LIFE-HISTORY TRAITS OF *CHRYSOMYA RUFIFACIES* (MACQUART) (DIPTERA: CALLIPHORIDAE) AND ITS ASSOCIATED NON-CONSUMPTIVE EFFECTS ON *COCHLIOMYIA MACELLARIA* (FABRICIUS) (DIPTERA: CALLIPHORIDAE) BEHAVIOR AND DEVELOPMENT

A Dissertation

by

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Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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August 2013

Major Subject: Entomology

ABSTRACT

Blow fly (Diptera: Calliphoridae) interactions in decomposition ecology are well studied; however, the non-consumptive effects (NCE) of predators on the behavior and development of prey species have yet to be examined. The effects of these interactions and the resulting cascades in the ecosystem dynamics are important for species conservation and community structures. The resulting effects can impact the time of colonization (TOC) of remains for use in minimum post-mortem interval (mPMI) estimations.

The development of the predacious blow fly, *Chrysomya rufifacies* (Macquart) was examined and determined to be sensitive to muscle type reared on, and not temperatures exposed to. Development time is important in forensic investigations utilizing entomological evidence to help establish a mPMI. Validation of the laboratory-based development data was done through blind TOC calculations and comparisons with known TOC times to assess errors. A range of errors was observed, depending on the stage of development of the collected flies, for all methods tested with no one method providing the most accurate estimation.

The NCE of the predator blow fly on prey blow fly, *Cochliomyia macellaria* (Fabricius) behavior and development were observed in the laboratory. Gravid female adult attraction was significantly greater to resources with predatory larvae rather than prey larvae and oviposition occurred on in the presence of heterospecific (predatory) and conspecific larvae equally. However, the life stages necessary for predation to occur

never overlapped and so these results may not be as surprising as they seem. Conversely, exposing prey larvae to predator cues through larval excretions/secretions led to larger prey larvae and faster times to pupariation when appropriate life stages overlapped. Differences in size and development times of prey larvae in the presence of predatory cues could lead to errors when estimating the mPMI. These data also partially explain the ability of *C. macellaria* to survive in the presence of *Ch. rufifacies*. Colonization of a resource with late instar *Ch. rufifacies* enhanced development and size of resulting larvae indicating that lag colonization, rather than being a primary colonizer, could become an alternate strategy for *C. macellaria* to survive the selective pressures of the predator, *Ch. rufifacies*. The differing effects of temperature on *Ch. rufifacies* and *C. macellaria* may also lend an advantage to *C. macellaria* over the predacious *Ch. rufifacies* in an environment with variable temperatures unlike what *Ch. rufifacies* is adapted for.

DEDICATION

First and foremost I would like to thank my Lord and Savior Jesus Christ for providing the means and the strength to accomplish all that I have done. I dedicate this dissertation to my family who has supported me in all that I have done. To my mother and father who have worked hard every day of their lives so that my brothers and I could attend college, I am truly grateful. I thank my brothers for maintaining my sense of humor in times when things seem overwhelming. Last but not least I would like to dedicate this work and my success to my beautiful wife Christina. Without whom I would never have finished this work and would certainly not have survived school. She has been my rock when times are hard and I know that she has put up with more during this time than should be required of a wife.

ACKNOWLEDGEMENTS

I would like to thank Dr. Jimmy K. Olson for giving me a chance to do work in his lab and help feed my interest in this field. Without his help I would not have been introduced to my major advisor, Dr. Jeffery K. Tomberlin. Without Dr. Tomberlin's immense guidance I surely would not be where I am today. He has gone above and beyond the role of a mentor and advisor and I consider him a great friend. He truly is a godsend.

I would also like to thank Dr. Aaron M. Tarone, my unofficial co-advisor, for his invaluable help with my research and trust in me as his teaching assistant for his course. Sincere thanks to Dr. S. Brad Vinson for his insights in insect behavior and his help whenever I needed it. Lastly and certainly not least of my committee members is Dr. Michael Longnecker with whom his support and guidance with my statistics was invaluable with data that are challenging to say the least.

To the members of the FLIES lab past and present thank you for your help and support especially during those long days and nights. I hope I was as much a help to you all as you all were to me.

Thanks also go to the Department of Entomology faculty and staff for making my time undergraduate and graduate work at Texas A&M University a great experience. I felt welcomed and encouraged in all that I did which is something that is lacking in larger departments.

NOMENCLATURE

TOC	Time of colonization
mPMI	Minimum post-mortem interval
ADH	Accumulated degree hour
ADD	Accumulated degree day
CE	Consumptive effect
NCE	Non-consumptive effect
ES	Excretions/Secretions
GAM	Generalized additive model

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Ephemeral Resources

Ephemeral resources are characterized as being in a state of decay (Elton and Miller 1954) and are available as a resource for a short period of time. It is a discrete habitat distributed across space and time (Beaver 1977) and because of its quick deterioration rate it usually supports only a single generation of species (Beaver 1977, Atkinson and Shorrocks 1981), which show little divergence on the resources they consume (Denno and Cothran 1975, Rathcke 1976). The appearance of such resources in an environment create a pulse of energy to be utilized across the community and throughout trophic levels (Yang et al. 2008).

Insects on Ephemeral Resources

Insects that utilize decomposing remains tend to follow a series of phases (Tomberlin et al. 2011). These phases include the death of an animal, the detection of that carrion resource in the environment, location of the resource, colonization of the resource and lastly dispersal from the fleeting resource. Blow flies (Diptera: Calliphoridae) are the primary invertebrate consumers of terrestrial carrion (Fuller 1934, Reed 1958, Payne 1965, Putman 1977, Braack and Retlef 1986, Peschke 1987, Wells and Greenberg 1992c). Competition on carrion can be between members of the same species (intraspecific) or between members of different species (interspecific) and occurs due to a regulating mechanism (i.e., the environment) (Klomp 1964). Intra- and

interspecific competition can be for space and therefore rely on density factors. However, interspecific competition can occur with one species lowering the numbers of the other species (i.e. predation) (Goodbrod and Goff 1990). Prey wanting to avoid predation will have to utilize different strategies to avoid predation/competition which will be discussed below as it relates to blow flies.

Factors Affecting Development

Development and growth of insects are driven by temperature but development rates can change based on species composition on a corpse, aggregations and tissue type consumed (Wells and Greenberg 1992b, Kaneshrajah and Turner 2004, Clark et al. 2006, Higley and Haskell 2009). Not only can these factors affect development rates, but size of insects produced which could alter age determinations using quantitative variables such as weight and length. Likewise, geographically distinct populations of the same species may have different development times for individual stadia or even variable sizes across temperatures when reared under similar conditions and caution should be taken when estimating the minimum post-mortem interval (mPMI) (Hwang and Turner 2009, Gallagher et al. 2010, Tarone et al. 2011). However, Cyr (Cyr 1993) (reference found in Picard and Wells (Picard and Wells 2009)) determined no difference in development rates for *Phormia regina* (Meigen) from different regions. Some of these development factors have relatively little effect on the development time/hours, while some can affect the development time by days to months (Villet and Amendt 2011).

***Temperature*:** Development data are available for forensically important blow flies giving hours necessary to finish a stage of life at various temperatures (Kamal 1958,

Greenberg 1991). With growth data, species/stage specific degree hours/degree days (DH/DD) (thermal heat summation units) can be calculated for the oldest flies collected to backtrack to the date of oviposition or the mPMI. Temperature experienced by insects on carrion is subject to ambient conditions and maggot masses (Higley and Haskell 2009) which are aggregations of larvae. Therefore, it is important when documenting a death scene to note the ambient, ground surface, body surface, underbody interface, maggot mass and soil temperatures for use in mPMI calculations (Byrd et al. 2009). Ambient temperatures during the days prior to discovery are determined by obtaining data from the nearest weather station and correlating it with data collected over several days at the death scene location (Haskell and Williams 1991, Catts and Goff 1992).

Ambient temperatures may be useful for determining mPMI for early larval instars, but maggot mass temperatures increase above ambient during the third larval instar, so ambient temperatures would overestimate the mPMI for older larvae who have developed in a maggot mass (Cianci and Sheldon 1990, Turner and Howard 1992, Higley and Haskell 2009). However, when determining whether or not to account for a maggot mass, one factor to consider is that the oldest individuals on the body could have developed prior to experiencing any aggregation (Wells and LaMotte 2001). Likewise, before using theoretical numbers for maggot mass temperatures to calculate a mPMI, it is important to look for evidence that a maggot mass has occurred (i.e. pupal cases in large numbers or disarticulation of the skeleton when no scavengers are thought to have been present) before your speculation of a maggot mass leads to an underestimation of the mPMI (Hall 2001).

VanLaerhoven and Anderson (1999) determined that buried carcasses in Canada did not form maggot masses (VanLaerhoven and Anderson 1999). The greatest difference between ambient and maggot mass temperatures is during periods of cold weather and the higher the maggot density, the higher the maggot mass temperature rises above ambient (Deonier 1940, Goodbrod and Goff 1990). Also, maggot mass temperatures in direct sunlight were determined to be directly correlated with ambient temperatures while maggot mass temperatures on shaded carcasses were not (Joy et al. 2006). However, Slone and Gruner (2007) determined contrary evidence that larval instar and density (based on the size of the larval mass and weight of the animal) did not affect the temperature of the maggot mass, but the size of the maggot mass along with ambient temperature affected the maggot mass temperature (Slone and Gruner 2007).

***Food*:** Blow flies are saprophagous meaning that they feed in decaying organic matter. However, some debate exists on whether or not the larvae feed on the actual organic matter, the liquids produced during decay by bacteria/larval activity or on the bacteria itself (Uvarov 1929). Work by Mackerras and Freney (1933) has observed small particles in the gut of sheep blow fly maggots feeding on liver which still does not rule out the possibility of other food items being eaten (Mackerras and Freney 1933).

Developing on decomposing matter varies across animal and even across tissue within the same animal. Clark et al. (2006) determined that larvae reared on pig grew faster than those on cow, and larvae fed lung and heart of both animals grew faster than larvae fed liver for the green bottle fly *Lucilia sericata* (Meigen) (Clark et al. 2006). Kaneshrajah and Turner (2004) determined similar results for *Calliphora vicina*

Robineau-Desvoidy, in that larvae reared on pig lung, kidney, heart or brain grew faster than larvae reared on pig liver (Kaneshrajah and Turner 2004). Both authors caution the use of development data for calculating degree days for use in mPMI estimates since differences can arise in development based on tissue or organ used.

***Study Design*:** Blow fly development has been shown to be dependent on environmental conditions experienced during rearing (Tarone and Foran 2006, Nabity et al. 2007). The above mentioned factors affecting development can be mixed and matched in various combinations when setting up a development study. Conversely, variations arise in development data likely due to the vast amount of combinations available for setting up experiments.

Tarone and Foran (2006) demonstrated that by manipulating conditions associated with rearing (i.e. moisture, handling and substrate type) could account for the discrepancies in development times across published data sets for *L. sericata* (Tarone and Foran 2006). Effects of photoperiod (light/dark cycle) was investigated by Nabity et al. (2007) and it was concluded that constant light versus cyclic light gave rise to more variation in development times (Nabity et al. 2007). Niederegger et al. (2010) observed different effects on growth time for different species reared at constant versus cyclic temperatures (Niederegger et al. 2010). Two of the four species observed developed faster under cyclic temperatures while the other two developed slower under cyclic conditions. Greenberg (1991) determined one out of four species of fly tested to have significantly slower development at alternating temperatures while the rest had equal development between alternating and constant temperatures (Greenberg 1991).

One possible remedy for discrepancies between controlled laboratory studies and flies collected from uncontrolled forensic cases is to conduct a validation experiment. In these experiments flies reared under field conditions can be aged with the laboratory data to determine how well the laboratory conditions estimate the field conditions.

***Competition*:** Blow fly competition on decomposing remains usually takes place in the larval stage as adults are not dependent on the carrion for sustenance (Nicholson 1957, Beaver 1977). Nicholson (1957) noted oscillations in adult population size with high densities of adults leading to high density of larvae which could not be supported by the constant supply of resource yielding smaller larvae and fewer adults (Nicholson 1957). Competition was therefore taking place between the larvae on the limited resource but some evidence for adult competition was observed among females trying to protein feed on liver which was difficult when adult density was high and lead to females not obtaining enough resource to produce eggs (Nicholson 1957).

Laboratory rearing of blow flies under mixed and pure cultures at various densities on a fixed amount of resource has been shown to affect development rate (Goodbrod and Goff 1990, Wells and Greenberg 1992b). Increasing intraspecific density led to decreased development times at various life stages (Goodbrod and Goff 1990) but was also determined to affect the size of the resulting larvae/pupae produced (Ullyett 1950, Goodbrod and Goff 1990). Increases in interspecific densities had various effects on development rates depending on the species involved (Wells and Greenberg 1992b) while size became smaller as seen with the increasing interspecific densities (Ullyett 1950, Goodbrod and Goff 1990).

By controlling the above factors affecting development researchers can examine the non-consumptive effects (NCE) of predators on prey growth and development. NCE are predator-mediated effects that alter prey character traits (e.g., behavior, growth and development) (Abrams 1984). Often times NCE, not consumptive effects (CE), can explain why lower level trophic cascades occur. The presence of multiple species of blow fly larvae on a carrion resource, some of which can act as a predator, lends to a great study system to observe species interactions.

Biology of the Secondary Screwworm

Cochliomyia macellaria (Fabricius) (Diptera: Calliphoridae) is commonly found on decomposing remains in the southeastern United States (Byrd and Butler 1996). The minimum temperature at which *C. macellaria* becomes active is between 12.8-15.6˚C with the minimum temperature at which it copulates and oviposits being 14.5˚C (Deonier 1940). Cool weather is the primary factor governing temporal niche duration in *C. macellaria* populations in the southwest with pupae unable to survive freezing soil temperatures, and its expansion to the north occurring only during the warm months of the year (Denno and Cothran 1975). *Cochliomyia macellaria* predominately occurs on large carcasses (e.g., sheep, goats, cattle and horses) (Deonier 1940, Denno and Cothran 1975). In Central Texas, it is one of the most common blow flies encountered at decomposing remains (Tenorio et al. 2003).

Cochliomyia macellaria eggs hatch at temperatures as high as 41.7˚C with no eggs hatching above 42.8˚C (Melvin 1934). Alternatively, Melvin (1934) determined that less than 10% of eggs hatched at approximately 15˚C (Melvin 1934). Greenberg

(1991) noted *C. macellaria* egg development to be relatively brief, as little as 10 h, with individuals laying between 75-150 eggs (Greenberg and Szyska 1984, Greenberg 1991). *Cochliomyia macellaria* has a short life span and can go through generations quickly during the warmer months of the summer and depending on the level of competition, can have periods of high population densities.

A description of the larvae as well as stage duration at constant temperature is given by Greenberg and Szyska (Greenberg and Szyska 1984). Laake et al. (1936) also describes the eggs, larval stages, pupae and adults, as well as comparing the percent *C. macellaria* visiting necrotic livestock wounds to the primary screwworm *Cochliomyia americana* (*hominivorax*) (Coquerel) (Diptera: Calliphoridae) (Laake et al. 1936). Greenberg (1991) also provided development times from egg to adult at both constant and cyclic temperatures with the cyclic regime taking approximately 12% longer than the constant temperature (Greenberg 1991). Byrd and Butler (1996) give a range of constant and cyclic temperatures at which *C. macellaria* was reared giving times for each individual stadia of growth in Florida (Byrd and Butler 1996). Boatright and Tomberlin (2010) have also looked at development time for individual stadia of growth for *C. macellaria* populations in Central Texas (Boatright and Tomberlin 2010). *Cochliomyia macellaria* is an ideal species to work with in that it has fast generation times and there are multiple data sets that observe the length of time it spends in various stages of its life.

Biology of the Hairy Maggot Blow Fly

Chrysomya rufifacies (Macquart) (Diptera: Calliphoridae) is an invasive species

first introduced to Central America in 1978 and is believed to be from tropical Australia and the Orient (Baumgartner 1993). *Chrysomya rufifacies* was the first of four Old World species of Chrysomya (Robineau-Desvoidy) to become established in the continental U.S. (Gagne et al. 1982). It has been collected throughout Texas (Richard and Ahrens 1983), Arizona (Baumgartner 1986), California (Greenberg 1988), Hawaii, Florida (Tantawi and Greenberg 1993), Louisiana (Martin et al. 1996), Colorado (De Jong 1994), Arkansas (Meek et al. 1998), Nebraska (Figarola and Skoda 1998), Michigan, Kentucky, Tennessee (Shahid et al. 2000), North Carolina (Tomberlin et al. 2006), Indiana, West Virginia (Joy and D'Avanzo 2007) and Canada (Rosati and VanLaerhoven 2007). *Chrysomya rufifacies* is considered to have a tropical temperature tolerance and is expected to expand along the tropical and subtropical temperate regions of the United States (Baumgartner 1993). Since *Chrysomya rufifacies* introduction into the U.S., it has spread rather quickly with little effort.

Eggs of this blow fly will not hatch at temperatures below 9˚C, and larvae will not pupate at temperatures below 15˚C (Baumgartner 1993). Eggs typically are deposited in the natural orifices of a carcass; however, females will also lay eggs in sheltered sites on the carcass surface rather than clean fresh wounds (O'Flynn and Moorhouse 1979). Byrd and Butler (1997) give time measurements for individual stadia of *Ch. rufifacies* growth over constant and cyclic temperature regimes while Greenberg (1991) gives accumulated degree hours (ADH) necessary for *Ch. rufifacies* to reach the adult stage at one constant and one cyclic temperature regime (Greenberg 1991, Byrd and Butler 1997). However, development data for *Ch. rufifacies* is lacking.

Second and third instar larvae of the hairy maggot blow fly are facultative predators and cannibalistic (James 1947, Goodbrod and Goff 1990, Baumgartner 1993). The larva is characterized by its sclerotized spines, fleshy processes and heavily sclerotized mouthhooks used to penetrate the bodies of other maggots for fluid extraction during predation (Baumgartner 1993). It is not known what circumstances are needed for predation to occur but it is not needed for complete development as noted from pure culture rearing in the laboratory without a prey species.

Reproduction and oviposition in *Ch. rufifacies* can occur very quickly. Adult *Ch. rufifacies* will mate two to ten days after emergence with the fastest mating occurring during the summer (Baumgartner 1993). Males are sexually mature at emergence following a teneral period, usually less than 24 hours based on mating success at various ages (Schmidt and Kunz 1985). Oviposition has been observed five days post emergence with females laying approximately 210 to 368 eggs (Baumgartner 1993). Knowing the life history of this species, one can observe how they change as *Ch. rufifacies* interacts with other species. Like *C. macellaria*, *Ch. rufifacies* has a quick generation time, multiple generations occurring throughout the year and development data sets for individual stadia which makes it an ideal species to study.

Baumgartner (1993) determined that *Ch. rufifacies* prefers large (e.g., rabbit, goat, sheep, kangaroos, and human cadavers) over small carcasses (e.g., mice, rats, birds, lizards, and guinea pigs) (Baumgartner 1993). Additionally, it is more active in open pastures as compared to forest habitats (Baumgartner 1993). In Central Texas it is one of the most common blow flies found at decomposing remains and is often one of the first

blow flies to show up at a carcass (Tenorio et al. 2003). The life history of *Ch. rufifacies* appears to be very similar to the life history of the secondary screwworm and the two species are often found on the same carcass in Central Texas.

Chrysomya rufifacies has some unique life history traits with females being able to selectively produce same sex offspring within a clutch of eggs (Ullerich 1977). This trait makes keeping a colony of this species rather difficult and requires that population numbers remain high to prevent colonies of all one sex. The trait also makes the fly an interesting species to study in the area of population genetics. Sex ratio in the wild has been observed as 50:50, but variability can occur from the females laying progeny of only one sex. There can be a possibility for unequal sex distributions which may help aid in *Ch. rufifacies* ability to spread throughout the U. S. so effortlessly. Areas where large numbers of individuals are emerging could lead to spikes in population sizes if there is a female bias in sex ratios. With males being ready to mate after only one day post emergence, even if the male ratio was lower a handful of males would most likely be able to fertilize many females. The population densities should dip back down as more females in the environment means more larvae laid on a resource increasing intraspecific competition. Likewise, competition for mates may increase as more females are being produced.

This fly is not a primary producer of myiasis (infestation of living animal tissue with fly larvae) in the U.S. but is useful in medico-legal entomology because of its tendency to favor human and animal carcasses (Baumgartner 1993). In Australia where *Ch. rufifacies* is native, it is a secondary myiasis producer on sheep (termed strike),

meaning that it cannot initiate the myiasis but can cause the wound to become more severe, increasing the sheep's chance of death (Hughes and Shanahan 1979). Tenquist (1977) noted that strike in New Zealand cost sheep industry farmers nearly $2 million in labor while later Heath and Bishop (1995) estimated the cost at around $37 million. In the U. S., *Ch. rufifacies* is known to be attracted to Swormlure-4 which attracts one of the worst pests from the U. S. the primary screwworm *C. hominivorax* (Tenquist 1977, Baumgartner 1993, Heath and Bishop 1995). It is not known if *Ch. rufifacies* will become a major myiasis producer in the U. S., but should be monitored closely to avoid such great losses in livestock.

C. macellaria and *Ch. rufifacies* Interactions

Interspecific competition has been studied between the two previously mentioned blow flies, *Ch. rufifacies* and *C. macellaria*. Intraspecific competition has been observed by Baumgartner (1993) but usually only when the resource is limited (Baumgartner 1993). Wells and Greenberg (1994a) note that *C. macellaria* was the native species whose resource use was most similar to that of *Ch. rufifacies* (Wells and Greenberg 1994a). Since *Ch. rufifacies* was introduced to the Americas, *C. macellaria* populations have been negatively affected (Wells and Greenberg 1994a, Byrd and Butler 1996) and is predicted to decline by Wells and Greenberg (Wells and Greenberg 1992a). Evidence of the Genus *Chrysomya* negatively affecting blow flies in the areas where they occur are found throughout the literature. For instance, *Chrysomya albiceps* (Wiedemann) is thought to be responsible for the eradication of *Lucilia caesar* (L.) in Madeira (Hanski 1977). In the laboratory *Ch. albiceps* reduced abundance of larvae in mixed populations

of *Ch. chloropyga* (Wiedemann) and *Phaenicia* (=*Lucilia*) *sericata* (Ullyett 1950). *Chrysomya albiceps*, *Ch. putoria* (Wiedemann) and *Ch. megacephala* (F.) are responsible for the lowering of *C. macellaria* in Santa Maria, Rio Grande do Sul, Brazil (Baumgartner and Greenberg 1984). *Chrysomya putoria* has almost completely replaced *C. macellaria* in Goiania and Campinas, Brazil (Guimaraes et al. 1979), perhaps because *Ch. putoria* arrives at a carcass faster and develops faster than *C. macellaria* (Baumgartner and Greenberg 1984). In Guam *Ch. megacephala* grows faster and crowds out its competitors (Baumgartner and Greenberg 1984). In Peru Baumgartner and Greenberg (1984) determined that through trapping over several years, *C. macellaria* was the most abundant blow fly trapped (89%) but 18 months later had become replaced by *Ch. putoria* and *Ch. albiceps* with *C. macellaria* making up only 0.19% (Baumgartner and Greenberg 1984). In Australia *Ch. rufifacies* outcompetes flies from the genera *Phaenicia* (=*Lucilia*) and *Calliphora* Robineau-Desvoidy (Norris 1959). Other predators may aid in the lowering of *C. macellaria* numbers as well, for instance, *Ch. rufifacies* numbers have been shown to not be affected by fire ants (*Solenopsis invicta* Buren) while *C. macellaria* numbers are affected (Wells and Greenberg 1994b). Only one study has been conducted in the U. S. evaluating the influence *Ch. rufifacies* has on *C. macellaria* in the field (Wells and Greenberg 1992c). Literature citing the decline of local *C. macellaria* populations due to the presence of *Chrysomya* species is purely anecdotal and provides no community abundance data to support these hypotheses. Some laboratory and field studies between *Ch. rufifacies* and *C. macellaria* are given below.

