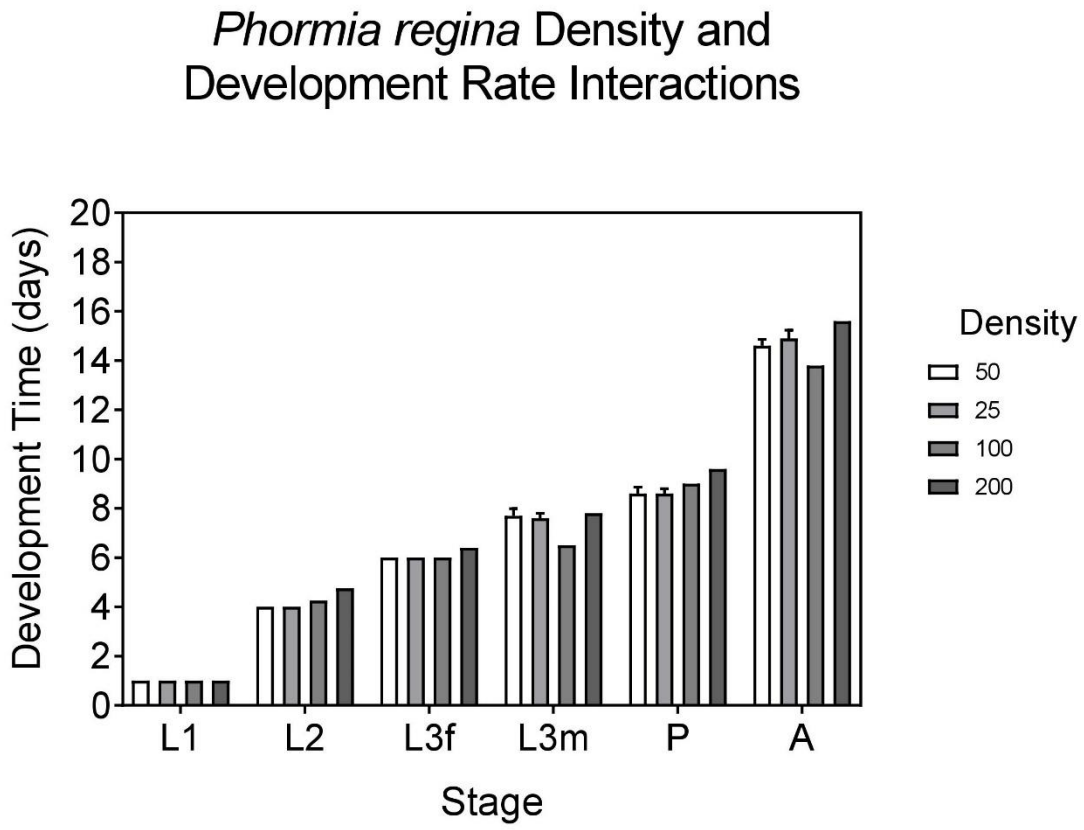

Figure 1. Density and development interaction of *Phormia regina*.

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Density	3	137	0.12	0.9469

Table 2. Proc Mixed table for all density treatments

Average temperatures for higher densities were greater than the set chamber temperatures at later instars. Higher temperatures were not significant though as p = .9469 according to the Proc Mixed test done. Figure 1 also illustrates this as density is correlated with mass effect based temperature increase and no significant difference is displayed.

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CHAPTER 3. MASS DENSITY AND SPATIAL ALLOWANCE'S EFFECT ON PHORMIA REGINA INTRODUCTION

Introduction

All organisms need space to develop and live. However, an ideal space that is not intruded upon by others is not realistic. An individual competes with members of its population and its community. For blowflies (Diptera: Calliphoridae) this competition is escalated by the ephemeral nature of their reproductive resource. Carrion is not a permanent fixture in an environment and blow fly adults and larvae need to exploit it as quickly as possible to develop. This is why the application of successional patterns for post mortem interval estimation is viable.