Wells and Greenberg (1992a) studied rates of predation of *Ch. rufifacies* on *C. macellaria* in the laboratory (Wells and Greenberg 1992a). They determined that predation occurred in 17 out of the 20 petri dishes between the 3rd instar stage of both species and only in 7 out of 20 petri dishes between 3rd instar *Ch. rufifacies* and 2nd instar *C. macellaria.* Second instar *Ch. rufifacies* were not found to attack any instar of *C. macellaria* (0 out of 20 petri dishes) which has been speculated previously (James 1947, Goodbrod and Goff 1990, Baumgartner 1993). Size of the 3rd instar *Ch. rufifacies* did not relate to the act of predation. Small (approx. 10.5 mm) and large (approx. 16.2 mm) third instar *Ch. rufifacies* equally predated upon mid-size (approx. 12.5 mm) *C. macellaria.* If an author was to want to perform an interaction study between these two species of blow flies, a good starting point would be with the 3rd instar stage of *Ch. rufifacies*.

Wells and Greenberg (1992c) demonstrated that *Ch. rufifacies* reduced the numbers of *C. macellaria* from rabbit carcasses in the field (Wells and Greenberg 1992c). Carcasses were exposed from 7:30-11:30 h and 15:00-19:00 h. During the exposed time periods, *Ch. rufifacies* adults were physically removed from the carrion using a battery operated aspirator while it was allowed to oviposit on the carcasses in the other treatment. When *Ch. rufifacies* were removed, on average a little over 2,000 adult *C. macellaria* emerged from the rabbit carcass while in the treatment where *Ch. rufifacies* were not removed, approximately 500 *C. macellaria* emerged from the carcass showing a negative relationship between the two species in the field. By looking at the local interactions of these two species, the authors were able to show a decline in

number of *C. macellaria* emerging off of carcasses.

Wells and Greenberg (1992b) looked at the influence density had between mixed and pure culture rearing of *Ch. rufifacies* and *C. macellaria* at two temperatures 22 and 29°C on survivorship, sex ratio and development rate (Wells and Greenberg 1992b). Mixed cultures consisted of 20, 100 and 500 flies of each species while pure cultures consisted of 40, 200 and 1,000 flies for each species. Survivorship in pure culture was not affected by increasing density in pure or mixed cultures. Survivorship however was higher for both species at the 29°C temperature, from 47 to 62% and from 29.5 to 33.5% for *C. macellaria* and *Ch. rufifacies* respectively. Sex ratio was unaffected by density or temperature. Development rate was influenced significantly by temperature with the 29°C taking less time to go from oviposition to adult for both species. Development of *Ch. rufifacies* was reduced significantly with increasing intraspecific (between *Ch. rufifacies*) density from 20 to 100 larvae per jar. Across all three interspecific (between *C. macellaria* and *Ch. rufifacies*) densities, *Ch. rufifacies* development was lengthened significantly from the egg to the adult. This study has shown a negative impact of *Ch. rufifacies* on *C. macellaria* in the laboratory. The authors have demonstrated that by changing density and species composition there is a difference in development times.

Similarities exist between *Ch. rufifacies* and *C. macellaria* and although *Ch. rufifacies* has negatively affected *C. macellaria* in Florida, by decreasing the frequency in which it is observed, it is still the predominate species found on human remains outdoors (Byrd and Butler 1996). Therefore, *C. macellaria* is able to avoid competition with *Ch. rufifacies* in some instances. One thought could be that by preceding *Ch.*

rufifacies in succession, *C. macellaria* is able to survive (Wells and Greenberg 1994a) (e.g., priority effects). Consequences of *C. macellaria* being placed on a resource at the same time as *Ch. rufifacies* in the laboratory led to a decrease in *C. macellaria* survival (Brundage, Personal Communication). Studies were conducted to determine how similar *Ch. rufifacies* is to *C. macellaria*, in terms of tissue and temperature effects on development. Following up on their ecological similarities if the two species are artificially "forced" to encounter one another under controlled laboratory conditions, what would the observed effects be on growth and development?

Objectives

Chapter 2 Development: The effects of temperature and muscle tissue type on the development of the hairy maggot blow fly *Ch. rufifacies* will be observed.

H_a: I expect that flies reared on various muscle tissue types and at various temperatures will show differences in growth times and growth parameters.

Chapter 3 Validation: Data obtained from the development experiment will be used to make age predictions for flies reared under controlled and uncontrolled field studies.

H_a: I expect the statistical age estimation method will yield more accurate (closer to true age) estimations for all field controlled and uncontrolled reared flies over single variable estimations alone (i.e., length, weight or instar).

Chapter 4 Growth Distributions: Length and weight larval variation will be observed across all larval instars for controlled field reared flies.

H_a: I expect larval weight and length variation to change as instars progress with

variable distributions depending on the age of the larvae.

Chapter 5 Non-consumptive Effects on Adult Behavior: Adult *C. macellaria* will be assessed for attraction and oviposition preference towards 3rd instar conspecific (*C. macellaria*) and/or heterospecific (*Ch. rufifacies*) larvae.

H_a: I hypothesize that adult *C. macellaria* will be attracted to conspecific (self) larvae and avoid heterospecific (predatory) larvae.

Chapter 6 Non-consumptive Effects on Larval Behavior: Larval instars of *C. macellaria* were inoculated with 3rd instar *Ch. rufifacies* predator cues (larval excretions/secretions (ES)) in all larval instars and time for individual stadia were recorded.

H_a: Larvae reared in the presence of predatory cues are hypothesized to develop differently from controls and differently depending on the instar treated and the type of predatory cues utilized.

Relevance

The purpose of the work is to better understand an invasive blow fly species to understand its effects on native blow fly species and the surrounding ecosystem. *Chrysomya rufifacies* has long been considered to be the ecological counterpart of *C. macellaria* (Baumgartner and Greenberg 1984) and by understanding the biology, ecology and behavior of this blow fly, as well as its competitive effects on *C. macellaria*, one may understand what effects it may have on other native fauna. Insights into the biology and predatory effects of this fly have broad applications to the fields of medical, veterinary and forensic entomology.

CHAPTER II

EFFECTS OF TEMPERATURE AND TISSUE ON *CHRYSOMYA RUFIFACIES* (DIPTERA: CALLIPHORIDAE) (MACQUART) DEVELOPMENT

Introduction

Blow fly (Diptera: Calliphoridae) development is a quantitative trait; a trait that is known to vary through genetic and environmental factors in many organisms (Conner and Hartl 2004). Studies on blow flies have demonstrated this variation (Kaneshrajah and Turner 2004, Tarone and Foran 2006, Gallagher et al. 2010), but more work is needed within blow flies to understand how single populations and multiple genotypes of the same species respond to such conditions (Tomberlin et al. 2011). In addition, the most critical abiotic factors affecting blow fly development are not known.

Temperature is a well-recognized abiotic factor that affects blow fly development. Warmer temperatures accelerate development while cooler temperatures have an inverse impact. This relationship has been documented in past growth studies on blow flies at varying temperatures (Greenberg 1991, Byrd and Butler 1996, 1997, Byrd and Allen 2001). However, the amount of daily variation in temperature (i.e., temperature fluctuation) experienced by blow fly immatures can also influence their rate of development, with cyclic temperatures increasing or decreasing development times, depending on the species (Greenberg 1991, Byrd and Butler 1996, 1997, Niederegger et al. 2010).

Type of tissue fed to immature blow flies also impacts their size and

development rate (Kaneshrajah and Turner 2004, Clark et al. 2006). Clark et al. (2006) determined that *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) larvae reared on porcine tissue grew faster and larger than those on bovine tissue (Clark et al. 2006). Larvae fed lung and heart of both tissue types grew faster and larger than larvae fed liver. Kaneshrajah and Turner (2004) recorded similar results for *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae) larvae with those fed pig lung, kidney, heart or brain growing faster and larger than those provided pig liver (Kaneshrajah and Turner 2004). Tarone and Foran (2006) showed that, even when fed only beef liver, *L. sericata* larvae could develop at different rates depending on the experimental conditions (specifically factors affecting liver moisture and the condition of the pupation substrate) (Tarone and Foran 2006). A Texas population of *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae), native to the Americas, was studied as to the effects of temperature and tissue type as it relates to its development (Boatright and Tomberlin 2010). *Cochliomyia macellaria* development was determined to be significantly ($P < 0.05$) affected by temperature rather than tissue type.

Chrysomya rufifacies (Macquart) (Diptera: Calliphoridae) is an invasive species from tropical Australia and the Orient first introduced to Central America in 1978 (Baumgartner 1993). It has steadily expanded its range across North America beyond its believed environmental tolerance. *Chrysomya rufifacies* has been documented throughout the contiguous United States, from California to Florida and as far north as southeastern Ontario, Canada (Baumgartner 1993, Rosati and VanLaerhoven 2007). *Chrysomya rufifacies*, like *C. macellaria*, is commonly collected from animal remains in

central Texas (Tenorio et al. 2003) and is frequently encountered on deceased humans as well (Jeffery K. Tomberlin, personal communication).

Both *Ch. rufifacies* and *C. macellaria* have been hypothesized to be ecological counterparts (Wells and Kurahashi 1997); however each fly has evolved in different ecological conditions as *Ch. rufifacies* is not native to the Americas. *Chrysomya rufifacies* being native to a habitat with less temperature variability (i.e., tropics region) may lead to flies less plastic (responsive) to temperature while *C. macellaria* may exhibit greater plasticity as it experiences a much more variable temperature range (i.e., temperate region). Additionally, the tropics are renowned for high species diversity (Stevens 1989), indicating that *Ch. rufifacies* may have evolved under conditions that require the ability to survive on more types of carrion. If these fly species truly are ecological counterparts not only will they share the same resources and environments, which have been documented previously in the literature (Wells and Greenberg 1994a), but they will also respond similarly to abiotic factors.

The objectives of my study were to determine the impact of temperature and muscle type on the development of a single population of *Ch. rufifacies*. I hypothesize that time for each stage of development will be affected by the muscle type flies were reared on and the temperatures that the flies were exposed to.

Materials and Methods

Fly Source: *Chrysomya rufifacies* larvae (> 500 individuals) were collected from decomposing animal remains located in College Station, Texas, USA during July and October of 2008, May of 2009 and August of 2011. Larvae were brought to the Texas

A&M University Forensic Laboratory for the Investigation of Entomological Sciences (FLIES Facility) to initiate colonies. Resulting adult flies were held in multiple 30 cm^3 BioQuip® (Rancho Dominguez, CA, USA) lumite screen collapsible cages in the FLIES Facility (~24.4°C, 50% RH and 14:10 L:D). Adult flies were provided a 50:50 mixture of table sugar and powdered milk or honey, as well as cotton balls soaked with deionized water (dH_2O) *ad libitum*.

Tissue Source: Striated muscle tissue from three animal species was used as a development medium in this study. Canine (*Canis spp.* Linnaeus) muscle tissue was obtained through an Austin-area (Austin, TX, USA) veterinarian from three separate canines (replicates). Equine (*Equus ferus* Boddaert) muscle tissue was donated by the Texas A&M University Veterinary Diagnostic Laboratory, College Station, TX, USA from three separate equines (replicates). Porcine (*Sus scrofa* L.) muscle tissue was obtained from a local grocery store from multiple packages of lean pork chops and separated into three groups (replicates). Bovine liver was obtained from an on campus meat processing plant (E. M. "Manny" Rosenthal Meat Science and Technology Center, College Station, TX, USA) and used as an oviposition medium and for rearing immature flies. For each animal, and pork chop package, muscle tissue samples were placed in individual Ziploc bags, labeled and stored in a -20°C freezer until use in the experiment. Muscle tissue acquisition protocols were approved by the Animal Welfare Assurance Program at Texas A&M University.

***Development*:** Methods for the development study were adapted from Byrd and Butler (1997) and identical to those used in the Boatright and Tomberlin (2010) study

(Byrd and Butler 1997, Boatright and Tomberlin 2010). Adults (7-10 d) from the F_1 generation were provided with approximately 200 g fresh beef (bovine) liver as an oviposition site. Hourly observations were made for egg clutches. Egg clutches less than one hour old were placed in dH_2O, broken apart and homogenized with a camel hair brush. For each temperature treatment individual plastic BioQuip® mosquito-breeding container bottoms (10 [h] x 12 cm [w]) were placed into an individual sterilite plastic shoe box container (35 [l] x 20 [w] x 13 cm [h]) (Townsend, MA, USA). Each plastic shoe box contained approximately 500 ml (850 g) of sand (Quikrete Premium Play Sand, Atlanta, GA, USA) as pupation medium. The mosquito-breeding container was placed on top of the sand in the center of the shoebox. Each mosquito-breeding container held 200 g of porcine, canine, or equine muscle tissue which had been cubed (~3 cm^3 or ~25 g). Approximately 200 eggs, representing multiple clutches, were placed on a moistened filter paper to prevent desiccation and then placed on the respective cubed muscle tissue in an order determined using a random number generator. Egg number was determined gravimetrically with an Adventure-Pro AV64 Ohaus scale (Pine Brook, NJ, USA). The three replicates of each muscle tissue were placed in three stand-up environmental chambers (136LLVL Percival Percival Scientific Inc., Perry, IA, USA) set at 21, 24, or 27°C with 14:10 L:D and 75-80% RH using a Latin square design which assigns each replicate to one of the three shelves without overlaps in animal tissue positions. A hobo data logger Onset (Onset Co., Pocasset, MA, USA) hobo U12-006 placed inside of each environmental chamber with probes placed on each of the three levels of the chamber to record temperature hourly.

Eggs were monitored hourly for hatch. Thereafter, observations were made every 12 h. During each observation after egg hatch, the three largest larvae were collected, placed in hot water at approximately 100°C for 30 s (Adams and Hall 2003) and then measured as described below. Feeding larvae observed wandering from a mosquito-breeding container were returned to the container with the muscle tissue.

Life stage, larval weight and length were recorded for each larva sampled as well as stage duration. Larvae were weighed using the scale previously described and length measured in millimeters with a Meiji Techno EMZ-8TR microscope (Santa Clara, CA, USA) and ruler. Larvae from each replicate were sampled until the cohorts had reached the pupal stage. For each replicate of muscle type, pupae were collected when they represented the oldest development stage present. The initial 30 pupae observed were sampled. Each of these pupae was placed individually in a 35 ml plastic container (Jetware, Hatfield, PA, USA) with approximately 10 ml of sand. Containers with pupae were labeled, returned to the appropriate growth chamber, and monitored for adult emergence. Time, date, and sex were recorded for each emergent adult. Resulting adults were provided 0.20 ml distilled water, every 24 h, via a 1 ml Kendall Monoject SoftPack Insulin Syringe (Mansfield, MA, USA) inserted through the lid and adult longevity was recorded. Stage duration was determined by observing when the first time an instar was observed to the next observation when no individuals of that instar were collected in the sample.

A preliminary study was conducted in August 2011 to determine time to complete the egg stage at the three temperatures studied. Two hundred eggs collected

from F_1 generation adults (same methods as above) were separated and weighed gravimetrically, placed on moistened filter paper to prevent desiccation and replicated six times. Care was taken to monitor how long eggs spent at room temperatures during weighing and at what point they were placed in their respective temperatures. Eggs were monitored hourly until hatch.

Statistics: A split plot design analysis of covariance (ANCOVA) was used to analyze the development data (SAS 9.2 for Windows, Carry, NC, USA) to determine the influence temperature and muscle types have on weight and length over time for each replicate. The whole plot was muscle type and the split plot was temperature with muscle tissue replicates set up as a random factor. The split plot design accounted for the variation which occurs in the data from having three temperatures nested within each of the muscle types being tested. *P*-values were considered statistically significant with $\alpha < 0.05$.

Results

Larvae failed to reach the 3rd instar in one of the canine muscle replicates. Therefore, results presented for canine are based on an $N = 2$. Mean weight over time as well as length over time are plotted for porcine, equine and canine muscle types (Figures 1-6 respectively). Muscle type was not a statistically significant predictor of weight ($F_2 = 0.41$; $P = 0.6835$) or length ($F_2 = 0.03$; $P = 0.9725$). Weight or length across temperatures did not differ significantly (weight; $F_2 = 0.01$; $P = 0.9899$: length; $F_2 = 0.33$; $P = 0.7234$). Time was a statistically significant predictor ($P \leq 0.0001$) for both weight and length. A significant interaction between time and muscle type was observed

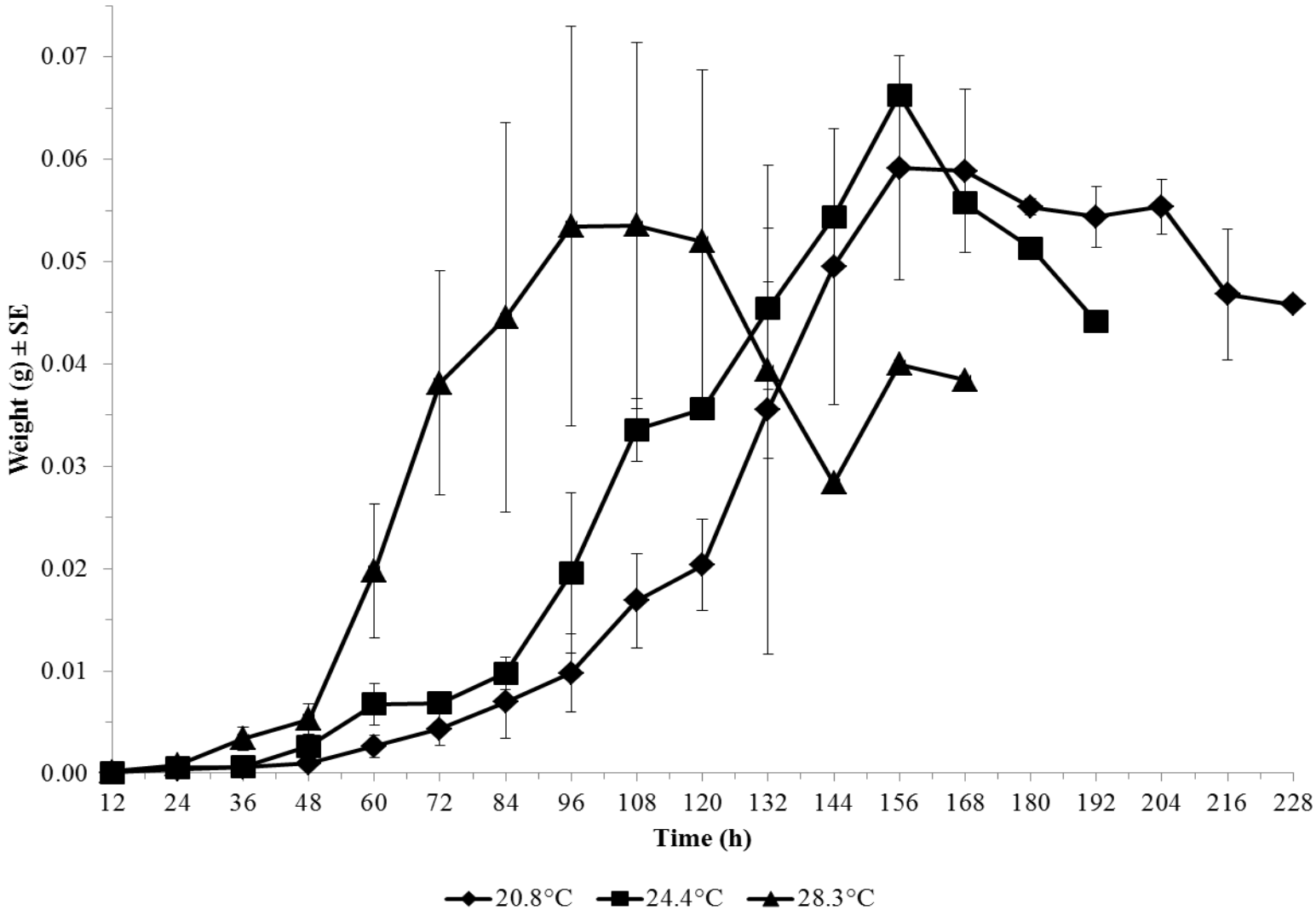

Figure 1. *Chrysomya rufifacies* larval weight (g) ± SE developing at three temperatures on porcine muscle (*N* = 3).