The temporary nature of carrion/corpses also leads to intraspecific and interspecific competition amongst blow flies and other necrophagous insects. Studies have primarily focused on interspecific competition under various population densities and their interactions (MacInnis 2018). However, few studies have looked at intraspecific competition.

Reigada and Godoy (2006) examined intraspecific competition at two densities, 200 and 1000, in *Chrysomya megacephala* on 50g of ground beef. There is no mention as to how the larvae were kept for the duration of the study besides being maintained at two different temperatures, 20 °C and 30 °C. They did report that there was an effect on the fecundity and body size of the adult flies, which is similar to what Ireland et al (2005)

report for *Calliphora vomitoria* but did not state whether there was a delay in development.

Goodbrod and Goff (1990) reared two different species of Calliphoridae, *C. megacephala* and *Chryosomya rufifaces*, in five-liter containers for their experiment. They examined intraspecific competition at densities of 1, 2, 4, 8, 10, and 40 on 25g of liver except for the 40 count which was on 12.5 grams. They report that at higher densities the development was faster, and adults and puparia weighed less due to the intraspecific competition.

Ireland and Turner (2006) reared *C. vomitoria* at densities of 1, 5, 10, 20, 50, 75, and 100 on 10g of liver for their experiment. The larvae and liver were placed in weigh boats and then in to 12 x 9.5 x 6.5cm plastic containers. They report that the larvae developed faster at higher densities, but their size and weight were less than those reared at the lower range.

The previously mentioned experiments either lack thoroughness in their methodology or detail that the containers in which they kept their larvae for the duration of their experiment were larger than what was used in chapter 2. From the experiment in chapter 2 it was observed that the lower densities did not have a delay in their development, which is similar to the aforementioned studies, whereas the higher numbers did, which is not. Even with adequate food the 100 and 200 counts did not appear to have enough room to feed, wander, and pupate in the 7 x 7 x 10cm plastic containers. The manner in which liver was provided also differs. A weigh boat, as Ireland and Turner (2006) used allows easier access than a condiment cup as was used in chapter 2.

Goodbrod and Goff (1990) and Reigada and Godoy (2006) do not state what their food source was in, but they do report the weights. This indicates that the mechanism which they used must be larger as well.

This led to several questions as to whether or not the containers were having an effect on the development rates and if the cups containing the liver were large enough for all individuals to feed. In the 100 and 200 counts there would often be a large mass of larvae outside of the cup containing the liver. As such the following experiment was done to examine the effect of increased spatial allowance for feeding and wandering behavior on two densities of *Phormia regina* at a constant temperature.

Material and Methods

Methods were similar to Chapter 2 with modification and are repeated for convenience. Larvae were counted in to groups of 25 and 200 eight times and placed on 5g of beef liver in 88.7 ml plastic cups. The cups were then placed in 1.42L plastic containers (Simply Done) with 2.5cm of pine shavings. The plastic container was then placed in one of four growth chambers (*DigiTherm*® 38-liter Heating/Cooling Incubators) set to 25.0°C. This was repeated four times for each count and counts were randomly assigned to one of the four growth chambers.

In the middle of the experiment 5g of beef liver was added to each of the 200 count containers as the original 5g was not sufficient for development. Containers were checked each day to ensure that there was adequate liver, the larvae were developing, and that the larvae were alive. Between 10 – 25% of each container was subsampled with

replacement for larval stage. Development rates were then compared to the rates from Roe 2014. Behavior was also noted during each check. Containers were monitored and maintained in the growth chambers until adult eclosion.

Analysis

Data were statistically analysed with SAS University Edition Software. Where data sets were balanced, analysis of variance (Proc ANOVA in SAS) was used.

Results

There was no difference in development time between the two densities at first and second instars ($F = 0.49$, $p = 0.7466$). Development time as displayed on figure 1 shows the time from egg to first instar. There was an effect on the development at the third instar stage between the two treatment densities ($F = 5.50$, $p = 0.0111$). Figures 4 and 5, and Tables 4 and 5 show the downstream effects of delay from the third instar on the following stages. Figure 6 shows the differences and lack thereof in development time between the two treatments.