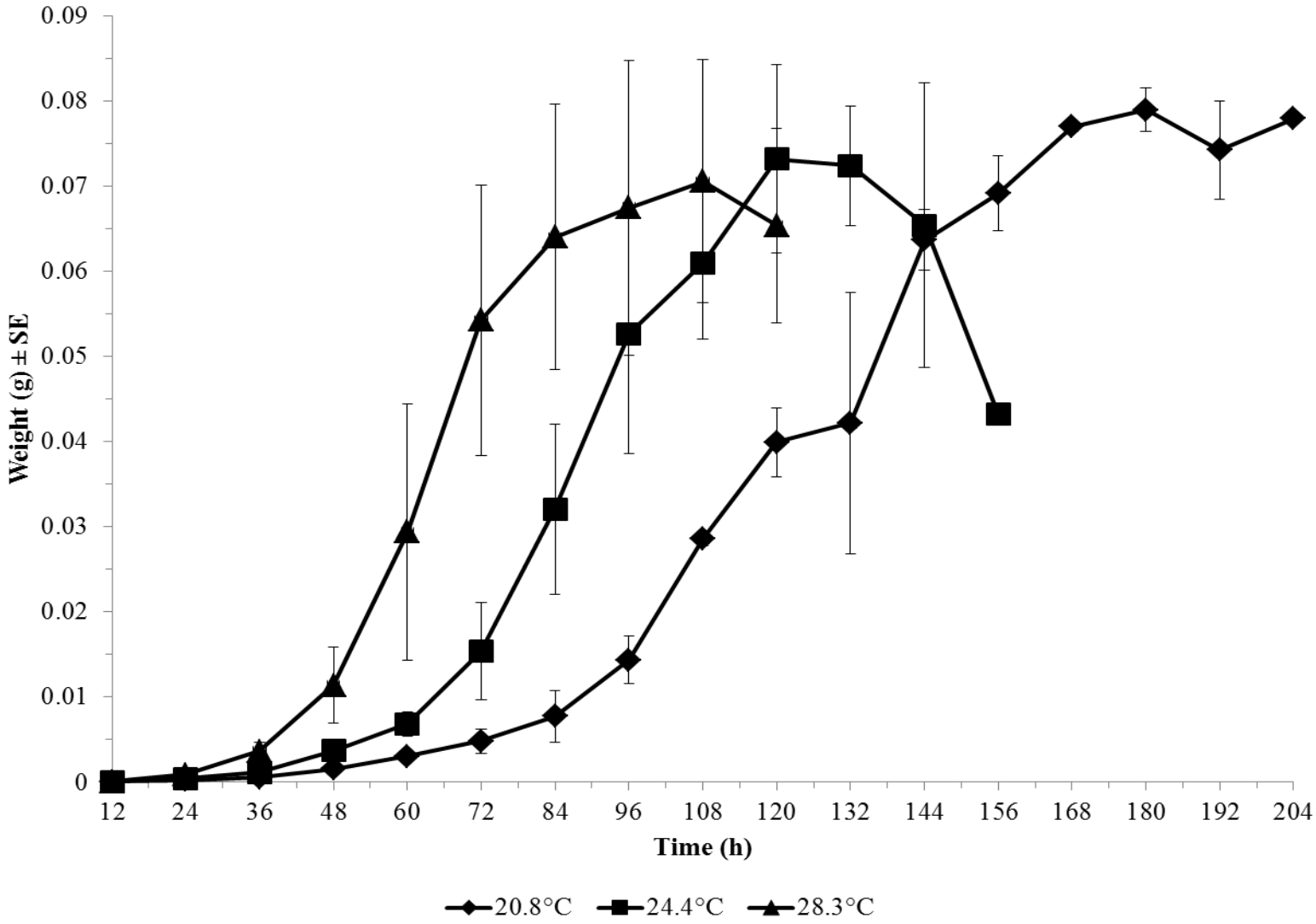

Figure 2. *Chrysomya rufifacies* larval weight (g) ± SE developing at three temperatures on equine muscle ($N = 3$).

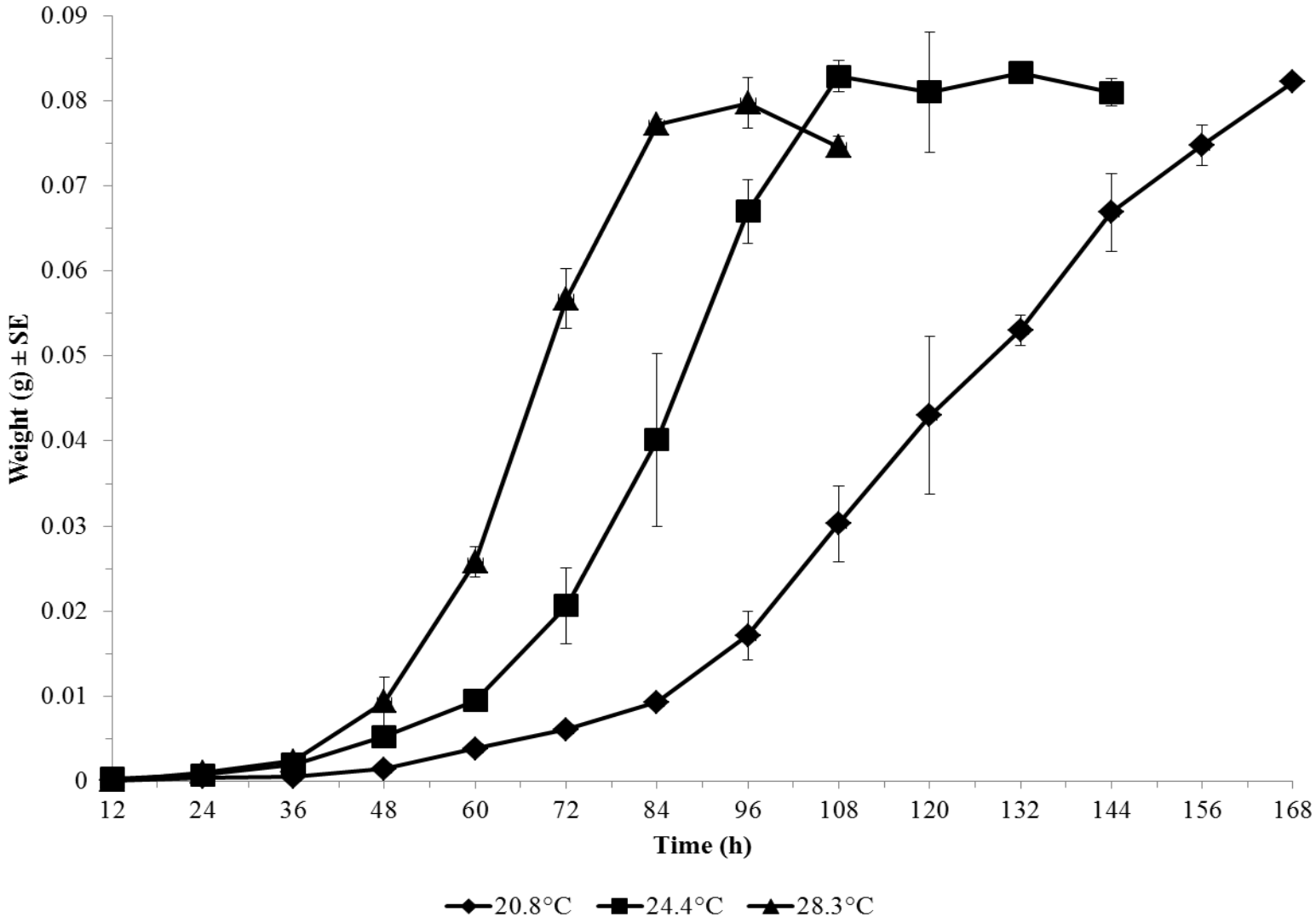

Figure 3. *Chrysomya rufifacies* larval weight (g) ± SE developing at three temperatures on canine muscle ($N = 2$).

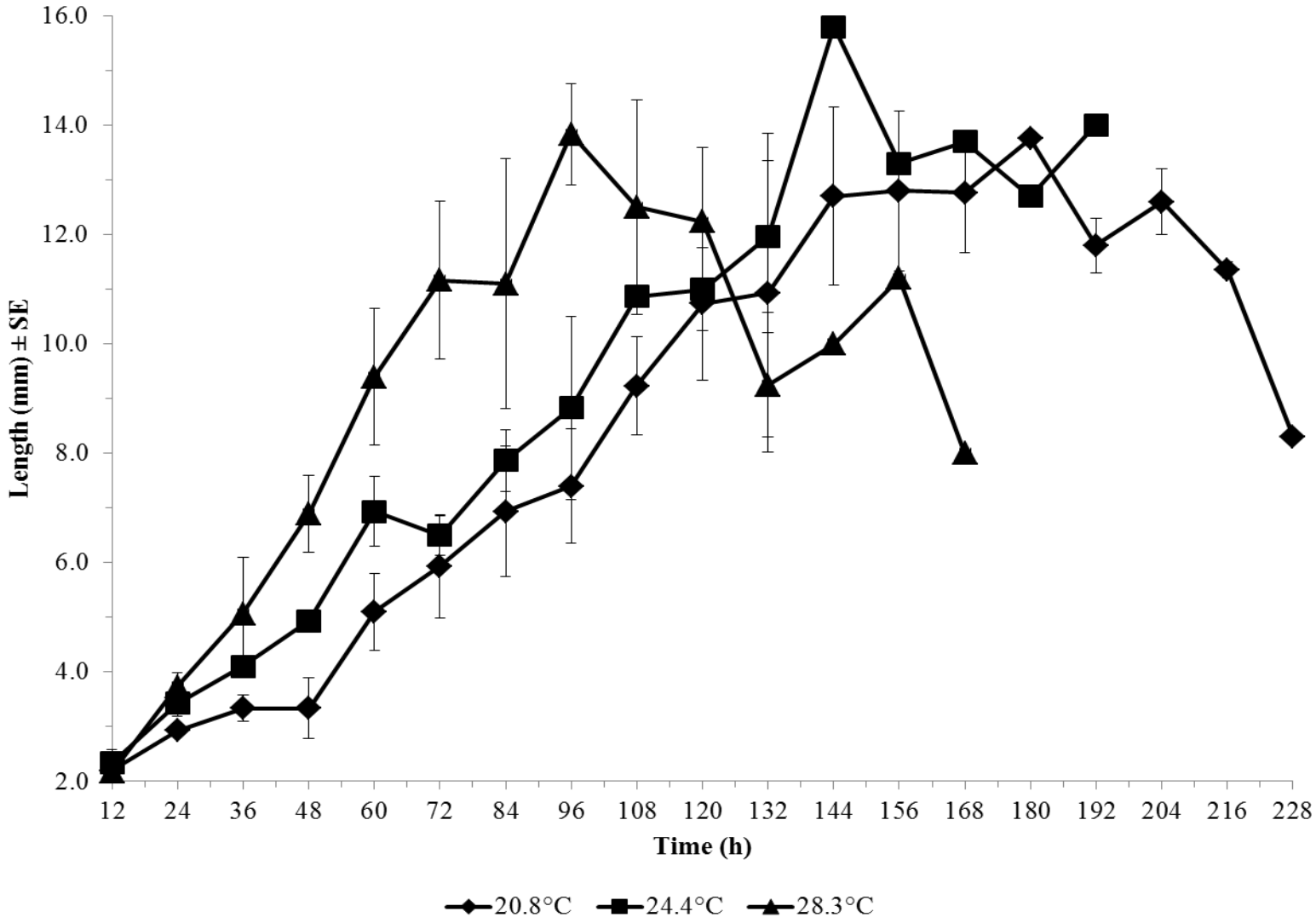

Figure 4. *Chrysomya rufifacies* larval length (mm) ± SE developing at three temperatures on porcine muscle ($N = 3$).

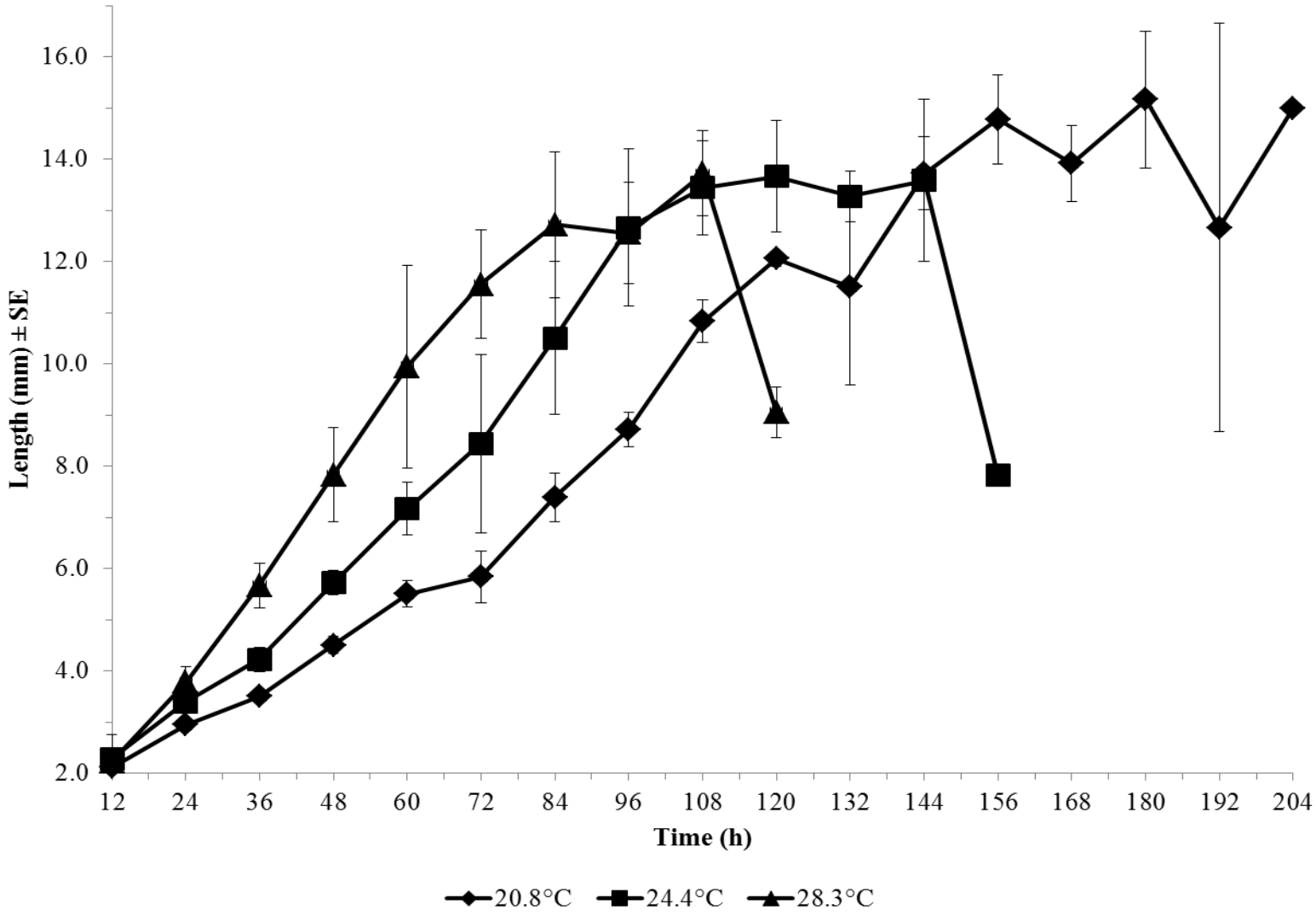

Figure 5. *Chrysomya rufifacies* larval length (mm) ± SE developing at three temperatures on equine muscle ($N = 3$).

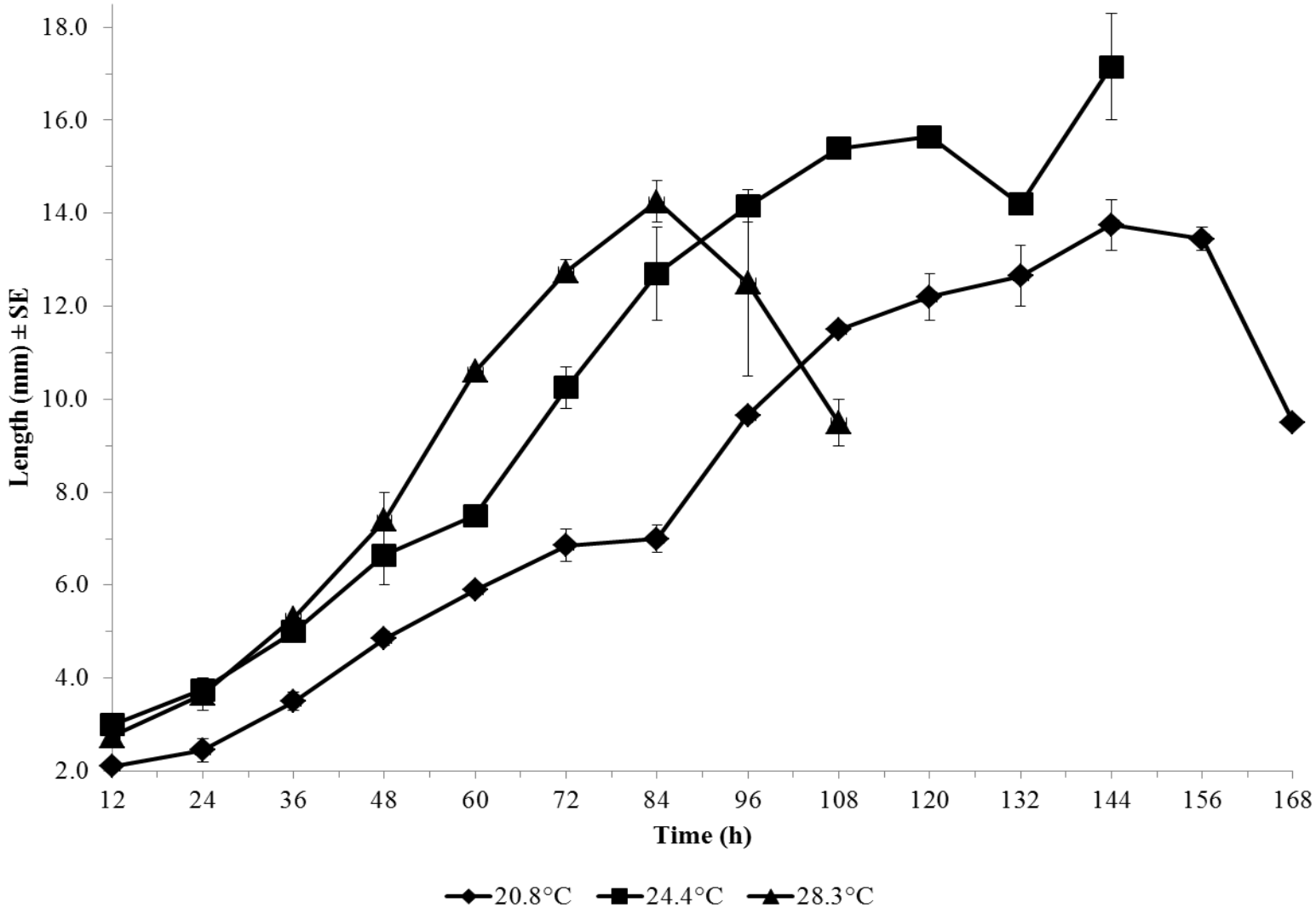

Figure 6. *Chrysomya rufifacies* larval length (mm) ± SE developing at three temperatures on canine muscle ($N = 2$).

for weight (F_2 = 15.87; $P \leq 0.0001$) and approached significance for length (F_2 = 2.49; P = 0.0847). The interaction between muscle type and temperature was not observed to be a significant predictor for weight (F_4 = 0.03; P = 0.9981) or length (F_4 = 0.04; P = 0.9959). An interaction between time and temperature approached significance with weight (F_2 = 3.01; P = 0.0511) but not length (F_2 = 1.61; P = 0.2013). The three way interaction between muscle type, temperature and time was not significant when predicting weight (F_4 = 0.16; P = 0.9594) or length (F_4 = 0.25; P = 0.9122).

Hours spent in each developmental stage were determined. Hours for egg hatch at the three temperatures are presented in Table 1. Results for stage durations are given in Table 2. Larvae needed 28 h longer to complete development to the adult stage on porcine and equine muscle tissue compared to the canine muscle tissue at the warmest temperature. At 24.4°C the porcine muscle tissue took the greatest amount of time to complete development to the adult stage by 12 h compared to the canine muscle and 20 h compared to the equine muscle. At the coolest temperature (20.8°C) the porcine muscle again took the longest to complete development to the adult stage by 44 h versus the equine muscle and 78 h versus the canine muscle. The largest larvae (length and weight) were recorded for the canine muscle (17.15 mm and 0.0833 g) at 24.4°C, porcine muscle (15.8 mm and 0.0663 g) at 24.4°C and equine muscle (15.17 mm and 0.0789 g) at 20.8°C.

Discussion

Excluding the current study, a single data set giving stage durations for *Ch. rufifacies* in the United States exists (Byrd and Butler 1997). I attempted to conduct my

Table 1. Mean hours ± SE needed for *Ch. rufifacies* to finish the egg stage at three temperatures.

Temperature (°C)	Egg (h)
28.3	15.7 ± 0.7
24.4	20.5 ± 0.2
20.8	25.2 ± 0.2

Table 2. Mean hours ± SE needed for *Ch. rufifacies* to complete each stage of development when raised on three muscle types and at three temperatures.

Muscle Type	Temperature (°C)	First instar	Second instar	Third instar	Pupa	Adult
Porcine	28.3	36.0 ± 6.9 A	32.0 ± 4.0 A	92.0 ± 8.0 A	84.0 ± 0.0 A	32.0 ± 8.0 A
	24.4	36.0 ± 0.0 A	44.0 ± 14.4 A	92.0 ± 26.2 A	128.0 ± 4.0 B	36.0 ± 6.9 A
	20.8	52.0 ± 4.0 A	64.0 ± 24.3 A	120.0 ± 12.0 A	172.0 ± 4.0 C	72.0 ± 6.9 B
Equine	28.3	32.0 ± 4.0 A	24.0 ± 0.0 A	84.0 ± 0.0 A	104.0 ± 8.0 A	24.0 ± 0.0 A
	24.4	40.0 ± 4.0 A	32.0 ± 4.0 A	88.0 ± 4.0 A	120.0 ± 12.0 A	52.0 ± 4.0 AB
	20.8	48.0 ± 0.0 A	40.0 ± 4.0 A	112.0 ±14.4 A	164.0 ± 4.0 B	68.0 ± 10.6 B
Canine	28.3	36.0 ± 0.0 A	18.0 ± 6.0 A	72.0 ± 0.0 A	90.0 ± 6.0 A	24.0 ± 0.0 A
	24.4	36.0 ± 0.0 A	30.0 ± 6.0 A	96.0 ± 0.0 A	126.0 ± 6.0 AB	60.0 ± 0.0 AB
	20.8	48.0 ± 0.0 A	42.0 ± 6.0 A	90.0 ± 6.0 A	150.0 ± 6.0 B	84.0 ± 0.0 B

No significant ($P < 0.05$) difference in development for larvae placed on different muscle types but at the same temperature was observed. Uppercase letters indicate significant ($P < 0.05$) difference in development for larvae placed on the same muscle types between temperatures.

research using similar temperatures (20.8, 24.4 and 28.3°C) as the other study (21.1, 25 and 26.7°C) but the lower (21.1°C) and higher (26.7°C) temperatures in their study were run with a cyclic amplitude of 5.5°C. All developmental stages at each temperature in the Byrd and Butler study required less time to complete development than observed in this study except for the 3rd instar stage when reared at the constant 25°C temperature (Byrd and Butler 1997). In their case, the larvae needed an additional 14 h to complete the 3rd instar stage than the average I observed, but this difference is encompassed in the standard error range and does not take into account the observation scale of 12 h employed in both of these studies.

In contrast to what Greenberg proposed, the constant and cyclic temperature times to pupation being similar, I determined in all cases the larvae developed slower than what was recorded by Byrd and Butler under these conditions (Greenberg 1991, Byrd and Butler 1997). Larvae reared on porcine tissue in my experiments, which is the same tissue used in the Byrd and Butler study, needed 40-70 h more to complete development (Byrd and Butler 1997). This relationship has been demonstrated previously for another blow fly species. A comparison between a central Texas population (Boatright and Tomberlin 2010) and a Florida population (Byrd and Butler 1996) of *C. macellaria* at 25°C determined the Texas population also required more time (~95 h) to complete development than the Florida population, both of which were run at a constant temperature and on porcine tissue.