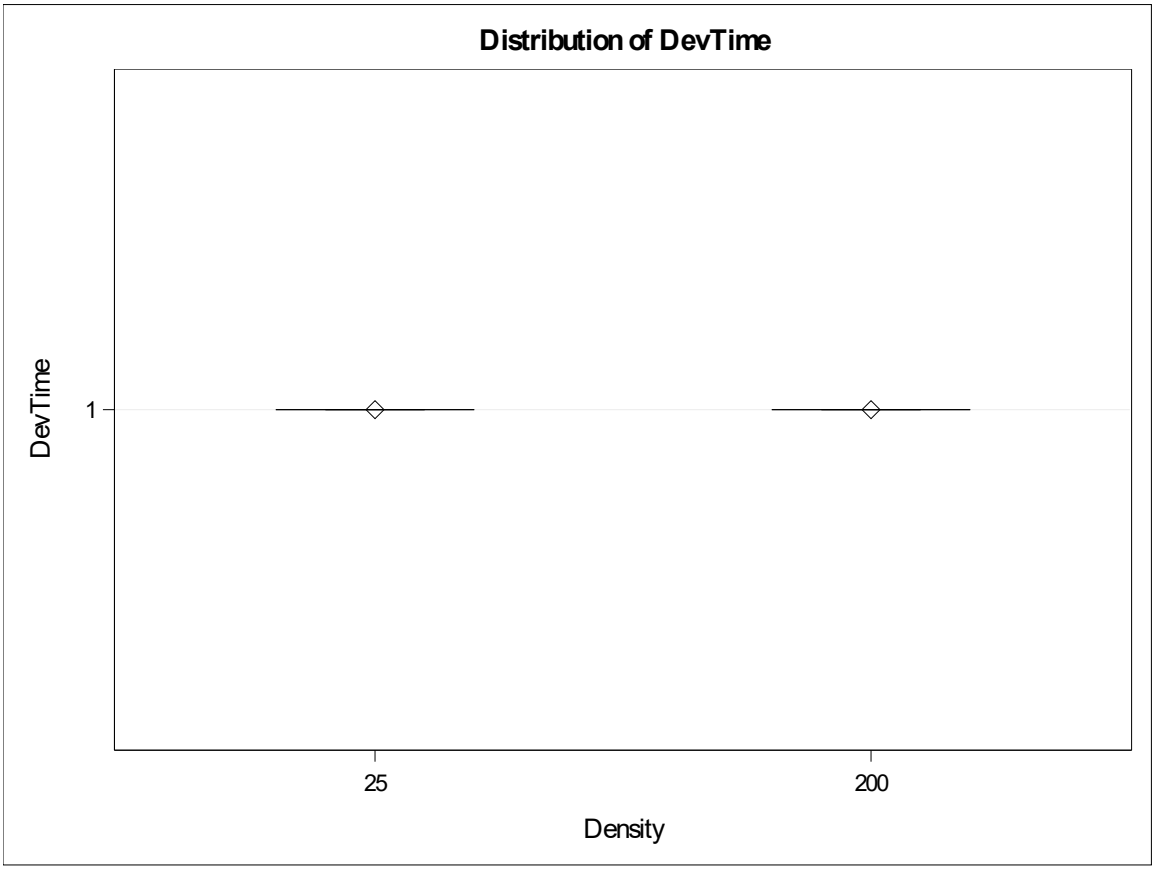

Figure 1. The effect of density on development time for first instars at densities of 25 and 200.