The discrepancies in development times between the Florida study and my study may be a case for genetic variation in *Ch. rufifacies*. However, in my study only a single

population was studied and the differences seen in time to complete stages of development across temperature are an example of environmental plasticity. Genetic variation in development time has been examined for *L. sericata* (Gallagher et al. 2010, Tarone et al. 2011). Gallagher et al. observed two populations from California, USA and one population from Massachusetts, USA in three environments (16.0, 26.0 and 36.0°C temperatures) (Gallagher et al. 2010). They determined faster development (~26 h) for flies from the Massachusetts population than in the California populations only in the middle temperature (26.0°C) examined. Tarone and Foran examined one population from California, USA, one population from Michigan, USA and one population from West Virginia, USA in two environments (20.0 and 35.5°C temperatures) and observed significantly smaller larvae (length and weight) for West Virginia and larger larvae for California, but all distributions exhibited considerable overlap with one another (Tarone and Foran 2008).

Differences in development could also be due to variation in experimental design like those present between my study and the Florida study. Tarone and Foran have demonstrated that altering experimental set up (e.g., larval feeding duration, food-substrate barriers and pupation substrate medium/method) can influence development times within populations of *L. sericata* (Tarone and Foran 2006). By varying environmental rearing conditions larval development times ranged from 329.0-505.5 h which encompassed observed larval development times in the published literature for *L. sericata*.

Design differences were present between my study and the Florida study (Byrd

and Butler 1997). For example, the blow fly egg to tissue weight ratio varied between the studies. I placed 200 eggs on 200 g of porcine tissue while they placed 100 eggs on 200 g of porcine tissue. Their study used a ratio of 1 maggot/2 g of tissue to prevent the heat generated by larvae to be a factor. Had this been the case in my study I would expect faster development as noticed in other studies on larval maggot mass heat generation (Goodbrod and Goff 1990, Turner and Howard 1992).

In my study I used a 14:10 L:D cycle while Byrd and Butler had a 12:12 L:D for the cyclic temperature regimes and a 24:0 L:D (Byrd and Butler 1997). Nabity et al. have demonstrated that constant light (24:0 L:D) in the black blow fly, *Phormia regina* Meigen (Diptera: Calliphoridae), led to an approximately 48 h longer development time when compared to a cyclic light cycle (12:12 L:D) across various temperatures studied (Nabity et al. 2007).

The Florida study also had cyclic temperature regimes (as previously discussed) while this study was conducted with the growth chambers set at a constant temperature. Fluctuating temperatures have been shown to have various effect on larval growth with some species taking a longer time to develop (*Ch. rufifacies*, *C. macellaria*, *P. regina*, *Phaenicia* (*Lucilia*) *sericata*, *Calliphora vicina* and *Ca. vomitoria* Linnaeus (Diptera: Calliphoridae)) and others taking less time to develop (*Sarcophaga argyrostoma* Robineau-Desvoidy (Diptera: Sarcophagidae) and *Lucilia illustris* Meigen (Diptera: Calliphoridae)) (Greenberg 1991, Niederegger et al. 2010).

Protocols for collecting larval samples also varied between studies. Byrd and Butler collected two of the largest larvae every 12 h, boiled them and placed them in

75% ethanol to be measured later (Byrd and Butler 1997). In my study I collected three of the largest larvae every 12 h, boiled the larvae and then measured them soon after that. Differences have been observed in the preservation of larvae after being boiled and placed in ethanol (Adams and Hall 2003). One or a combination of these factors could attribute to the differences observed between these two studies.

Chrysomya rufifacies growth was more impacted by the tissue provided rather than the temperature experienced. This trend was opposite of what was observed for a *C. macellaria* population (Boatright and Tomberlin 2010) even though both species of flies were collected in the same area and around the same time. These differences in response to uncontrollable factors in the field could lead to the coexistence of the two species in this eco-region, with one species at the advantage when temperatures fluctuate and the other at the advantage when resource types are more varied.

This research is also important to the field of forensic entomology which is the application of arthropod science in the judicial system. Forensic entomologists assist in criminal cases by estimating the time of insect colonization of human, or other animals remains (Keh 1985). In order to make these estimates, forensic entomologists rely on laboratory development data for the species in question. Given that colonization by many of these arthropods occurs after death, these estimates are synonymous with the minimum postmortem interval (mPMI). The need for development data for these forensically important species from various eco-regions is necessary as they might be significantly different (Tarone and Foran 2006, Boatright and Tomberlin 2010). Accounting for this variation could help reduce error associated with estimating a mPMI

(Tarone et al. 2011). By researching different populations of flies and their ecological similarities and differences, forensic entomologists can partially explain the variation associated with the consumption phase of the post-colonization interval and infer a mPMI (Tomberlin et al. 2011).

Temperature and tissue type can both contribute to error associated with mPMI estimates based on larval growth or stage duration, by impacting the development of the immature flies. By assessing larval growth and development under various conditions one can help determine all potential factors leading to mPMI errors. Accounting for this error will support the use of this evidence in a court of law and meet the Daubert standard (Daubert, et al. v. Merrell Dow Pharmaceuticals 1993 (509 U.S. 579)). By accounting for these errors in mPMI estimates, forensic entomologists can better understand the limitations of their methods. Validation of laboratory development data with field studies should accompany all new development data (Tarone and Foran 2008, VanLaerhoven 2008, Núñez-Vázquez et al. 2013). Doing so will result in establishing error rates with estimates of mPMI assigned to human death investigations.

CHAPTER III

BLIND FIELD VALIDATION OF TIME OF COLONIZATION OF HUMAN AND PIG REMAINS BY *CHRYSOMYA RUFIFACIES* (MACQUART) (DIPTERA: CALLIPHORIDAE)

Introduction

Forensic entomologists analyze age and assemblage patterns of arthropods recovered from human and other animal remains to estimate the time of colonization (TOC) (Benecke 2001, Campobasso et al. 2001). In many instances, blow flies (Diptera: Calliphoridae) are the primary insects to colonize remains soon after death. Consequently, their age is estimated in order to determine the TOC and thus infer a minimum-postmortem interval (mPMI).

Estimating the age of blow fly larvae associated with decomposing remains can be based on their length, weight and/or stage of development (Wells and LaMotte 1995, Byrd and Butler 1996, 1997, Byrd and Allen 2001, Grassberger and Reiter 2001). For many of these estimates, quantifying heat accumulation over time (i.e., accumulation degree hour (ADH) degree day (ADD)) which accounts for temperatures experienced by organisms (e.g., insects) above lower developmental thresholds (Arnold 1959, 1960) is used. Such an approach allows for a more accurate estimate of how long the immature fly was potentially associated with the remains in question and for a unit of comparison between expected development times under laboratory conditions and experienced field conditions at the site of the collection of the flies (Higley and Haskell 2009).

While development data sets for blow flies, and other forensically relevant arthropods, are commonly used to estimate a mPMI, few have been validated (Tarone and Foran 2008, VanLaerhoven 2008, Núñez-Vázquez et al. 2013). Consequently, the accuracy in such estimates is not known and no standard operating procedures (SOP) exist to state which methodology to implement (Tarone and Foran 2008, VanLaerhoven 2008). Tarone and Foran (2008) predicted the age of larval *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) collected from rat, *Rattus norvegicus* (Berkenhout), carcasses under laboratory conditions using generalized additive models (GAM) in association with growth data (Tarone and Foran 2008). By applying the best GAM, predictions made were within 5% of true age for most larvae collected (Tarone and Foran 2008). Following up on this study gene expression of immatures collected across development on the rats in the previous study were used to make predictions of age to further validate the same growth data (Tarone and Foran 2011). Inclusion of the gene expression data in the GAM prediction models increased precision in the larval estimates 3-8% but no increase was seen in the pupal age predictions. Validation of development data for *Phormia regina* Meigen (Diptera: Calliphoridae) were recently published demonstrating the variability in age estimates assessed using size (e.g., length and weight) but observed the ability to encompass the age of the validated flies within instar estimations (Núñez-Vázquez et al. 2013). In a blind field study, ADD was used to predict the age of *P. regina* collected from decomposing pigs in the field (VanLaerhoven 2008). Depending on the fly development data set (Kamal 1958, Nishida 1984, Greenberg 1991, Anderson 2000, Byrd and Allen 2001), ADD method and lower development threshold, select

predictions were within one day of the true postmortem interval for each set of pig remains examined. Validation of the carrion beetle, *Necrodes littoralis* Linnaeus (Coleoptera: Silphidae), determined an inverse relationship between the pre-appearance interval and ground temperatures for predicting larval and adult beetle arrival (Matuszewski 2011). Error estimates for the adult stage were approximately 25% while larval estimates were more accurate at about 9%. A recent push in the literature calls for the use of more statistically produced age estimations of larvae as another tool for producing mPMI (Tarone and Foran 2008, Ieno et al. 2010).

Chrysomya rufifacies (Macquart) has great importance for forensic application in North America as it is commonly used in estimating the period of insect activity on a corpse as it rapidly colonizes fresh remains (Byrd and Butler 1997, Tenorio et al. 2003). This species is found in Texas during the warmer months of the year (Tenorio et al. 2003), frequently observed in forensic investigations (J.K. Tomberlin personal communication) and can be the sole species found at a death scene (Byrd and Castner 2009).

The objective of this study was to determine the accuracy of estimating the time of field placement of vertebrate remains (e.g., human and pig) when applying *Ch. rufifacies* data presented in chapter 2. Instar, length and weight of larvae collected from decomposing remains are current methods implemented in forensic entomology to make such TOC estimates (Wells and LaMotte 1995, Byrd and Butler 1996, 1997, Byrd and Allen 2001, Grassberger and Reiter 2001) while GAM are more recently being implemented (Tarone and Foran 2008, Ieno et al. 2010). I hypothesized that the GAM

statistical methods will be more accurate than current implemented methods (length, weight and instar estimates).

Materials and Methods

Fly source: *Chrysomya rufifacies* larvae (> 500 individuals) were collected from decomposing animal remains located in College Station, Texas, USA. Larvae were brought to the Texas A&M University Forensic Laboratory for the Investigation of Entomological Sciences (FLIES Facility) to initiate colonies. Resulting adult flies were held in multiple 30 cm^3 BioQuip® (Rancho Dominguez, CA, USA) lumite screen collapsible cages in the FLIES Facility (~ 24.4°C, 50% RH and 14:10 Light-Dark cycle (L:D)). Adult flies were provided table sugar and cotton balls soaked with deionized water (dH_2O) *ad libitum*.

Experiment 1 Controlled Field Validation with Porcine Tissue: For the field validation study, 14 widemouth mason jars (79 mm x 178 mm; 946 ml, Ball Inc., Daleville, IN, USA) containing 100 ml of sand, a moist paper towel and 200 g porcine muscle tissue (obtained from a local grocery store from multiple packages of lean pork chops) were inoculated with approximately 200 homogenized eggs (multiple female egg clutches broken apart with a camel hair brush in dH_2O). Number of eggs was determined gravimetrically with an Adventure-Pro AV64 Ohaus scale (Pine Brook, NJ, USA). Eggs resulting from F_1 *Ch. rufifacies* adults were used in the experiment. The tops of the jars were covered with a Wypall (Kimberly-Clark Global Sales LLC, Roswell, GA, USA) cloth that was secured to the mason jar via the lid to prevent contamination and larval escape. All 14 jars were set up within a 24 h period and placed in a cage made out of an

aluminum frame (90 x 60 x 76.5 cm) covered in BioQuip® Lumite screen (Rancho Dominguez, CA, USA) on a table (70 cm off the ground) to prevent contamination and aid in protecting the jars from the elements. A U12-012 hobo data logger (Onset, Bourne, MA) was placed in the center of the group of jars on the table to record temperature, humidity and light intensity every ten minutes. The experiment was conducted from 21 June 2010 through 4 July 2010 in College Station, TX, USA.

Two jars were randomly selected at two set times (1100 and 1600 h) daily and all larvae present were placed in hot water at approximately 100°C for 30 s (Adams and Hall 2003) and transferred to vials containing 70% ethyl alcohol (Amendt et al. 2007). Eggs present during the first collection were placed directly into a vial filled with 70% ethyl alcohol. This process continued until pupae were observed, in which case collections ceased and earliest adult emergence was monitored at the set times. Weight, length and life stage were recorded for each larva collected. The study was set up blind to prevent any bias as to the age of the larvae. Total immature flies collected from each jar were analyzed to obtain the developmental variation present in each jar.

Weight, length and life stage were used to estimate age of the immature flies collected. Development data for *Ch. rufifacies* fed porcine muscle tissue at 28.3°C (from chapter 2) herein referred to as the reference data set, were used to analyze the field results, as this temperature was closest in temperature to the outside field cyclic conditions (28.8°C; Appendix A). Length (y-axis) over ADH (x-axis) and Weight (y-axis) over ADH (x-axis) were plotted for the reference data set. Average and maximum lengths/weights were determined for each jar and plotted horizontally across the

reference data set (y-axis). The corresponding x-value (ADH) from where the horizontal average or maximum weight/length intersected the reference data set line was determined as the age of the individuals from the jar (termed the estimated ADH). This estimate was made for each jar containing larvae. Maximum length/weight values were chosen for predicting estimations as this is thought to represent the oldest individuals on a carcass (Byrd et al. 2009). Average was also chosen to incorporate the variation that is found within larval cohorts of all the same age.

True age was estimated with instar by using the oldest instar present in a jar. Using ADH necessary to begin the instar observed (calculated from reference data set) and ADH necessary to complete the instar observed (calculated from reference data set) resulted in a range for the estimated ADH.

ADH for the controlled field validation were calculated (summed hourly temperature minus minimum development threshold, assumed to be 10°C as no development was determined to occur for *Ch. rufifacies* at this temperature (Byrd 1998)) for each hour point of each day during the study. True ADH for each jar were calculated by summing the degree hour at each hour point from the time the flies were collected as eggs in the laboratory to the time the jar was randomly selected out in the field and recorded on a temperature spreadsheet. ADH estimates were then compared to true ADH and percent over- and underestimations determined.

Statistics: GAM, using the mgcv library in the R (Vienna, Austria) statistical package, were used to determine the best model which explains the highest percent deviance for the laboratory data set (Wood 2006). Model selection was determined by

observing the percent deviance explained (used rather than R^2 for non-linear models) for each of the models as well as the generalized cross-validation (GCV) score. Lower GCV scores indicate models that better predict the estimated parameter (development proportion). GAM can relate non-linear parameters, such as larval length and weight to the value being predicted (ex. development proportion) using smoothed non-linear mathematical functions (Tarone and Foran 2008). Likewise the relationship of these two terms could also be tested (length by weight). These non-linear parameters are denoted by s(measured variable) in R, either length (s(length)), weight (s(weight)) or the relationship term of length and weight (s(length, weight)). Distributions of the reference data as well as a link function are required in the GAM and were determined to be best represented by a Gamma distribution (rather than normal) with a log-link based on the observed residuals of the compared distributions (data not shown).

Nineteen model comparisons using different variables and/or their interactions and determining which best predicted development proportion (true age) were examined. Development proportion is defined as the hour at which the larvae were collected in the experiment divided by the minimum total time it took that cohort to emerge as adults (Tarone and Foran 2008). In the case of the controlled field validation study, all larvae from a single jar are killed so time to emergence was determined by the first un-sampled jar with pupae to have adult emergence. Development proportion was necessary to compare individuals reared at different temperatures. This method has been used previously for predicting age of a forensically relevant fly (Tarone and Foran 2008).

Once the best model was selected, development proportion predictions for each

jar with larvae were made for the average or maximum model parameters combined (e.g., average length + average weight + average instar). Predictions were determined for each jar using the entire laboratory data set (for all times (development proportions), muscle tissue type, temperatures, replicates and larvae collected) as the reference data set with which to predict development proportion from. Although, the best model may not include all of the variables from the laboratory data set. Estimations are given in the form of development proportion and can be transformed to estimated ADH by multiplying predicted development proportion to the number of ADH required for flies from the prediction data set (same as above) to complete development to the adult stage. This is similar to a forensic case in which a published growth data set (prediction data set) is necessary to calculate the ADH necessary (Higley and Haskell 2009). The estimated ADH are then compared to the true ADH to calculate the percent over- or underestimation for each jar based on the maximum or average GAM estimates.

Experiment 2 Uncontrolled Field Validation with Porcine and Human Remains: Time of field placement for pig and human remains, with known time of death and placement in the field, were determined for *Ch. rufifacies*. In this validation experiment true age (time of colonization) of the flies is not known but is assumed to be close to time of field placement. This information is critical as it demonstrates time of colonization might not coincide with time of placement or actual time of death of the decedent.

These remains were placed in the field in Texas, USA during September-November of 2011, May of 2012 and July of 2012. Each set of pig remains were covered

with a metal cage (built out of cattle panel ~152 x 122 x 61 cm and covered in poultry netting) to prevent vertebrate scavenging. Three pigs (~36 kg) were euthanized by a single 0.22 caliber shot to the head at 0825-0829 h and were placed in open fields on 15 September 2011. Two sets of pig remains were placed at the Texas A&M University Rangeland Science Park in College Station, TX, USA, at 0919 h for the left pig (pig 1) and 0922 h for the right pig (pig 2), approximately 30 m apart. One pig was placed in an open field in Snook, TX, USA at 0953 h (pig 3) which is approximately 25 km Southwest of College Station. All of the pigs were placed in the field with their snouts facing east and belly facing south. A single pig (pig 4) (19.1 kg) was euthanized by a single 0.22 caliber shot to the head at 1156 h on 30 October 2011 and stored in a cooler on ice until placed out in a field at the Forensic Anthropology Research Facility (FARF) in San Marcos, TX, USA at 1529 h on 2 November 2011. San Marcos, Texas is approximately 206 km Southwest of College Station. The snout of the animal was placed pointing north and the belly facing east. A single pig (pig 5) (~77 kg) was euthanized by a single 0.22 caliber shot to the head at 1215 h on 22 May 2012 and stored in a body bag on ice until placed out in a field at the FARF at 1656 h on 22 May 2012. The snout of the animal was placed pointing north and the belly facing east. Use of pig remains was approved by the Animal Welfare Assurance Program at Texas A&M University. The remains of an 84-year-old African American male (human 1) weighing ~47.6 kg were stored in a morgue from the time of death (1152 h on 25 October 2011) until they were brought to the FARF on 02 November 2011 and placed in the field at 1529 h. The remains were covered with an approximately 4.98 m (l) x 4.98 m (w) x 2.44 m (h) large

steel pole frame cage covered in 87% shade cloth (Easy Gardener, Waco, TX, USA) to prevent desiccation and also acted to exclude scavengers. The remains of a 59-year-old Caucasian male (human 2) weighing ~153 kg were stored in the morgue/ FARF cooler from the time of death (0845 h on 15 May 2012) until placed out in the field at 1812 h on 22 May 2012 at the FARF. The remains were covered with a large secondary cage as previously described. The remains of an 18-year-old Caucasian male (human 3) weighing ~66 kg were stored in the morgue from the time of death (1722 h on 18 May 2012) until placed out in the field at 1200 h on 23 May 2012 at the FARF facility and covered with an approximately 2.13 m (l) x 1.22 m (w) x 0.61 m (h) cattle panel wire cage to prevent scavenging. The remains of a 58-year-old Caucasian female (human 4) weighing ~68 kg were stored in the morgue from the time of death (1855 h on 05 July 2012) until moved to the FARF cooler at 4°C (1628 h on 11 July 2012) and finally placed out in the field at 1500 h on 14 July 2012. The remains were covered with a 2.13 m (l) x 1.22 m (w) x 0.61 m (h) cattle panel wire cage to prevent scavenging.

This study was conducted blind. Larvae collected were placed directly in 70% ethyl alcohol. Larvae were returned to the FLIES Facility and instar, length and weight recorded for each specimen. Larvae were collected at 1515 h for pigs 1 and 2 and at 1536 h for pig 3 on 19 September 2011. Larvae were collected for pig 4 at 1205 h on 8 November 2011. Larvae were collected for pig 5 at 0720 h on 26 May 2012. Larvae were collected from human 1 on 8 November 2011 at 1115 h. Larvae were collected from human 2 on 26 May 2012 at 0932 h. Larvae were collected from human 3 on 26 May 2012 at 1700 h. Larvae were collected from human 4 at 2007 h on 24 July 2012.

ADH estimates for each set of remains were determined using the traditional methods of average or maximum length, weight or instar (begin and finish) as mentioned above to predict time of field placement. Estimated ADH using the laboratory data as the reference data set ($GAM_{Lab)}$, as mentioned above were also done to predict time of field placement. Daily ADH were calculated using weather data obtained from www.wunderground.com for the cities in which the studies were performed (College Station, Snook and San Marcos, TX) (Scala and Wallace 2009). Instar(s) was determined by observing the number of posterior slits present in the posterior spiracles. Percent error was calculated by dividing the estimated ADH by the true ADH and subtracting 100. Percentages were converted to hours for comparison, as percentage values represent different ADH for each jar, since each jar experienced variable temperatures depending on how long it was exposed while in the field. In the traditional estimation of ADH with length and weight, when field-collected larvae were too large (heavier or longer than any of the laboratory reared larvae) to intersect the laboratory growth curve (reference data set) the age was estimated using the age of the largest individuals observed on the growth curve. When the field collected larvae did intersect the laboratory growth curve there were instances when it intersected the curve numerous times. In this case ranges were calculated for the first and last intersections and reported as percentages.

As a follow up to the current study, data collected from the controlled field validation study (i.e., data from larvae in the mason jars) were used as the reference data set rather than the laboratory data set. GAM estimates of ADH for time of field placement of the remains previously described also were made, herein referred to as the

GAM_{Field}.

Results

Overall, development rate of blow flies in the laboratory study (chapter 2 porcine reared flies at 28.3°C) and the controlled field validation on porcine muscle (Table 3) were similar with regards to the number of hours necessary to complete development to the adult stage, 259.7 h and 262 h respectively. However, larval instars took less time (125 h) to develop in the field than in the laboratory (160 h) but the pupal stage was 36 h longer in the field. Hours needed to complete each stage of development were calculated by observing when the first time an instar was observed to the next observation when no individuals of that instar were collected in the sample (Table 3). A minimum temperature of 24.6°C and a maximum temperature of 33.6°C were recorded during the experiment with an average of 28.8°C.

Table 3. Hours needed to finish stage of development for the blind field study for flies reared on porcine muscle.

Temperature (°C)	Egg (h)	First instar (h)	Second instar (h)	Third instar (h)	Pupa (h)
28.1	17	24	29	72	120

Percent over- and underestimation of true age were calculated for each of the jars (presented in the order they were collected) for average and maximum larval length and weight ADH estimations (Table 4) and larval beginning and ending of oldest instar observed ADH estimations (Table 5). GAM_{Lab} ADH estimations required a model (Table 6) before estimations which is explained further below. GAM_{Lab} ADH

estimations of average model parameters (length, weight and stage) and maximum model parameters of the controlled field validation are given in Table 7.

For the uncontrolled field study using the laboratory development data set to predict true age (GAM_{Lab}), percent over- and underestimation for each of the whole animal remains are given for average and maximum larval length and weight ADH estimations, larval beginning and ending of oldest instar observed ADH estimations and GAM ADH estimations of average model parameters (length, weight and stage) and maximum model parameters (Table 8).

The uncontrolled field study using the controlled field study (reference data set) to predict percent over- and underestimations for true age (GAM_{Field}) of the whole animal remains are given for average model parameters (length, weight and stage) and maximum model parameters (Table 9).