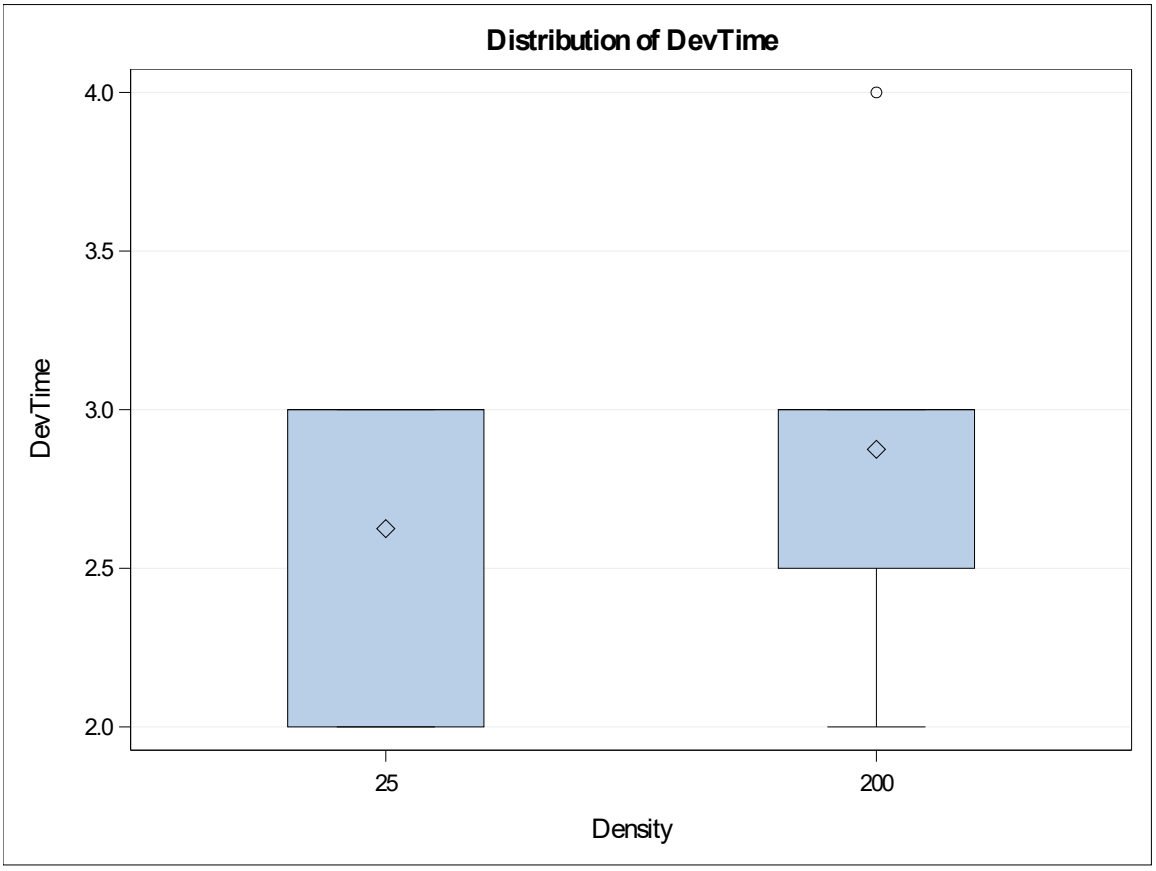

Figure 2. The effect of density on development time for second instars at densities of 25 and 200.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.75000000	0.18750000	0.49	0.7466
Error	11	4.25000000	0.38636364		
Corrected Total	15	5.00000000			

Table 2. Analysis of variance (ANOVA) table for 2nd instars where F = 0.49 and p = 0.7466.