Percent deviance explained for development proportion along with GCV scores were used to select the best GAM (Table 6). The relationship term of length and weight (s(length, weight), model 4, explained the most percent deviance alone (90%) while Tarone and Foran (2008) determined stage of development to predict the most deviance as a single factor (determined to be 80.3% in this study, model 1) (Tarone and Foran 2008). However, when s(length, weight) was added to the traditional estimate factors (instar, length and weight), model 14, it did not add anything more to the model as indicated by no change in the GCV score, model 15. The best model (model 14) was determined to be the model which included instar, s(length) and s(weight). Adding more variables to this model did Adding more variables to this model did not improve the

Table 4. Percent over- or underestimation of true age using length and weight ADH estimations for flies collected from controlled field validation.

Jar	*N* =	Model	Length Percent	Hours	Model	Weight Percent	Hours	Instar(s) Observed
1	67	Maximum	9.3	2	Maximum	-27.2	4	1
		Average	-21.1	3	Average	-27.2	4	
2	189	Maximum	25.0	8	Maximum	-10.7	3	1 and 2
		Average	7.1	3	Average	-28.6	10	
3	122	Maximum	21.0	8	Maximum	13.4	5	2
		Average	0.8	<1	Average	-16.9	7	
4	139	Maximum	58.5-80.7† (4) ‡	32-44	Maximum	24.4-121.6† (2) ‡	12-65	2 and 3
		Average	15.9-169.3† (2) ‡	9-92	Average	4.0	3	
5	170	Maximum	46.4-57.2† (4) ‡	29-34	Maximum	15.6-98.8† (2) ‡	9-58	3
		Average	4.8-143.5† (2) ‡	4-85	Average	-2.9	1	
6	180	Maximum	13.8*	10	Maximum	13.8*	10	3
		Average	13.8*	10	Average	13.8*	10	
7	175	Maximum	5.6*	5	Maximum	5.6*	5	3
		Average	5.6*	5	Average	5.6*	5	
8	183	Maximum	-13.2*	16	Maximum	-13.2	16	3
		Average	-13.2*	16	Average	-13.2	16	
9	210	Maximum	-17.9*	22	Maximum	-17.9	22	3
		Average	-17.9*	22	Average	-17.9	22	

*Percent was estimated for average or maximum length or weight as they did not intersect with the laboratory data
†Ranges are given when length or weight values intersect the graph at the first and last point in the growth curve, upper range is always positive
‡ (value) number of times the length or weight values intersect the laboratory growth curve

Table 5. Percent over- or underestimation of true age using time to begin and finish the oldest instar observed for flies collected from the controlled field validation.

Jar	*N* =	Model	Instar Percent	Hours	Instar(s) Observed
1	67	Finish	116.7	17	1
		Begin	-4.7	<1	
2	189	Finish	77.7	25	1 and 2
		Begin	6.2	3	
3	122	Finish	50.4	18	2
		Begin	-10.1	4	
4	139	Finish	165.4	89	2 and 3
		Begin	1.8	2	
5	170	Finish	140.0	83	3
		Begin	-8.0	5	
6	180	Finish	84.6	65	3
		Begin	-29.2	27	
7	175	Finish	71.2	59	3
		Begin	-34.4	35	
8	183	Finish	40.8	41	3
		Begin	-46.0	53	
9	210	Finish	33.2	35	3
		Begin	-48.9	60	

Table 6. Generalized additive models showing proportion of development assessed.

Model	Development Proportion =	Percent	GCV
1	Stage	80.3	0.045
2	s(length)	84.3	0.037
3	s(weight)	86.8	0.031
4	s(length, weight)	90.0	0.024
5	Temperature	0.5	0.229
6	Muscle type	0.1	0.230
7	Stage + temperature	80.4	0.045
8	Stage + muscle type	80.4	0.045
9	Stage + s(length, weight)	90.6	0.023
10	Stage + temperature + muscle type	80.5	0.045
11	Stage + s(length)	85.8	0.033
12	Stage + s(weight)	89.5	0.025
13	s(length) + s(weight)	91.3	0.021
14	Stage + s(length) + s(weight)	91.6	0.020
15	Stage + s(length) + s(weight) + s(length, weight)	91.7	0.020
16	Stage + s(length) + s(weight) + temperature	91.6	0.020
17	Stage + s(length) + s(weight) + muscle type	92.0	0.019
18	Stage + s(length) + s(weight) + muscle type + temperature	92.0	0.019
19	Stage + s(length) + s(weight) + s(length, weight) + muscle type + temperature	93.1	0.018

Table 7. Percent over- or underestimation of true *Ch. rufifacies* age using the adjusted GAM model for flies collected from the controlled field validation.

Jar	*N* =	Model	Adjusted GAM Percent	Hours	Instar(s) Observed
1	67	Maximum	-44.9	8	1
		Average	-65.4	11	
2	189	Maximum	2.0	1	1 and 2
		Average	-11.3	4	
3	122	Maximum	-1.6	<1	2
		Average	-12.7	5	
4	139	Maximum	47.7	27	2 and 3
		Average	-1.4	<1	
5	170	Maximum	35.5	22	3
		Average	-2.1	1	
6	180	Maximum	14.6	11	3
		Average	11.8	9	
7	175	Maximum	5.3	5	3
		Average	3.7	4	
8	183	Maximum	-21.3	26	3
		Average	-16.2	19	
9	210	Maximum	-21.2	27	3
		Average	-21.6	28	

Table 8. Percent over- or underestimation of remains time of field placement using traditional and adjusted GAM methods with laboratory data as a reference for flies collected from the uncontrolled field validation.

Remains	*N* =	Model**	Length Percent	Hours	Weight Percent	Hours	Instar Percent	Hours	Adjusted GAM Percent	Hours
Pig 1	11	Maximum	-5.1-12.3† (2)‡	4-13	-2.0*	2	58.9	51	-7.5	6
(College Station Left)		Average	-11.2-22.5† (2) ‡	9-20	-12.2-24.5† (2) ‡	10-22	-39.1	38	-7.3	6
Pig 2	17	Maximum	-13.2-24.5† (2) ‡	11-22	-30.6-53.1† (3) ‡	30-46	58.9	51	-16.2	14
(College Station Right)		Average	-40.8-68.4† (2) ‡	40-61	-41.8	41	-39.1	38	-36.7	35
Pig 3	18	Maximum	1.9*	2	1.9*	2	65.2	58	0.1	<1
(Snook)		Average	-8.8-29.4† (2) ‡	7-26	-30.0-57.0† (3) ‡	28-49	-36.7	34	-18.4	16
Pig 4	53	Maximum	16.4-159.1† (5) ‡	18-218	3.3	2	-2.1	1	10.8	11
(San Marcos 2011)		Average	-8.2-173.8† (2) ‡	5-232	-9.8	7	-41.5	88	-4.9	3
Pig 5	88	Maximum	20.3-37.3† (2) ‡	19-32	25.5*	23	103.5	98	17.2	16
(San Marcos 2012)		Average	-15.0-109.1† (4) ‡	15-102	-17.7	18	-22.0	21	-8.0	6
Human 1	10	Maximum	-40.9	87	-45.9	92	-2.1	<1	-47.9	94
(San Marcos 2011)		Average	-50.8	96	-47.5	94	-41.4	87	-53.3	98
Human 2	7	Maximum	-22.0-110.7† (4) ‡	21-103	-19.4	19	102.5	97	-6.6	5
(San Marcos 2012)		Average	-32.4-118.5† (2) ‡	29-115	-27.2	25	-22.4	21	-15.3	16
Human 3	4	Maximum	19.0-58.2† (2) ‡	19-51	29.8*	26	110.6	101	20.1	20
(San Marcos 2012)		Average	17.7-63.6† (2) ‡	19-59	19.0-67.7† (2) ‡	19-63	-19.3	14	23.5	22
Human 4	20	Maximum	-61.8-56.0† (2) ‡	158-144	-60.2*	153	-35.4	93	-62.4	160
(San Marcos 2012)		Average	-64.3-49.4† (2) ‡	164-128	-63.1-49.8† (2) ‡	162-129	-75.2	189	-61.8	158

**For instar estimates maximum equates to finishing the instar and minimum equates to beginning the instar
*Percent was estimated for average or maximum length or weight as they did not intersect with the laboratory data
†Ranges are given when length or weight values intersect the graph at the first and last point in the growth curve, upper range is always positive
‡ (value) number of times the length or weight values intersect the laboratory growth curve

Table 9. Percent over- or underestimation of remains time of field placement using adjusted GAM methods with field data as a reference for flies collected from the uncontrolled field validation.

Remains	*N* =	Model	Field GAM	Hours
Pig 1	11	Maximum	-8.4	7
(College Station Left)		Average	-12.0	9
Pig 2	17	Maximum	-30.7	30
(College Station Right)		Average	-37.3	36
Pig 3	18	Maximum	-1.5	1
(Snook)		Average	-31.2	29
Pig 4	53	Maximum	-1.3	1
(San Marcos 2011)		Average	3.2	2
Pig 5	88	Maximum	-13.4	14
(San Marcos 2012)		Average	-53.0	46
Human 1	10	Maximum	-40.8	86
(San Marcos 2011)		Average	-42.4	88
Human 2	7	Maximum	-53.0	47
(San Marcos 2012)		Average	-47.1	43
Human 3	4	Maximum	-9.1	5
(San Marcos 2012)		Average	-13.7	9
Human 4	20	Maximum	-72.3	181
(San Marcos 2012)		Average	-73.3	184

GCV scores or percent deviance explained.

After model selection the data were assessed for accuracy in predictions. The laboratory development data set (reference) was used to make predictions of itself in order to determine any errors in the prediction estimates. The data were not centered on zero (Figure 7), which was contrary to what was expected, as the reference data set and "unknown" data set were both the same. Therefore, the chosen models' predictions of development proportion were adjusted by subtracting 0.1 to center the predicted minus true data on zero (Figure 8). This model is herein referred to as the adjusted GAM.

Plots of predicted age versus true age are given for each set of jars (as evident from the 9 stacked predictions) for three models (models 18, 11 and 14: found in Table 6) using GAM prediction estimates (Figures 9-11). Plots were also made for predicted age versus true age in the uncontrolled field validation, using only model 14, for pig (Figure 12) and human (Figure 13) remains. However, one set of human remains (human 4) had a predicted versus true age that was greatly different than the other remains and skewed the graph. Therefore, this human was removed from the graph to allow for better resolution of human 1-3 remains (Figure 14).

Controlled Blind Field Validation of Ch. rufifacies for Weight, Length and Instar: A range of 0.79% at the closest to true age and 58.52% at the furthest using the length model for the jars, represented an overestimation of <1 h (2^{nd} instar) to ~32 h (early 3^{rd} instar) overestimated respectively. Early 3^{rd} instar is defined here as the first two instances where 3^{rd} instar larvae were observed in the 6 jars containing 3^{rd} instar larvae. Length model estimations were fairly accurate for 1^{st}, 2^{nd} and early 3^{rd} instars (<1-9 h) when using the average length prediction estimate. For mid to late 3^{rd} instar larvae the predictions were more variable (5-22 h) and were estimated as the field larvae were longer than the laboratory reared larvae. Mid 3^{rd} instar is defined here as the two 3^{rd} instar collections between the two early and two late 3^{rd} instar larval collections while late 3^{rd} instar refers to the last two collections of 3^{rd} instar larvae prior to the appearance of pupae.

The range for the weight model estimations for the jars collected was -2.89% at the closest to true age and -17.88% at the greatest with an underestimation representing

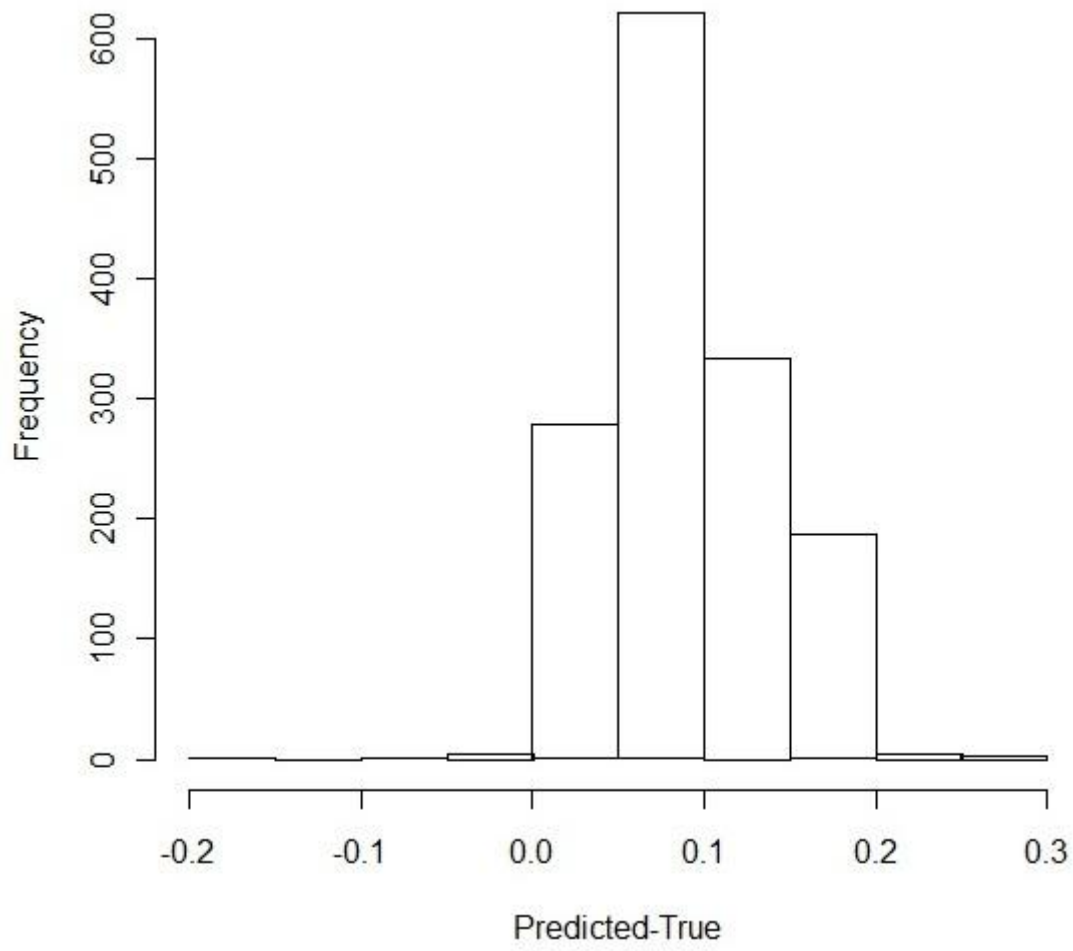

Figure 7. Histogram of predicted proportion of development minus true proportion of development to determine how close model 14 predictions are to true age of unknown age larvae (predicted-true=0).

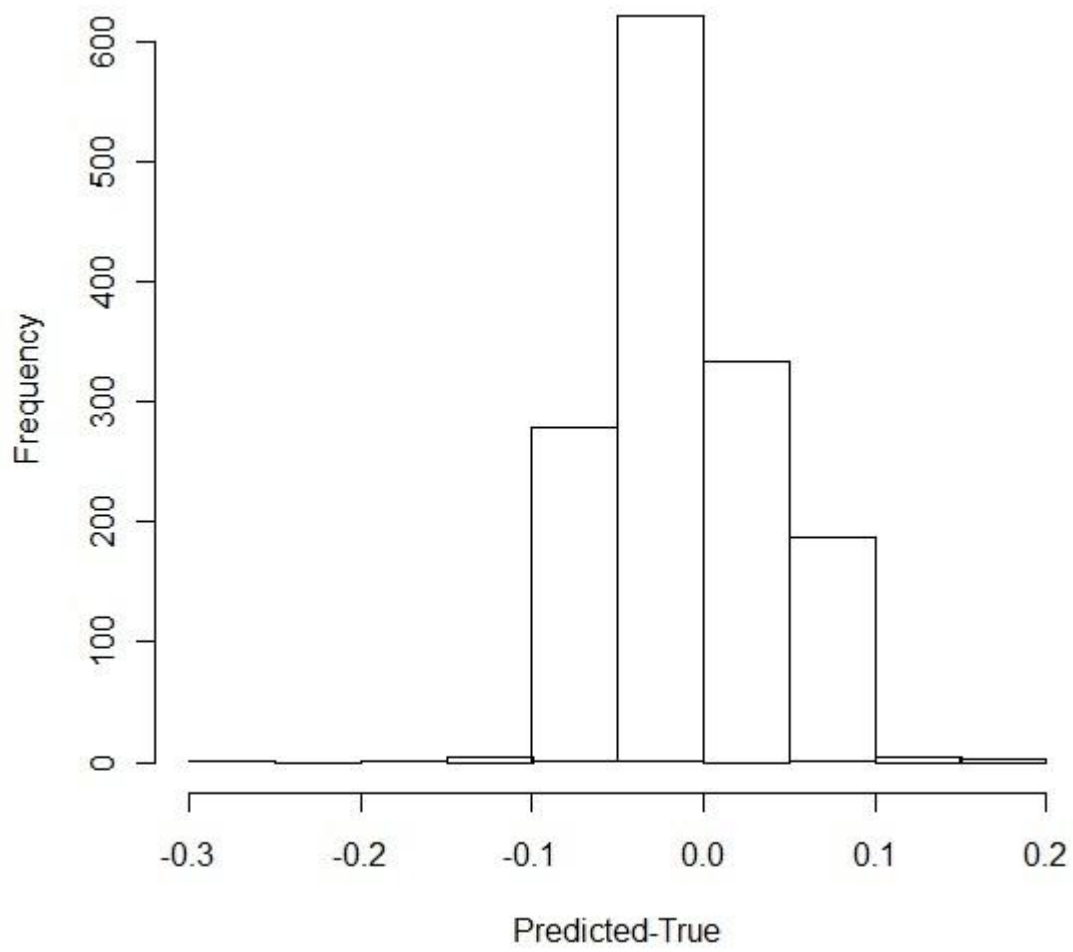

Figure 8. Histogram of predicted proportion of development minus true proportion of development to determine how close model 14 predictions are to true age of unknown age larvae (predicted-true=0) using predictions adjusted by subtracting 0.1.

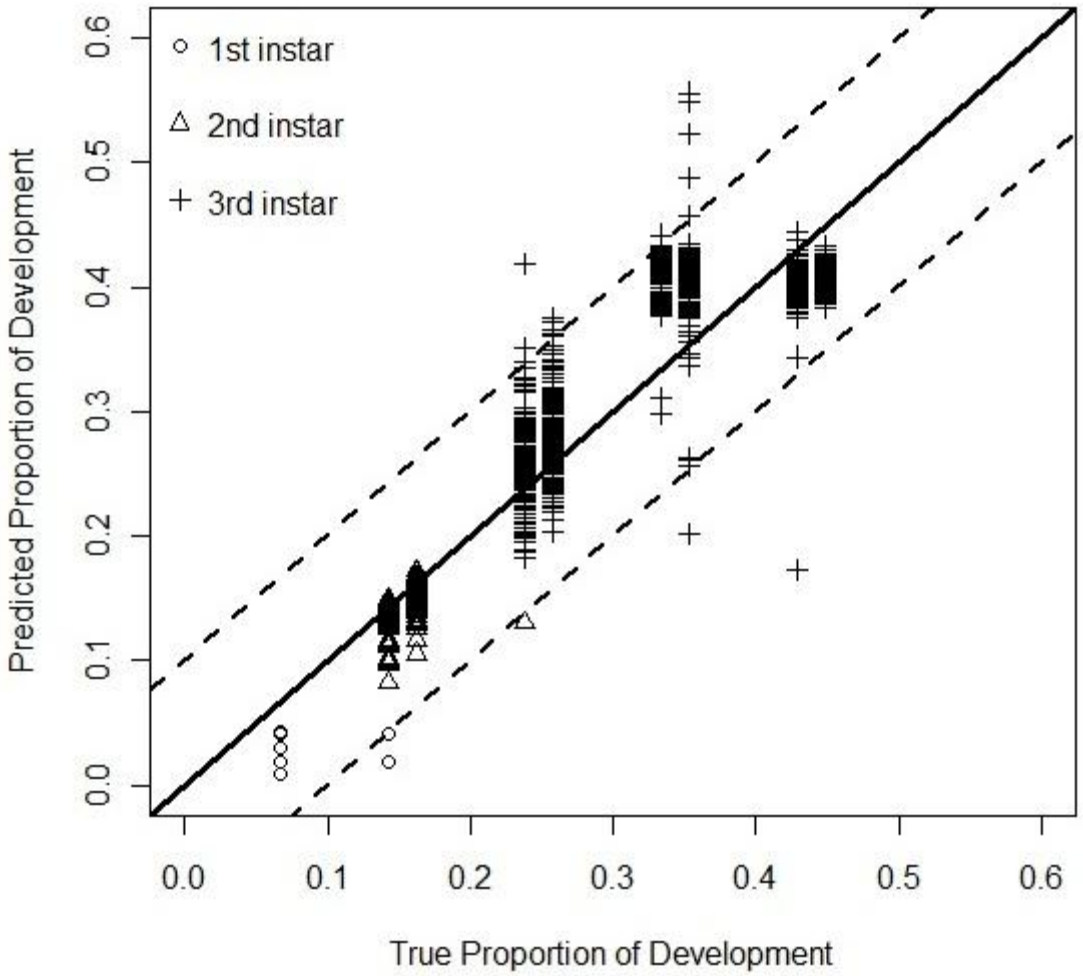

Figure 9. Plot of predicted proportion of development versus true proportion of development using model 18 (stage, s(length), s(weight), tissue and temperature) for predictions on the controlled field reared *Ch. rufifacies* larvae. Solid line represents a 1:1 line (true proportion of development predicted correctly) with the dashed lines representing 10% above and below true proportion of development. 1st instar ($N = 69$), 2nd instar ($N = 310$) and 3rd instar ($N = 1056$).