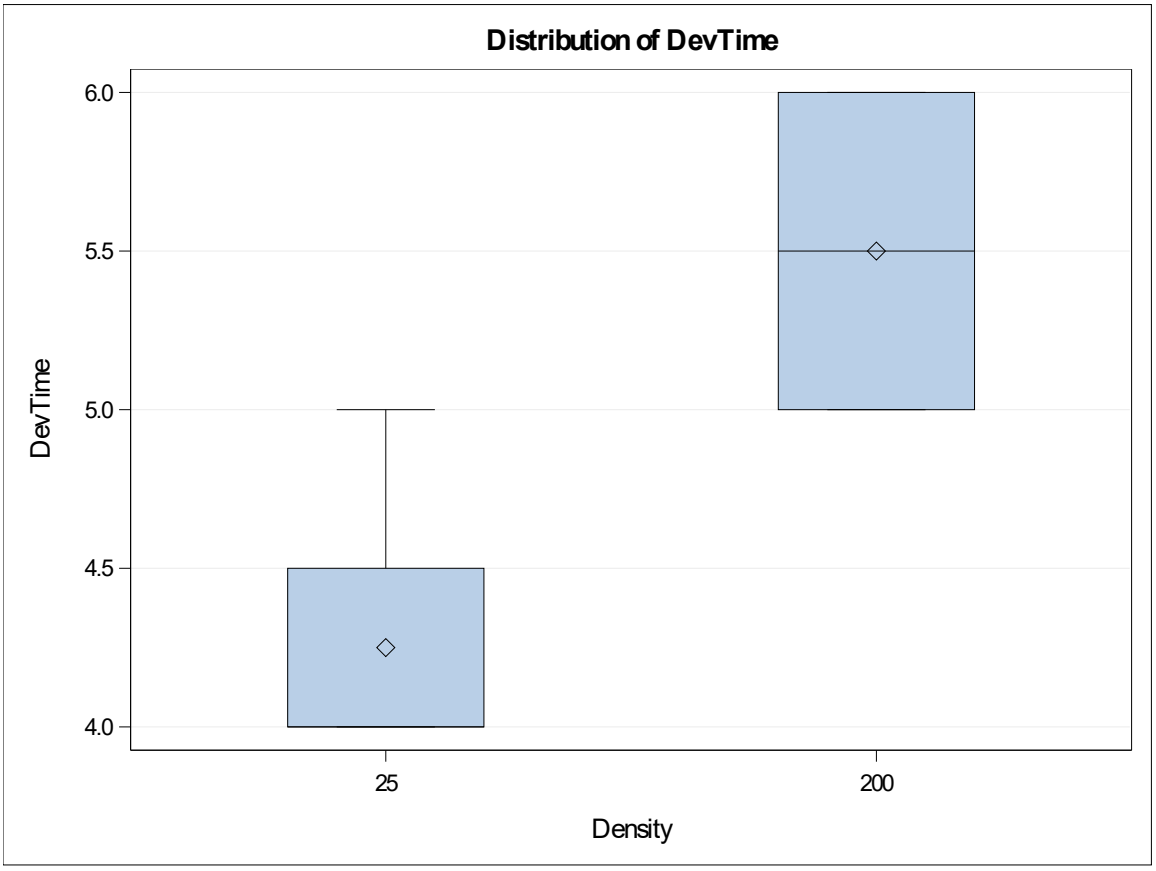

Figure 3. The effect of density on development time for third instars at densities of 25 and 200.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	6.50000000	1.62500000	5.50	0.0111
Error	11	3.25000000	0.29545455		
Corrected Total	15	9.75000000			

Table 3. Analysis of variance (ANOVA) table for 3rd instars where F = 5.50 and p = 0.0111.