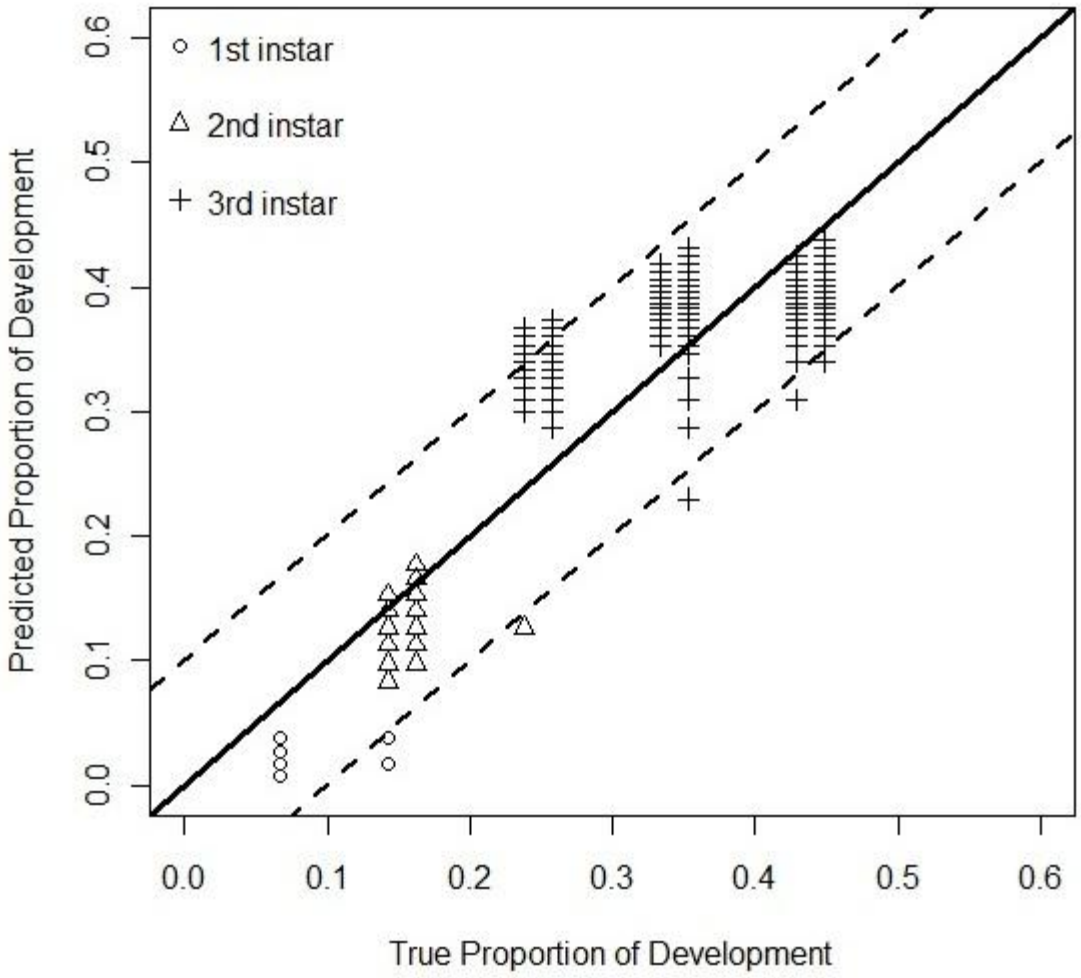

Figure 10. Plot of predicted proportion of development versus true proportion of development using model 11 (stage and s(length)) for predictions on the controlled field reared *Ch. rufifacies* larvae. Solid line represents a 1:1 line (true proportion of development predicted correctly) with the dashed lines representing 10% above and below true proportion of development. 1st instar ($N = 69$), 2nd instar ($N = 310$) and 3rd instar ($N = 1056$).

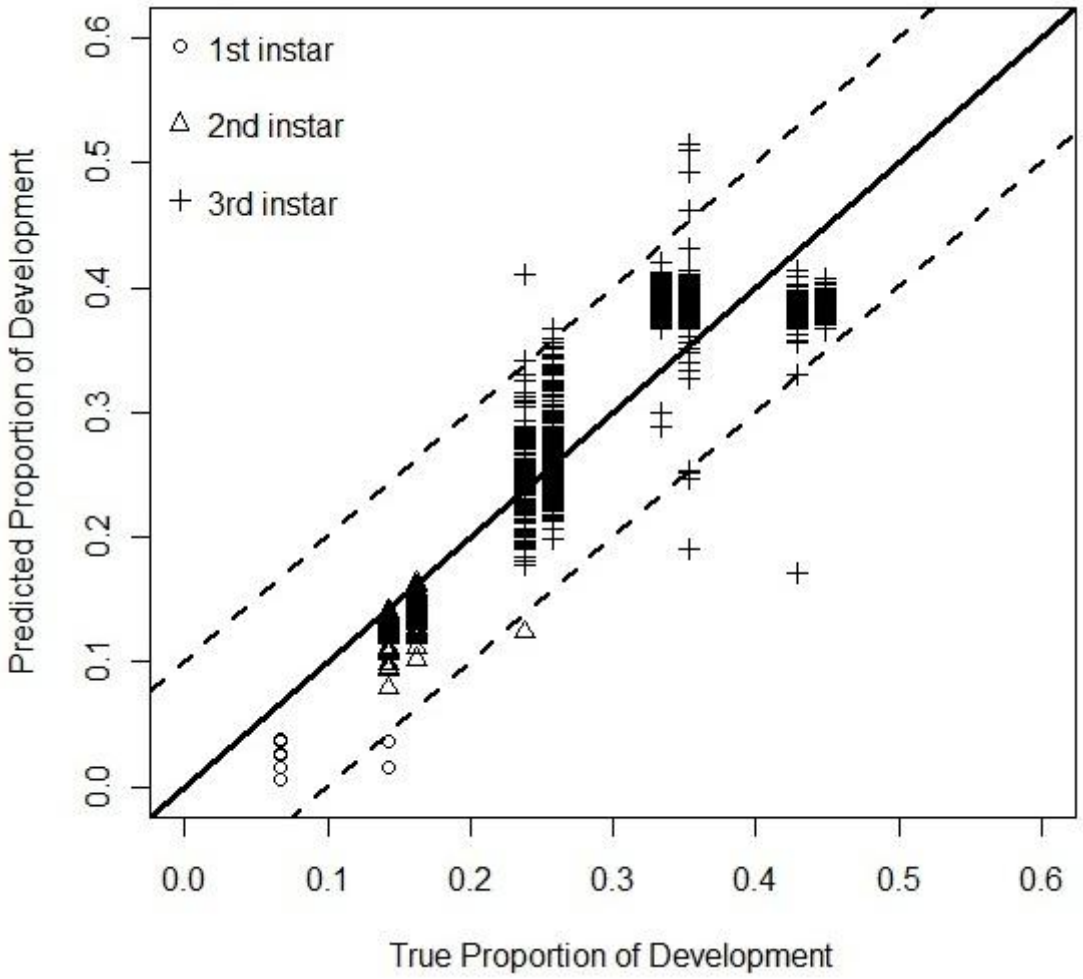

Figure 11. Plot of predicted proportion of development versus true proportion of development using model 14 (stage, s(length) and s(weight)) for predictions on the controlled field reared *Ch. rufifacies* larvae. Solid line represents a 1:1 line (true proportion of development predicted correctly) with the dashed lines representing 10% above and below true proportion of development. 1st instar ($N = 69$), 2nd instar ($N = 310$) and 3rd instar ($N = 1056$).

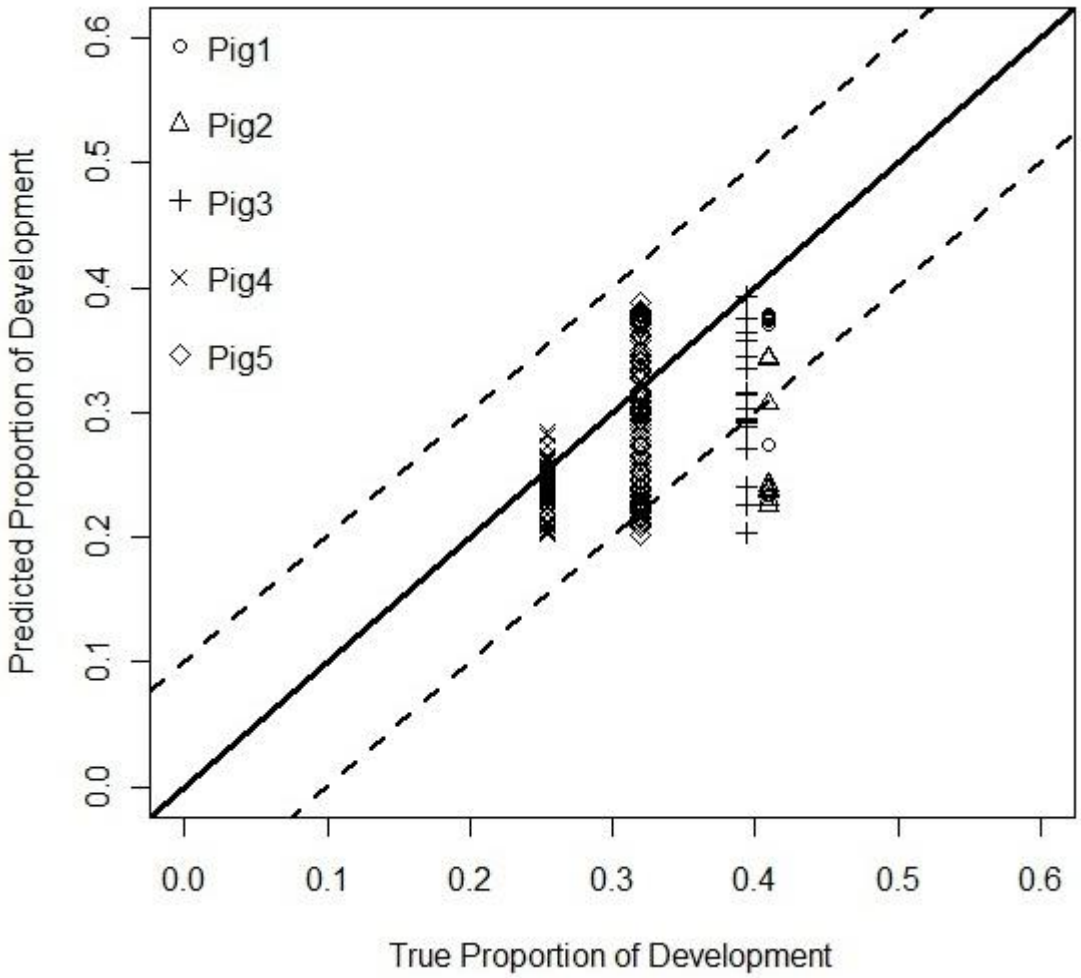

Figure 12. Plot of predicted proportion of development versus true proportion of development using model 14 (stage, s(length) and s(weight)) for predictions on the uncontrolled field *Ch. rufifacies* collected from pig remains. Solid line represents a 1:1 line (true proportion of development predicted correctly) with the dashed lines representing 10% above and below true proportion of development. Pig 1 (N = 11), Pig 2 (N = 17), Pig 3 (N = 18), Pig 4 (N = 53) and Pig 5 (N = 88).

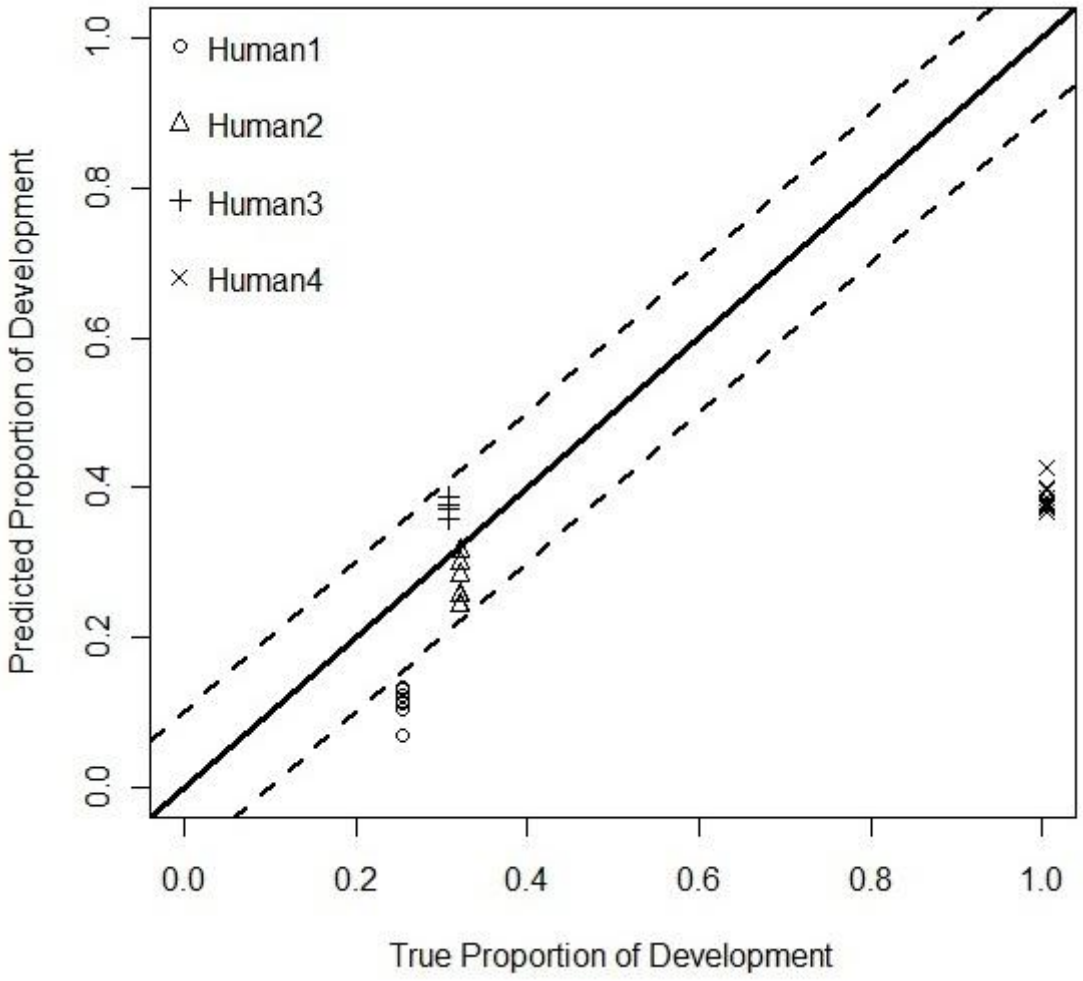

Figure 13. Plot of predicted proportion of development versus true proportion of development using model 14 (stage, s(length) and s(weight)) for predictions on the uncontrolled field *Ch. rufifacies* collected from human remains. Solid line represents a 1:1 line (true proportion of development predicted correctly) with the dashed lines representing 10% above and below true proportion of development. Human 1 (N = 10), Human 2 (N = 7), Human 3 (N = 4) and Human 4 (N = 20).

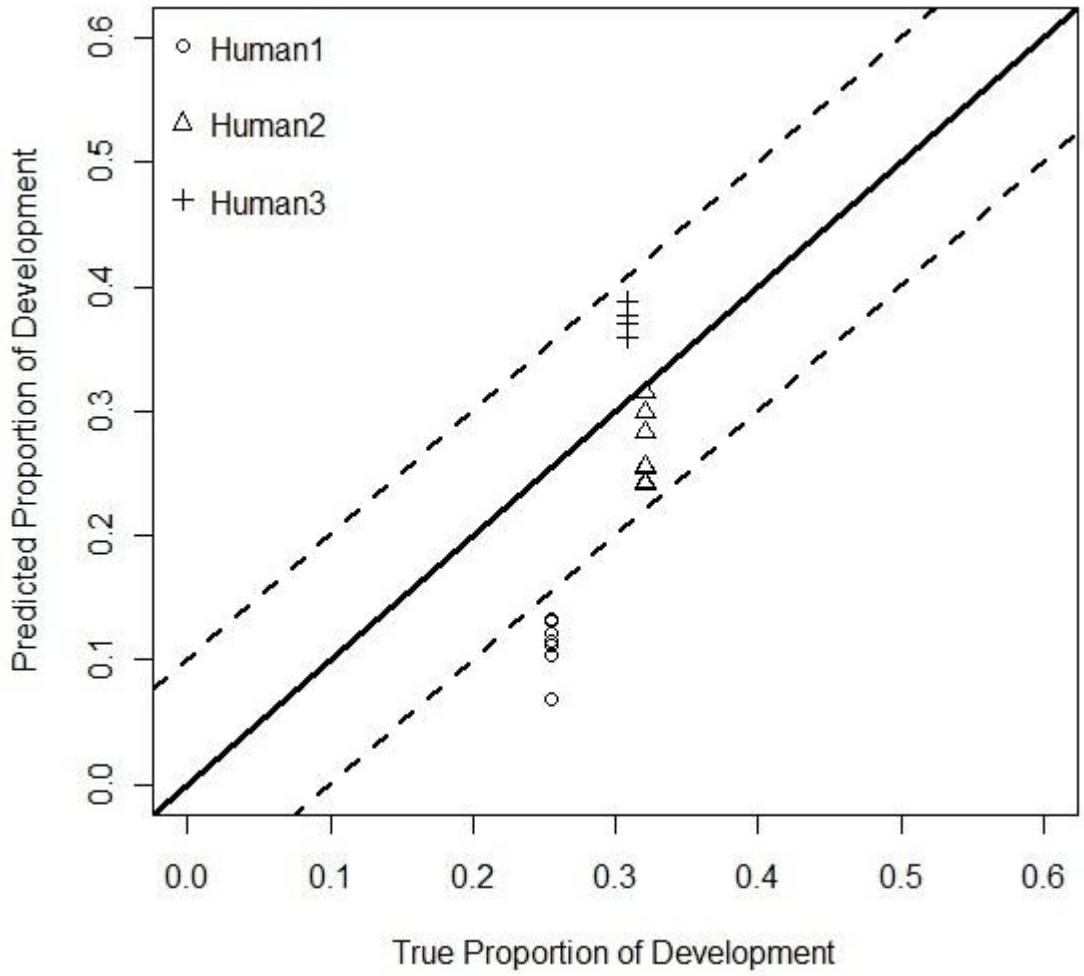

Figure 14. Plot of predicted proportion of development versus true proportion of development using model 14 (stage, s(length) and s(weight)) for predictions on the uncontrolled field *Ch. rufifacies* collected from human remains, minus Human4. Solid line represents a 1:1 line (true proportion of development predicted correctly) with the dashed lines representing 10% above and below true proportion of development. Human 1 (N = 10), Human 2 (N = 7) and Human 3 (N = 4).

~1 h (early 3rd instar) to ~22 h (late 3rd instar) respectively. For weight model estimations on 1st and 2nd instars using the maximum weight estimation ranged from 3-5 h while early 3rd instars the average weight model had a range of 1-3 h. Mid 3rd instar predictions were estimated as these larvae were heavier than laboratory larvae up until the late 3rd instar stage when predictions could be made (larval sizes reduce at this stage) and were the same for both average and maximum weight methods with a range of 1-22 h.

Length model estimations were never the most accurate prediction method examined while weight model estimations were more accurate for the last two jars collected (late 3rd instar). More than half of the length model estimates were overestimated while just over half were underestimated for the weight model.

The instar model estimations are provided in a range (begin and finish). For this study, the range encompasses the true age for all of the jars except 2 and 4 in which case the most it was off by was ~3 h. A range of -4.70% at the closest to true age and 165.39% at the furthest for instar model estimations were observed, representing <1 h (1st instar) to ~89 h (early 3rd instar) respectively. Over half (11 of 18 jars) of the instar models for the controlled field data were overestimated. Estimates for time to finish the instar were only more accurate than estimates to begin the instar on the last two jars representing larvae finishing the 3rd instar stage. A range of error for 1st and 2nd instars (<1-25 h) was less than that determined for 3rd instar larvae (2-89 h) depending on what portion of the 3rd instar the larvae were in. The instar model prediction method was the most accurate predictor of all the methods tested for jar 1 when the larvae were just hatching from eggs.

Controlled Blind Field Validation of Ch. rufifacies with GAM: No trend was observed for a model (maximum or average) that more accurately predicted true age from the adjusted GAM estimates. More than half of the estimates predicted were underestimated with the range of estimates for the adjusted GAM models on true age between -1.41% at the closest to -21.59% at the furthest representing <1 h to ~28 h respectively. The error range for the adjusted GAM estimates was largest for the 3rd

instar stage (<1-28 h) when compared to the 1st and 2nd instar range (<1-11 h). The adjusted GAM prediction method was the more accurate predictor compared to the other models for jars 2-7 representing flies in the 2nd instar and early to mid-3rd instar.

Overall for the controlled field validation the instar model was accurate for jar 1 (1st instar), the adjusted GAM model for jars 2-7 (1st instar-mid 3rd instars) and the weight model for jars 8 and 9 (late 3rd instars). For the jars where length and weight were estimated, because the average or maximum length/weight of the larvae collected did not intersect the growth curve, there was always a more accurate model (instar or adjusted GAM) to predict true age. Adjusted GAM, length and weight estimates were within 24 h of one another with a range of 0-20 h.

Uncontrolled Blind Field Validation of Ch. rufifacies for Weight, Length and Instar: All pig remains had third instar *Ch. rufifacies*, human 1 remains had second instar *Ch. rufifacies* larvae and human 2, 3 and 4 had third instar larvae. Larvae in the 3rd instar were preferred for this portion of the study as this is the most variable stage of growth (length and weight) measured in the laboratory study (chapter 2). The range of estimates for the length model predictions were from 1.85% at the closest to -64.32% at the furthest representing 2 h (pig 3) to 164 h (human 4) respectively. Weight model predictions had a range from 1.85% at the closest to -63.07% at the furthest representing 2 h (pig 3) to 162 h (human 4) respectively. The instar error predictions ranged from -2.05% (human 1) at the closest and -75.23% (human 4) at the furthest representing <1 h to 189 h respectively.

In 6 out of the 9 remains, the instar predictions encompassed the time of field

placement with 2 remains being off by at most 1 h and the last by 158 h for time of field placement (most likely due to the temperatures experienced early in the trial being below the minimum threshold for this fly to accumulate degree days). Weight and length were the most accurate prediction models for pig 1 (2 h) and pig 2 (11 h) respectively however for pig 1 the prediction for weight was estimated. For pig 4, human 1 and human 3 the instar model predictions were the most accurate representing 1, <1 and 14 h of time of field placement respectively. Of the accurate models the maximum estimate worked for 7 of the 9 remains (3 out of 4 humans). Predictions based on maximum length were more accurate for 7 out of 9 remains rather than the average. Likewise, the same results were generated for weight where field placement determination was more accurate using the maximum estimates (7 out of 9 remains). The literature recommends using the largest individuals on remains as they are the oldest individuals and in this case may help yield better estimates (Byrd et al. 2009).

Pig 4 and human 1 were both placed out in the field at the same time, larvae collected within 1 h of one another. In both cases the instar model predicted within ~1 h time of placement of the remains. Pigs 1, 2 and 3 were placed out in the field within 1 h of each other while only pig 1 and 2 were accurately predicted by the same model (length) (if I exclude the estimated weight prediction). Pig 1 and 2 were both placed out in College Station, TX and pig 3 in Snook, TX with the larvae being collected within 1 h of each other.

Uncontrolled Blind Field Validation of Ch. rufifacies with Laboratory Prediction Data Set for GAM_{Lab}: For the adjusted GAM_{Lab} predictions, the range of

estimates was from 0.06% (pig 3) at the closest and -62.42% (human 4) at the furthest representing <1 h to 160 h respectively. Adjusted GAM_{Lab} predictions were accurate for pig 3 (<1 h), pig 5 (6 h) and human 2 (5 h). Pig 5 and human 2 were both placed in the field within ~1.25 h of one another and were accurately predicted by the adjusted GAM_{Lab} model. However pig 5 was more accurately predicted by the average parameters of the adjusted GAM_{Lab} while human 2 was the maximum parameters of the adjusted GAM_{Lab} which is perhaps due to a ~2.25 h gap in collections from each remains. Pigs 1, 2 and 3 were placed out in the field within 1 h of each other; however, the adjusted GAM_{Lab} only accurately predicted pig 3 and the other two pigs accurately predicted by the traditional length method.

More than half of the estimates for the whole vertebrate estimations for all models (length, weight, instar and adjusted GAM_{Lab}) were underestimated. This is most likely since I was predicting TOC and not time of field placement which in some instances may be very close to one another. However, TOC was not tested as the remains were allowed to be colonized naturally without disturbance. In all instances, except one (human 4), the length, weight and adjusted GAM_{Lab} estimates were within 24 h of one another with a range of 1-21 h.

Uncontrolled Blind Field Validation of Ch. rufifacies with Controlled Field Prediction Data Set for GAM_{Field}: Using the controlled field data to predict time of field placement using adjusted GAM_{Field} models was more accurate than adjusted GAM_{Lab} predictions for pig 4 (average and maximum), pig 5 (maximum), human 1 (average and maximum) and human 3 (average and maximum). The predictions were more accurate

by a range of 1-15 h which might not be large enough to consider using the adjusted GAM_{Field} over the adjusted GAM_{Lab}.

Discussion

Only four validation studies have been conducted to date (Tarone and Foran 2008, VanLaerhoven 2008, Matuszewski 2011, Núñez-Vázquez et al. 2013) despite the 2009 criticisms of the forensic sciences put forth by the National Research Council (NRC) indicating a need for such work (Committee 2009). Validation studies aid in understanding the error associated with predicting insect age in forensic investigations. Within the USA, known error rates are one of the standards for the admissibility of scientific evidence in the court of law as set by Daubert, et al. v. Merrell Dow Pharmaceuticals (509 U.S. 579 [1993]). Failure to meet these criteria can result in evidence not being admitted to court. Consequently, more research is needed to further validate forensic entomology and demonstrate its reliability and relevance within the USA judiciary system.

This work examined error associated with ADH estimations of the time of colonization and postmortem interval in the validation portion examining whole remains based on both traditional and more recently applied statistical techniques. Of the three validation studies on blow flies one (VanLaerhoven 2008) focused on a traditional ADH estimation method (instar), one (Núñez-Vázquez et al. 2013)on the traditional measurements of (length, weight and instar) and the other (Tarone and Foran 2008) on the modern statistical ADH estimation method (GAM). Although no one method was ever the most accurate at predicting true age or time of field placement this work has

helped bring to light variations in these methods.

All methods from the current study were highly variable in the time of field placement predictions for whole animal remains. Traditional methods had a range for length (2-164 h), weight (1-162 h) and instar (<1-189 h) and modern methods had a range for GAM (<1-160 h). However for the instar method, 6 of the 9 predictions encompassed time of field placement, and should be recommended as a method for predicting mPMI in forensic cases.

Variation in methods can be compounded by variation in population differences in development for a given species as demonstrated before for *L. sericata* (Tarone and Foran 2008, Gallagher et al. 2010). As mentioned in chapter 2, both authors observed variation in development time and sizes of different populations of *L. sericata*. Both development and size would influence ADH estimation predictions and is more reason for developing local development data sets for forensic species encountered in areas where forensic cases often arise. It is recommended that future development studies make their raw data available for use by forensic entomologists either in regards to research or application in the analysis of entomological evidence. Furthermore, if a practitioner would like to apply a GAM to estimate ADH elapsed; such efforts can only be made by having the raw data as the reference data set.

Time of field placement was controlled in my field study which is why it was predicted, instead of time of death, for the ADH estimations. When assisting with a forensic case, TOC is usually predicted which often times can coincide with time of field placement and occasionally with the actual time of death. In most cases, forensic

entomologists predict a minimum time since death (mPMI) which encompasses both time of field placement and time of colonization. In the case of human 1, the individual was stored in a cooler for 8 d until placement in the field during winter temperatures which would have unnecessary error inferred when predicting time of death. A delay in colonization was evident between the human remains (human 1) and a set of pig remains (pig 4) placed out at the same time. Pig 4 had late 3rd instar larvae present while the human 1 had late 2nd instar larvae. For these remains, estimating time of death would result in error not only from cold storage but also from a delay in colonization. However, if estimating a mPMI, the forensic entomologist is being more conservative and accurate.

Although an attempt was made to standardize methods between studies, slight methodological variations did exist. Intervals between experiment measurements, with laboratory observations made every 12 h, and observations in the field made at the same time twice every day with unequal gaps from one observation to the next but 24 h between every other observation (i.e., 17, 36, 41, 60, 65, 84, 89, 108 and 113). However, as this would likely only affect time spent in individual stages, those values are averaged across replicates and do not adhere to the 12 h gaps in the laboratory data.

Fluctuating temperatures significantly affect blow fly immature development. Greenberg (1991) observed slightly longer development (but not significant) for *Ch. rufifacies*, *Cochliomyia macellaria* (Fabricius) and *P. regina* reared at alternating temperatures as opposed to constant temperatures, each of which had the same average temperature (22.5°C) (Greenberg 1991). Conversely, longer development at alternating

temperatures was observed for *Phaenicia* (*Lucilia*) *sericata* (significantly different). Niederegger et al. (2010) observed similar results in that two cold weather blow flies, *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae) and *Calliphora vomitoria* L. (Diptera: Calliphoridae), developed slightly faster at constant temperatures while two warm weather flies, *Sarcophaga argyrostoma* Robineau-Desvoidy (Diptera: Sarcophagidae) and *Lucilia illustris* Meigen (Diptera: Calliphoridae), developed significantly faster at fluctuating temperatures (Niederegger et al. 2010). When making TOC estimations precautions should be taken depending on the fly species being studied and how much it is influenced by temperature. In the case of *Ch. rufifacies*, both Greenberg (1991) and I determined temperature to not significantly affect larval growth to adulthood (Greenberg 1991).

Temperature data for uncontrolled field remains were obtained from nearby weather stations and so no temperature sensors were placed at the site of the remains to best mimic true clandestine remains locations (Scala and Wallace 2009). This differs from the laboratory and controlled field studies where temperature was monitored and may have led to some errors in calculating ADH during the exposure period of the remains. The controlled laboratory experiment recorded temperature data correlated with the nearby weather station data. Error in correlating weather data recorded from a crime scene after the removal of remains to local weather station data have been previously demonstrated (Archer 2004). However, these errors are associated with correlating temperatures during seasons different from that experienced during the time the remains were in the environment. Since the duration of the experiments in this study were

known, weather data from the appropriate time can be obtained thus minimizing errors in temperature data.

Larval preservation methods were also different between the two studies as they were parboiled in the laboratory study and placed directly in 70% ethanol (usually done by crime scene investigators) in the field study. Preservation method has been shown to affect larval size and weight (Adams and Hall 2003) but has been shown to not affect size of *Ch. rufifacies* larvae (Sanford et al. 2011).

Larval weight and length plotted over time form the laboratory data was variable and nonlinear with observed bimodal humps. Therefore, the field-collected larvae's measured values used for predictions can cross the laboratory length or weight lines multiple times resulting in multiple ADH predictions. It is unclear in these instances which estimate should be used when predicting larval age based on length or weight variables. Similar larval length growth curves for *Ch. rufifacies* with multiple humps in the plots near the end of the 3rd instar stage have been observed previously (Byrd and Butler 1997). GAM estimations, in this study, took into account length, weight and stage of development to predict the estimate of larval age, and provide an estimate that is produced statistically thus removing the potential errors associated with multiple estimations for a single prediction.

When predicting time of field placement based on larvae collected from human and pig remains, the factor most influencing the accuracy of an estimate of ADH depended on when the flies actually colonized the remains. The GAM method in the 3rd instar stage was just as good at predicting true age in the controlled field study without

any of the problems of estimates ages or multiple estimations of age. Therefore it can be a more accurate estimator for TOC estimates of remains regardless of factors slowing or preventing immediate colonization after death. Until these precolonization factors (Tomberlin et al. 2011) can be quantified, the limitation with GAM estimates is that one will only be potentially predicting TOC and not time of field placement or time of death. For the time being a more conservative method that will more often encompass true time of field placement should be utilized (e.g., the instar method). Forensic entomology practitioners should be aware of the assumptions being made for stating time of field placement and/or the post-mortem interval (PMI). Presenting findings as TOC or mPMI removes these assumptions, does not confuse people unaware of these assumptions and leaves room for other evidence to help narrow the true PMI.

The current validation study is the first of its kind for this species and the first to combine all current computational methods being used by forensic entomologists to make mPMI estimations. This work has highlighted the variation in estimates associated with size. It is evident from this study that the limitations to my predictions are to give TOC estimates, or minimum time of field placement/mPMI, as even with fresh remains my predictions of field placement varied (<1 to 189 h) across all models. Instar range estimations are conservative and often encompass time of field placement, something that has been observed previously in *P. regina* (VanLaerhoven 2008, Núñez-Vázquez et al. 2013) but should be further evaluated in other species. Future research should focus on seasonality differences in colonization and how they affect prediction estimations so validation of GAM estimates can be further evaluated. Likewise, validation of published

data sets to predict true age/time of field placement, and understand errors associated with these, this can be done by developing SOP by forensic entomologists (such as the North American Forensic Entomology Association) for conducting future development studies.

CHAPTER IV

SIZE DISTRIBUTIONS IN THE LARVAE OF *CHRYSOMYA RUFIFACIES* (DIPTERA: CALLIPHORIDAE) AND THE IMPLICATIONS FOR FORENSIC ENTOMOLOGY

Introduction

Studies on inter- and intraspecific variation in size have been conducted for a number of insect species (Chown and Gaston 2010). Most of this work is mainly focused on adult size variation or sexual dimorphism. Chown and Gaston (2010) also make note of factors, such as season, latitude, altitude and temperature, affecting size distributions in populations (Chown and Gaston 2010). Tanaka (1981) studied the variation in pronotal shield measurements for head size throughout development of the German cockroach, *Blattella germanica* (Linnaeus) (Blattodea: Blatellidae), and observed increasing variation as the immature cockroaches matured (Tanaka 1981).

Size variation within developmental stages is an important source of error when estimating time of colonization of human remains as related to forensic investigations. Growth studies on blow flies examine relatively few individuals, 20 at the maximum (Anderson 2000) and 2 at the fewest (Byrd and Butler 1997), when numbers observed on remains can be in the thousands (personal observation). Search results in the literature as to development variation among cohorts at individual time points across their growth were absent. Wells (personal communication) urges the need for this work to further understand the variations which may affect size-based estimations of age.

Estimation of insect age in blow flies (Diptera: Calliphoridae) has been based on a number of ways including length, weight, stage of development, gene expression or a combination of some or all of those growth parameters (Wells and LaMotte 1995, Tarone and Foran 2008, 2011) (chapter 3), which are thought to usually have fewer factors affecting them and therefore fewer chances of error (Wells and LaMotte 1995). However, little is known about the variation in these variables occurring in larval population across individual time points in development.

Evaluation of the variation associated with mPMI factors such as length and weight in blow flies has only been investigated briefly at best (Wells and LaMotte 1995, Tarone and Foran 2008). Wells and LaMotte (1995) studied the variation associated with *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae) larval weight for potential use in inverse predictions of unknown aged larvae collected from forensic investigations (Wells and LaMotte 1995). Although specifics were not given, they determined size variation was greatest for 3-4 d old larvae due to low numbers of small larvae and low numbers of larger larvae. Age predictions were possible, though problems distinguishing larvae close in age were observed. They also inferred that combining data for multiple variables such as instar would provide greater resolution.

Tarone and Foran (2008) assessed errors associated with predicting age of the blow fly *Lucilia sericata* (Meigen) through generalized additive models (GAM) to select the growth parameters best for estimations (Tarone and Foran 2008). While larval length and weight (chapter 3) were useful in estimating age in the first and second instar stages, the variation associated with length and weight in the third instar yielded wide TOC

estimates. When utilizing GAM or inverse predictions for estimations of age no set protocols exist for how many larvae to sample and which to include in your estimate. Should the mean of the individuals measured be used or a range from the minimum and maximum values observed? A better understanding of the variation and potential errors associated with growth variables is necessary to best address concerns with mPMI estimations put forth by the National Research Council (NRC) (Committee 2009). The goal of this study was to determine how much variation is associated with larvae of different ages and immature stages.

Materials and Methods

Fly source: *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) larvae (> 500 individuals) were collected from decomposing animal remains located in College Station, Texas, USA. Larvae were brought to the Texas A&M University Forensic Laboratory for the Investigation of Entomological Sciences (FLIES Facility) to initiate colonies. Resulting adult flies were held in multiple 30 cm^3 BioQuip® (Rancho Dominguez, CA, USA) lumite screen collapsible cages in the FLIES Facility (~ 24.4°C, 50% RH and 14:10 light-dark cycle (L:D)). Adult flies were provided deionized water (dH_2O) in a 250 ml Erlenmeyer flask (VWR, Radnor, PA, USA) with paper towels as a wick, and table sugar *ad libitum*.

Experiment: Fourteen widemouth mason jars (79 mm x 178 mm; 946 ml, Ball Inc., Daleville, IN, USA) containing 100 ml of sand, a moist paper towel, and 200 g porcine muscle tissue (obtained from a local grocery store from multiple packages of lean pork chops) were inoculated with approximately 200 homogenized eggs (multiple

female egg clutches broken apart with a camel hair brush in dH_2O). Number of eggs was determined gravimetrically. Eggs resulting from F_1 *Ch. rufifacies* adults were used in the experiment. The tops of the jars were covered with a Wypall (Kimberly-Clark Global Sales LLC, Roswell, GA, USA) cloth that was secured to the mason jar via the lid to prevent contamination and larval escape. All 14 jars were set up within a 24 h period and placed in a cage made out of an aluminum frame (90 x 60 x 76.5 cm) covered in BioQuip® lumite screen (Rancho Dominguez, CA, USA) on a table (70 cm off the ground) to prevent contamination and aid in protecting the jars from the elements. A U12-012 hobo data logger (Onset, Bourne, MA) was placed in the center of the group of jars on the table to record temperature, humidity and light intensity every ten minutes. The average temperature, relative humidity and light intensity experienced by the developing flies in the field was 28.8°C, 85.2% and 367.05 lum/m^2 respectively. Two jars were randomly selected at two set times (1100 and 1600 h) daily and all larvae present were placed in hot water at approximately 100°C for 30 s (Adams and Hall 2003) and placed in 70% ethyl alcohol for later age estimations (Amendt et al. 2007). Weight, length and life stage were recorded for each individual larva. The experiment was conducted from 21 June 2010 through 4 July 2010 in College Station, TX, USA.

Statistics: A Kolmogorov-Smirnov (KS) test in R statistical package (Vienna, Austria) was run to assess if the distributions for first instars was similar to the distributions of second or third instars, and the similarities in distributions of second instars compared to the distributions of third instars. Shapiro-Wilks (SW) test for normal distribution was run for all length and weight larval instar distributions.

Results

Nine jars contained larvae and were evaluated for their variation in weight and length while the other 5 jars contained eggs ($N = 1$) or pupae ($N = 4$). The KS test of all weight comparisons (first v second, first v third and second v third) and length comparisons observed significantly different ($P \leq 0.0001$) distributions amongst all instars for both parameters. All SW tests for normality rejected the null hypothesis of normal distributions ($P \leq 0.0001$).

Boxplots of weight and length (Figure 15 and 16) were plotted over development for each jar showing the distributions for each variable at each larval time point collected. Dot plots of weight and length (Figures 17 and 18) over development for all individuals are is presented by jar. Average weights (Table 10) and lengths (Table 11) are given for each jar with standard errors, instar(s) observed, percent individuals in each instar and number of individuals sampled (N) at that time point.

Jar 7 (3^{rd} instar) larvae had the greatest variance observed for weight while jar 9 (3^{rd} instar) larvae had the greatest variance for length. Length appears to have more variation than weight in the observed ages across time.

Discussion

Body size has been demonstrated to be controlled through an oxygen limiting mechanism due to fixed tracheal systems in insects that cannot support growing body sizes (Callier and Nijhout 2011). As the insect grows within each instar it molts in order to expand the size of its fixed tracheal system to support the larger body size. The

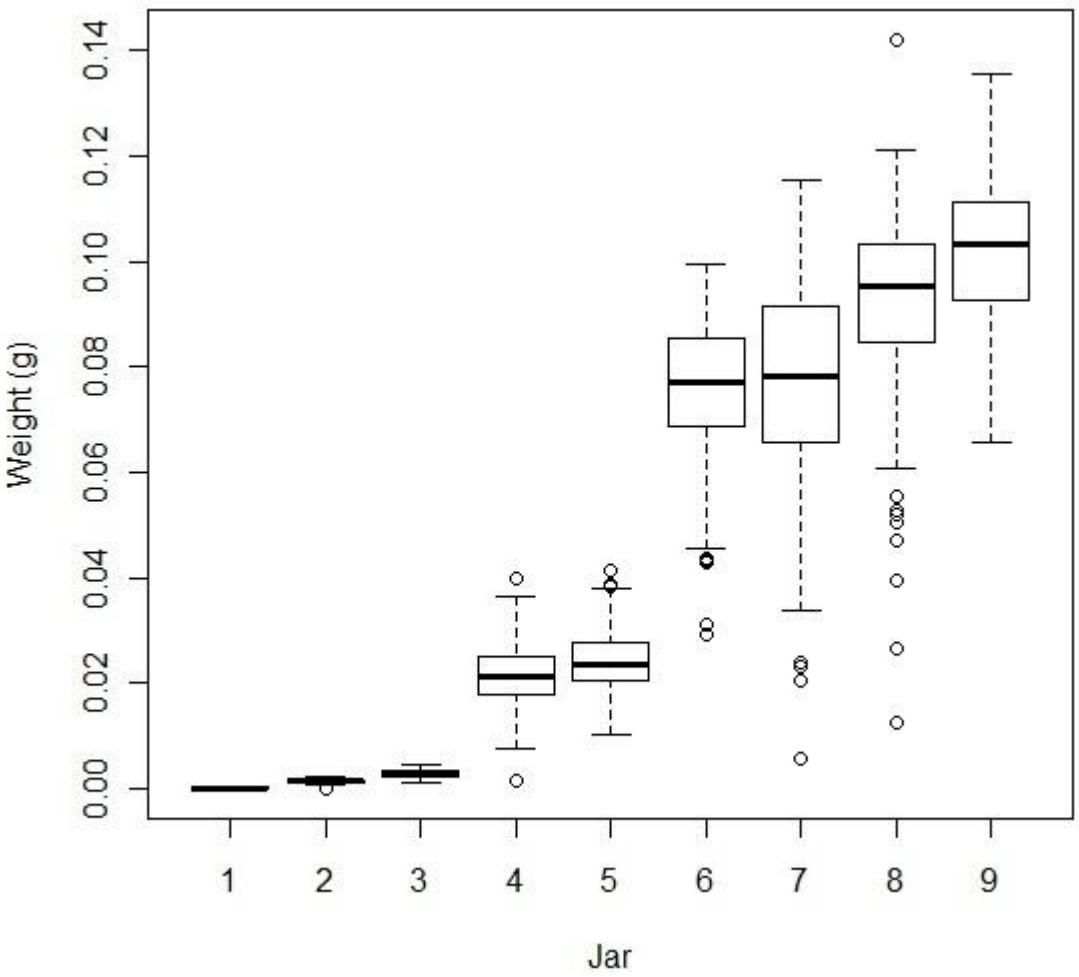

Figure 15. Distribution of *Ch. rufifacies* larval weight separated by jar, in the order they were collected, in the controlled field validation. Jar 1 (*N* = 67), Jar 2 (*N* = 189), Jar 3 (*N* = 122), Jar 4 (*N* = 139), Jar 5 (*N* = 170), Jar 6 (*N* = 180), Jar 7 (*N* = 175), Jar 8 (*N* = 183) and Jar 9 (*N* = 210).

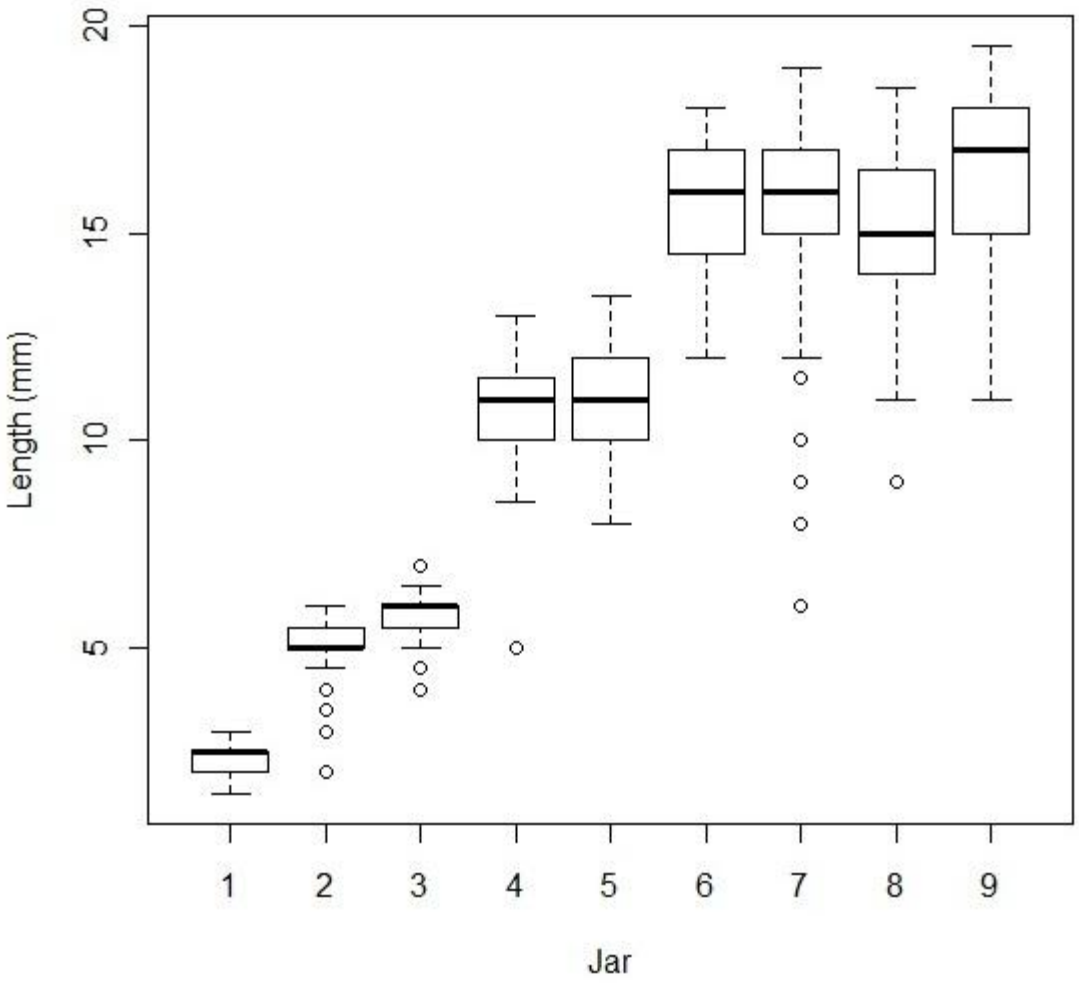

Figure 16. Distribution of *Ch. rufifacies* larval length separated by jar, in the order they were collected, in the controlled field validation. Jar 1 (*N* = 67), Jar 2 (*N* = 189), Jar 3 (*N* = 122), Jar 4 (*N* = 139), Jar 5 (*N* = 170), Jar 6 (*N* = 180), Jar 7 (*N* = 175), Jar 8 (*N* = 183) and Jar 9 (*N* = 210).