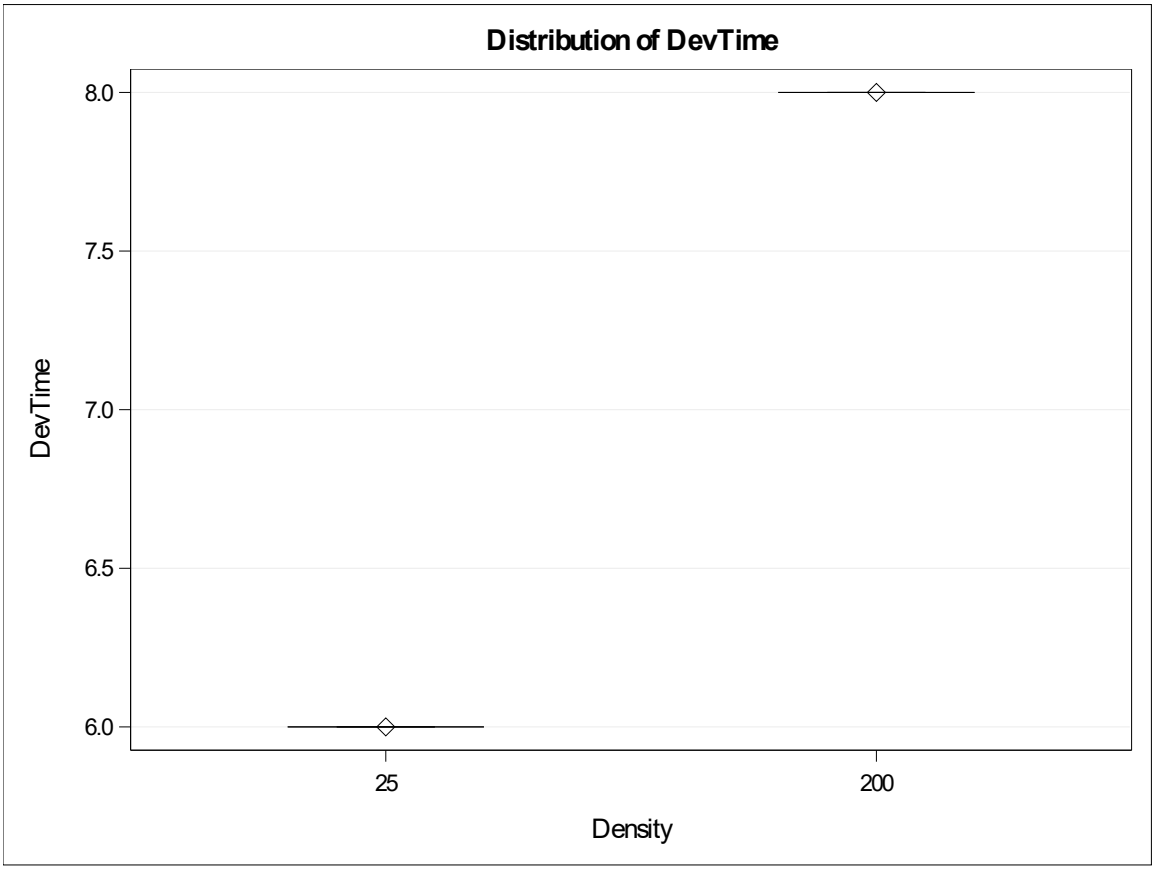

Figure 4. The effect of density on development time for wandering phase at densities of 25 and 200.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	16.00000000	4.00000000	Infty	<.0001
Error	11	0.00000000	0.00000000		
Corrected Total	15	16.00000000			

Table 4. Analysis of variance (ANOVA) table for 3rd meandering/wandering instars where downstream effects are observed in the F and P values.

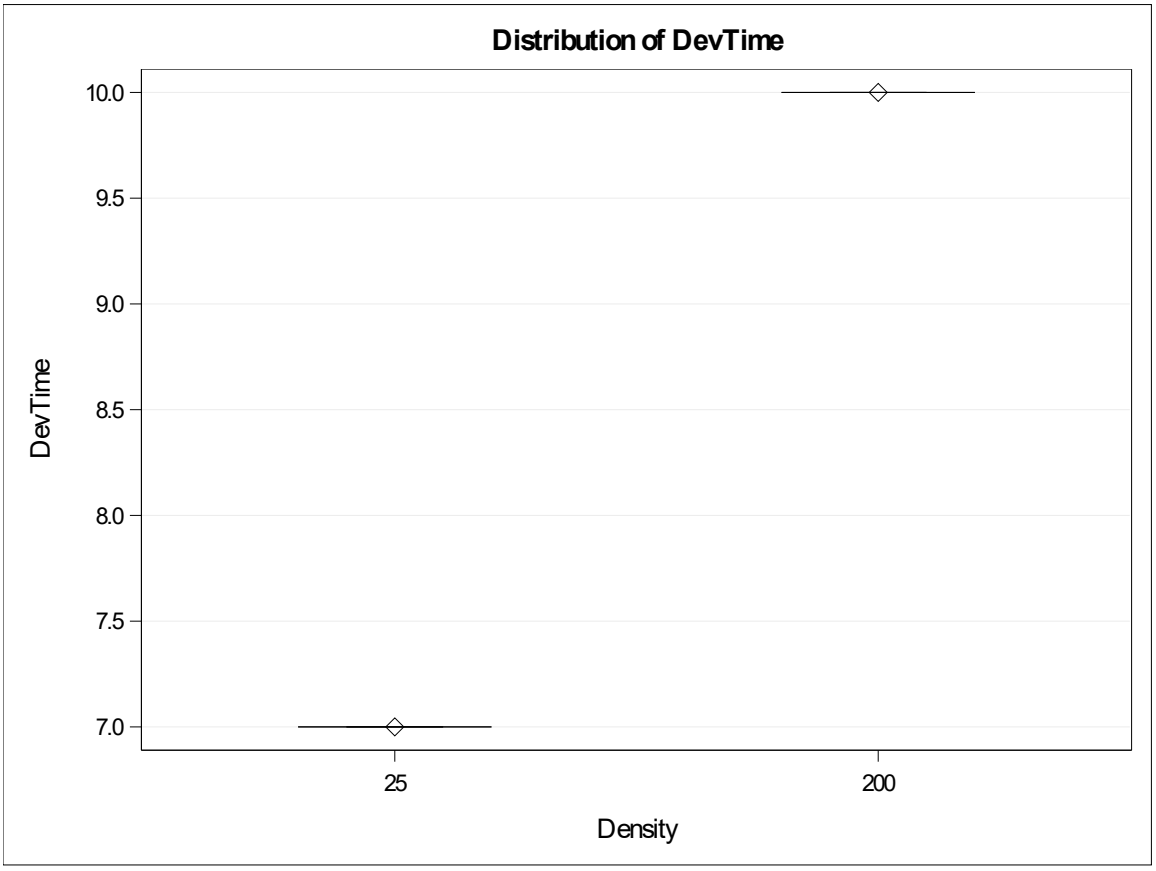

Figure 5. The effect of density on development time for pupae at densities of 25 and 200.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	36.00000000	9.00000000	Infty	<.0001
Error	11	0.00000000	0.00000000		
Corrected Total	15	36.00000000			

Table 5. Analysis of variance (ANOVA) table for pupal stage where downstream effects are observed in the F and P values.

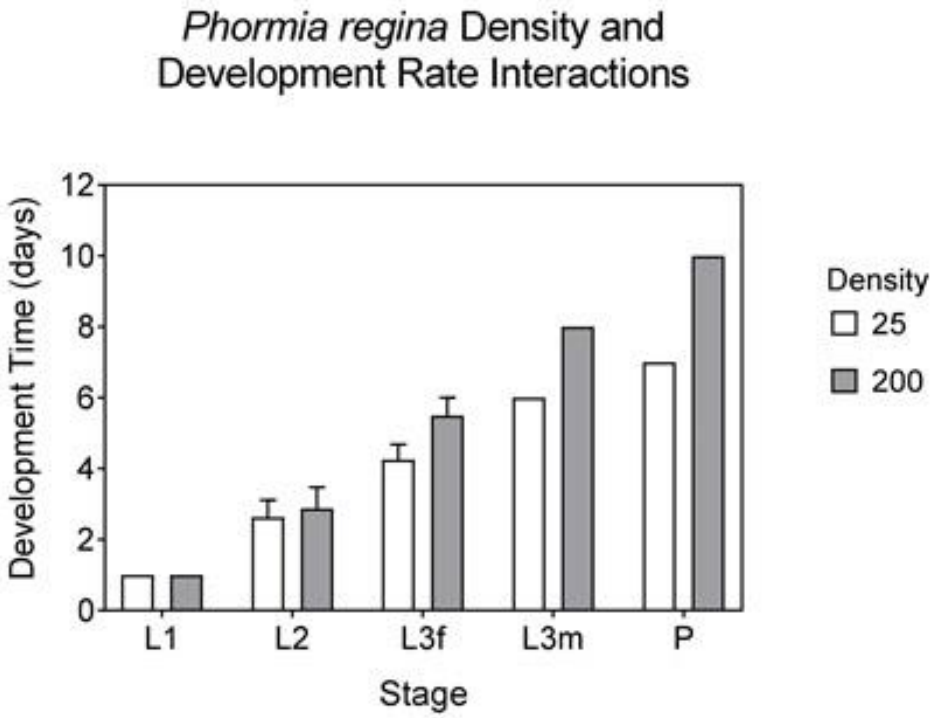

Figure 6. Differences in development time (days) between the two treatment densities.

Conclusion

For the duration of the first experiment it was observed that the low counts, 25 and 50, would have larvae aggregate in masses. Only the 50 counts had an average temperature that was above the set 25°C but not by a significant amount. The high counts, 100 and 200, did exhibit 1-1.5°C higher temperatures than the growth chambers with individual containers getting as high as 28.0°C. However, it was observed that the larvae in these containers would not all be in an aggregate. Often there would be a mass on the food and a large portion of larvae would either be wandering or in a separate mass

somewhere in the container. This behavior was exhibited more often in the 200 counts than in the 100s and not in the 25 and 50s.

The results in this experiment also show that the 100 counts were producing an average temperature greater than the 200s. This is in direct contrast with the reported literature and caused the development of the second experiment. The results for the latter indicate that something unknown is having an effect as the data also contradicts what is reported in the literature that an aggregate should reduce the amount of time it takes for development. There are a few possibilities that may have caused this, but the most likely ones will be examined here.

One possibility is the methodology used to rear the larvae and feed them. In Ireland and Turner (2006) it states that they used weigh boats with dimensions of 8 x 6.5 x 1.5cm with 10g of beef liver. This means that the surface area of the liver available to the larvae is greater than that of the plastic cups that were used in this experiment. Goodbrod and Goff (1990) and Reigada and Godoy (2006) do not report what they used to hold their food source in their containers for the duration of their experiments. However, it can be inferred that any apparatus they used provided enough surface area for adequate feeding due to the large amounts of food they used; 12.5g, 25g, and 50g.

The difference between these studies and the data shown here indicate that adequate access to the food source may cause a delaying effect, regardless of the mass. While massing does increase feeding efficiency due to the increase in proteolytic enzyme production this effect would become null if individuals cannot access the food. The same goes for the temperature effect that should increase the rate of development. If food

access is limited, then any beneficial behavior to increase development rate is canceled out.

However, Goodbrod and Goff, Ireland, and Turner, and Reigada and Godoy all state that along with shortened development times there were also decreases in weight and size for pupa and adults. While these two things were not measured for this experiment, the increase in development time that was observed indicates that there may be a minimum threshold for larvae to develop in to viable smaller adults. Intraspecific competition may therefore be preventing individuals from reaching the minimum at an increased rate when access to food is limited by a physical barrier.

Another factor that may have influenced the results is the method in which developmental stage data were collected. This data was collected once per day throughout the duration of the experiment, which means that the stage transition was most likely missed for all juvenile stages. Roe (2014) detailed that transition from 1st instar to 2nd can happen within hours, as well as 2nd to 3rd. The longest development time reported by Roe (2014) and the rest of the literature is for 3rd instars. As such the window of time in which the transition to the wandering/meandering phase may also have been missed. This is further confounded by the behavior of *P. regina* to wander throughout all juvenile stages and that said phase does not have a defining characteristic to differentiate it beyond the lack of food in the crop. Repeating the experiment with a greater number of replicates and adhering to an intensive methodology to check stage development may help identify some of the unknown variables that caused this outcome.

For forensic entomologists this effect needs to be taken in to account when

developing a post mortem interval (PMI) estimate. While maggot massing behavior can reduce the time to adulthood in most cases, the inverse is also possible. If a scene has physical barrier that might prevent larvae from spreading out as they feed on a corpse and in turn cause a delay in development. This may also be true for cases of dismemberment where a barrier is present as well. As such the practitioner could possibly underestimate the age of the maggots present. Furthermore, this study indicates that the space provided for a food source and for wandering behavior during an experiment may have a significant effect on development regardless of mass effect and future research should take this in to account with the appropriate intensive checking of stage transition time.

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