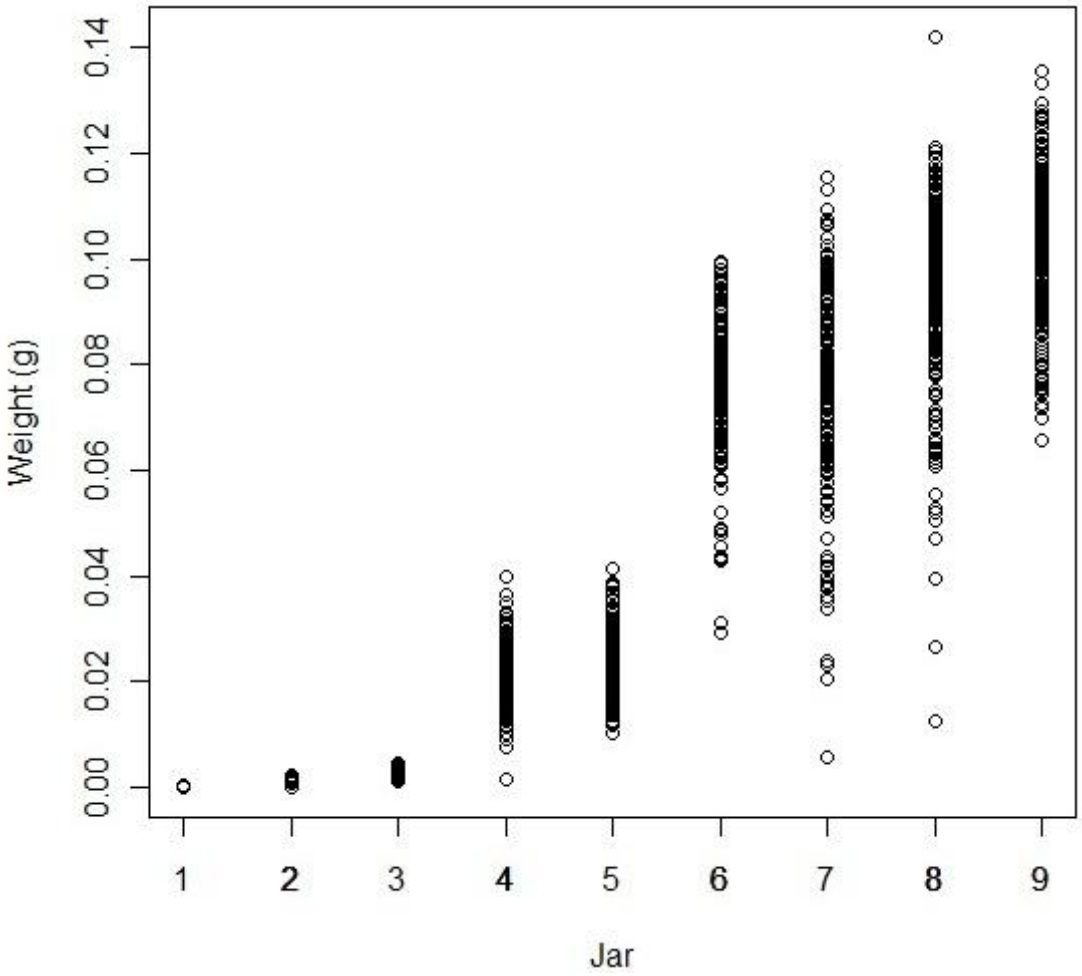

Figure 17. Length of individual *Ch. rufifacies* larvae for each jar, in the order they were collected, for the controlled field validation. Jar 1 ($N = 67$), Jar 2 ($N = 189$), Jar 3 ($N = 122$), Jar 4 ($N = 139$), Jar 5 ($N = 170$), Jar 6 ($N = 180$), Jar 7 ($N = 175$), Jar 8 ($N = 183$) and Jar 9 ($N = 210$).

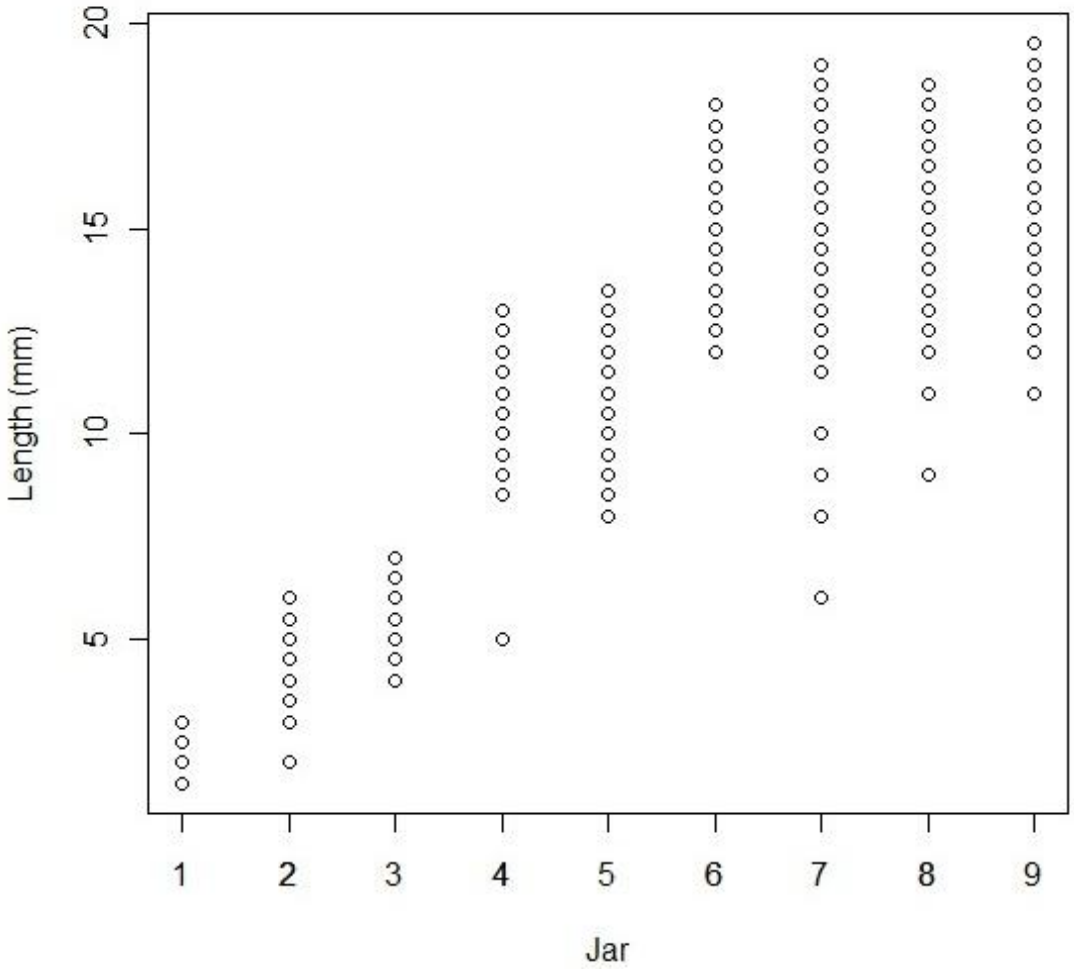

Figure 18. Weight of individual *Ch. rufifacies* larvae for each jar, in the order it was collected, for the controlled field validation. Jar 1 (*N* = 67), Jar 2 (*N* = 189), Jar 3 (*N* = 122), Jar 4 (*N* = 139), Jar 5 (*N* = 170), Jar 6 (*N* = 180), Jar 7 (*N* = 175), Jar 8 (*N* = 183) and Jar 9 (*N* = 210).

Table 10. Average weight of *Ch. rufifacies* larvae (*N*) ± SE for all jars reared under field conditions at 28.8°C during 21 June 2010 through 4 July 2010.

Jar	Average Weight (g) ± SE	Instar(s) Observed (Percent of Total Larvae)	*N*
1	$1x10^{-4} \pm 1x10^{-5}$	1 (100%)	67
2	$1.4x10^{-3} \pm 2 x10^{-5}$	1-2 (1.1-98.9%)	189
3	$2.6x10^{-3} \pm 1 x10^{-4}$	2 (100%)	122
4	$2.1 x10^{-2} \pm 1 x10^{-3}$	2-3 (0.8-99.2%)	139
5	$2.4 x10^{-2} \pm 1 x10^{-3}$	3 (100%)	170
6	$7.6 x10^{-2} \pm 1 x10^{-3}$	3 (100%)	180
7	$7.6 x10^{-2} \pm 1 x10^{-3}$	3 (100%)	175
8	$9.3 x10^{-2} \pm 1 x10^{-3}$	3(100%)	183
9	$0.1 \pm 1 x10^{-3}$	3 (100%)	210

Table 11. Average length of *Ch. rufifacies* larvae (*N*) ± SE for all jars reared under field conditions at 28.8°C during 21 June 2010 through 4 July 2010.

Jar	Average Length (mm) ± SE	Instar(s) Observed (Percent of Total Larvae)	*N*
1	2.4 ± 0	1 (100%)	67
2	5.1 ± 0	1-2 (1.1-98.9%)	189
3	5.9 ± 0.1	2 (100%)	122
4	10.9 ± 0.1	2-3 (0.8-99.2%)	139
5	10.8 ± 0.1	3 (100%)	170
6	15.6 ± 0.1	3 (100%)	180
7	15.6 ± 0.2	3 (100%)	175
8	15.0 ± 0.1	3(100%)	183
9	16.4 ± 0.1	3 (100%)	210

minimal size at which growth to the pupal stage will commence is referred to as the critical weight (Davidowitz et al. 2003), however more recent work suggests that individual instars may have critical sizes at which they initiate molting to the next stage (Callier and Nijhout 2011). Likewise the development time of smaller individuals, who may have pupated sooner, may give *C. macellaria* an evolutionary advantage in time to avoid consequential (i.e., predator-prey) stage overlaps. Previous work has been done to observe the size variations during the growth of immature calliphorids for use in age estimations (Wells and Kurahashi 1994, Wells and LaMotte 1995).

These studies (chapters 2, 3 and 4) on the effects of abiotic factors on the growth and development of *Ch. rufifacies* have demonstrated its sensitivity to tissue more than

temperature. The reverse was determined for a Texas population of *C. macellaria* (Boatright and Tomberlin 2010). In areas where temperature is constant (S. America and the native habitats of *Ch. rufifacies*), *C. macellaria* would not have an ecological advantage of quicker development and could potentially overlap with the predacious state of *Ch. rufifacies* leading to the lowering of its abundance as evident in S. American native and introduced blow fly interactions (Baumgartner and Greenberg 1984). The subsequent work will study the potential biotic effects (interspecific competition) of *Ch. rufifacies* on *C. macellaria* growth and development while controlling for abiotic factors in the laboratory.

Research examining the development of forensically relevant Diptera is common. In many instances, the effects of temperature on the growth of insects examined (Byrd and Butler 1996, 1997, Grassberger and Reiter 2001); however, in most of these studies, not many individuals are measured at a given time point. For instance in Byrd and Butler's study on *Ch. rufifacies* only two larvae were collected while Grassberger and Reiter's (2001) study on *L. sericata* only four were collected (Byrd and Butler 1997, Grassberger and Reiter 2001). Thus a true appreciation of the variation associated with a given time point the development of the target arthropod is not known. Consequently, using data that might not be developed to incorporate true variation could lead to inaccurate age estimations and therefore error prone mPMI estimations. Ways to improve these data would be to increase the number of larvae sampled or increase the frequency of observations.

Recent work has demonstrated that size and development time differences occur

intraspecifically between sexes of *L. sericata* (Picard et al. 2013). Not only should care be taken to observe variation in sexually dimorphic development times but also precise timing on transitions to the next stage. The more time points sampled within an individual stage may help break up stages where longer periods of time are spent. For instance, the third instar stage, longest of the three larval instars, yield large TOC estimates which may be able to be narrowed down with a closer investigation of time points within such a stage. Anderson (2000) measured development with increasing frequency around expected molting times to help narrow down the transition from one stage to another (Anderson 2000).

Known error rates are one of the standards for the admissibility of scientific evidence in the court of law as set by Daubert, et al. v. Merrell Dow Pharmaceuticals (509 U.S. 579 [1993]). Errors associated with predicting TOC with fly development data have been demonstrated (Tarone and Foran 2008) (chapter 3). Incorporating a second variable, like larval size, into estimates based on development can help refine TOC estimates (chapter 3). Understanding the variation surrounding larval size across instars can be beneficial to forensic entomologists for determining appropriate sample sizes of larvae collected from death scenes. The TOC can infer a minimum post-mortem interval (mPMI) which in human remains is based on calculations of the time to elapse since death based on insect succession (Greenberg 1991) or development data (Higley and Haskell 2009). However, development data tend to be more commonly used to estimate the mPMI (Haskell 2007).

CHAPTER V

NON-CONSUMPTIVE EFFECTS OF PREDATORY *CHRYSOMYA RUFIFACIES* LARVAL CUES ON ADULT *COCHLIOMYIA MACELLARIA* ATTRACTION AND OVIPOSITIONAL RESPONSES

Introduction

Multiple arthropod species colonize and utilize vertebrate carrion remains over time (Payne 1965) with the arthropod community pattern shifting (i.e., succession) temporally in a quantifiable fashion. These patterns are also relatively conserved and thus predictable due to the close association of select arthropods with specific ranges of biological criteria in the decomposition process (Wells and LaMotte 2001). With regards to forensic entomology non-reoccurring taxa should be used for estimating time of death of the decedent since these arthropods are only associated with the remains during a particular stage such as skin beetles (Coleoptera: Dermestidae) being associated with the dry remains stage for remains late in the decomposition process (Schoenly 1992).

The cues that alert a given arthropod to the presence of a resource that is in an appropriate state conducive for the survival of their offspring (i.e., nutrients are present, and predators are absent) are not known for many necrophagous arthropods. Studies with parasitoids (Hymenoptera) have demonstrated their responses (e.g. attraction and repellence) to semiochemicals produced in the environment and from their prey are influenced by their age, experience, egg load and hunger, and external influences (e.g., environment, food, competitors and predators) (Vinson 1998). It is hypothesized that

such effects could also play a role in blow fly (Diptera: Calliphoridae) attraction or repellence to decomposing remains (Tomberlin et al. 2011).

Similarly, blow flies (Diptera: Calliphoridae), which are the primary invertebrate decomposers of terrestrial carrion (Fuller 1934), also are influenced by the same physiological factors (Dethier 1961, Bowdan 1982, Ashworth and Wall 1995, Giao and Godoy 2007, Tomberlin et al. 2012). However, cues from the resource also play a role. For instance the breakdown of remains through decomposition by bacteria and other insects produce a number of semiochemicals by which blow flies could use to find such a resource (LeBlanc and Logan 2010).

Cochliomyia macellaria (Fabricius) and *Chrysomya rufifacies* (Macquart) are blow flies commonly observed co-inhabiting decomposing vertebrate remains in Texas (Tenorio et al. 2003) and consequently encountered on decomposing human remains in death investigations (J. K. Tomberlin, personal communication). *Ch. rufifacies* is an invasive species of blow fly while *C. macellaria* is native to the new world (Baumgartner 1993, Byrd and Butler 1996). Their use in forensic entomology to estimate the time of colonization (TOC) is primarily based on available development data generated in the laboratory for pure cultures of a targeted species (Higley and Haskell 2009). How development in mixed culture impacts development is a crucial question for understanding the ecology of vertebrate carrion decomposition as well as when applying such data to TOC estimate (Wells and Greenberg 1992b). All published data sets used by forensic entomologists are single species data sets that do not take into account possible variations due to species interactions in mixed growth conditions.

Oviposition by blow flies has been demonstrated to be impacted by the presence of eggs from competing blow fly species. Giao and Godoy (2007) observed the individual ovipositional preference of three blow flies, *Ch. megacephala* (F.), *Ch. albiceps* (Wiedemann) and *Lucilia eximia* (Robineau-Desvoidy), to three simultaneous choices of ground beef and ground beef inoculated with pure cultures of interspecific 3rd instar larvae of the other two species (Giao and Godoy 2007). All flies preferentially laid eggs on the ground beef only. The predatory fly, *Ch. albiceps*, laid secondarily on ground beef with *Ch. megacephala* and then on ground beef with *L. eximia*. *Ch. megacephala* preferred ground beef with *L. eximia* and lastly ground beef with *Ch. albiceps*, while, *L. eximia* laid eggs on ground beef with *Ch. megacephala* and then on ground beef with *Ch. albiceps*. In essence, the non-predatory flies both laid the least amount of eggs on the ground beef containing *Ch. albiceps* larvae which are predatory.

Although arthropod succession is a predictable series of events on vertebrate carrion, little is known about the impact of one species on another especially when dealing with novel predator-prey interactions (i.e., invasive species biology and non-consumptive effects). In the case of the invasive blow fly *Ch. rufifacies* and native blow fly *C. macellaria*, does the mere presence of the predator influence the behavior of the prey? Such responses are known as non-consumptive effects (NCE: aka nonlethal/trait mediated effects) which are predator mediated effects that alter prey character traits (e.g., behavior, growth and development) (Abrams 1984). In the previous experiment (Giao and Godoy 2007) the authors observed decreased egg laying on resources containing larvae (predatory and non-predatory). However, to date, no study has

investigated similar interactions between *Ch. rufifacies* and *C. macellaria*. The objective of my study was to test the hypothesis that the presence of 3rd instar *Ch. rufifacies* larvae, which may contain larval predatory cues in the form of larval excretion/secretions (ES), influences the attraction and oviposition responses of *C. macellaria*.

Materials and Methods

Fly Source: *Chrysomya rufifacies* and *C. macellaria* larvae (> 500 individuals) were collected from decomposing animal remains located in College Station, Texas, USA. Larvae were brought to the Texas A&M University Forensic Laboratory for the Investigation of Entomological Sciences (FLIES) Facility to initiate colonies. Resulting adult flies were held in multiple 30 cm^3 BioQuip® (Rancho Dominguez, CA, USA) lumite screen collapsible cages or bugdorm cages in the FLIES Facility (~24.4°C, 50% RH and 14:10 L:D). Adult flies were provided deionized water (dH_2O) in 250 ml Erlenmeyer flasks (VWR, Radnor, PA, USA) with paper towels as wicks and table sugar *ad libitum*. Adult flies were provided approximately 30 g beef liver as a protein source changed every 2 d for the first 8 d of life.

Cochliomyia macellaria (between F_0 and F_{10}), 9-12-d old were used in the behavior experiment described below. This age range was selected as these flies have a greater likelihood of being gravid (9 d old from preliminary observations), and they have greater odds of responding (Tomberlin et al. 2012). Only flies from the initial 10 generations were used in order to prevent populations from differentiating greatly from wild type flies (Conner and Hartl 2004). *Ch. rufifacies* and *C. macellaria* (between F_0

and F_{10}) larvae were maintained at ~24.4°C, 50% RH and 14:10 L:D on beef liver in mason jars (79 mm x 178 mm; 946 ml, Ball Inc., Daleville, IN, USA) containing coarse vermiculite (Sungro Agriculture, Agawam, MA), as a pupation medium, and covered with a Wypall cloth (Kimberly-Clark Global Sales LLC, Roswell, GA, USA) held on with the cap. Larvae reaching the active feeding 3rd instar stage (as determined by size and food in the crop) were used in the assay.

Attraction and Oviposition Assays: The behavioral assay was conducted in a 45 cm^3 Plexiglas cage (as previously described (Tomberlin et al. 2012)). The oviposition assay was conducted in a 30 cm^3 BioQuip® bugdorm cage previously described. Cages were cleaned with liqui-nox liquid detergent (White Plains, NY, USA) and water to remove fly specs and odors between each experiment and allowed to dry overnight.

The Plexiglas cage was used to test the response of adult *C. macellaria* for 8 h to all two-way combinations of the following treatments (blank, 50 g of beef liver, 50 g of beef liver with 100 3rd instar *C. macellaria* larvae or 50 g of beef liver with 100 3rd instar *Ch. rufifacies* (predator) larvae). Approximately 200 adult *C. macellaria* flies were aspirated from a rearing cage with a hand vacuum and released in the center of the Plexiglas behavior cage. A 250 w halogen light bulb was placed directly over (~25 cm) the center of the Plexiglas behavior cage during the duration of the experiment to provide the only source of light in the room. The treatments in the two-choice experiment are housed within 90° elbowed polyvinyl chloride (PVC) pipes that were attached to the Plexiglas cage via holes on the left and right bottom sides of the cage (Figure 19). A straight PVC pipe coming directly out of the Plexiglas cage contained a PVC funnel that trapped flies

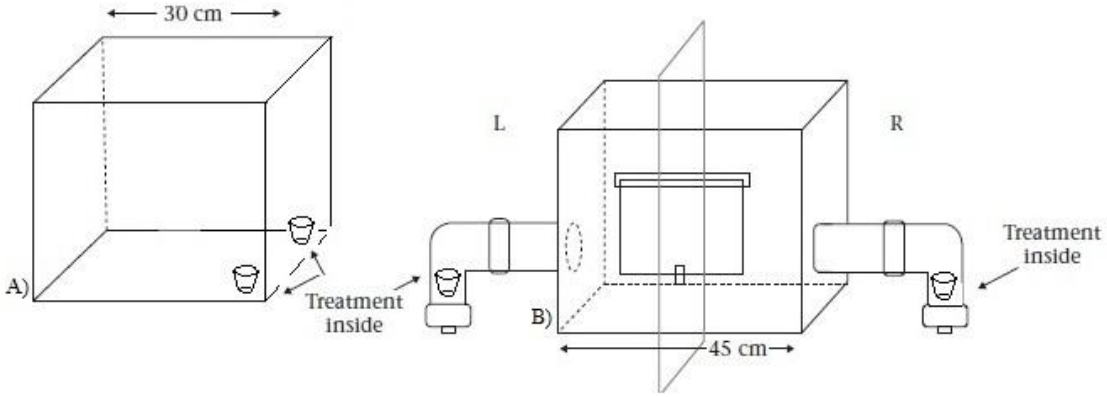

Figure 19. A) Oviposition cage. B) Plexiglas behavior cage. Image not drawn to scale.

that entered the straight pipe. Inside the straight pipe just after the funnel were two unscented sticky glue boards, (Trapper Max Free Bell Laboratories Madison, WI, USA) that acted to collect flies. Chiffon mesh was placed between the distal end of the straight PVC pipe and the elbowed PVC pipe which contained the treatments. The mesh prevented the flies from accessing the treatments. White bath cups (~89 ml) (Great Value San Bruno, CA, USA) were used to hold the treatments as well as a kim wipe (Kimberly-Clark Roswell, GA, USA) placed over the top of the bath cup to prevent larvae from escaping. The kim wipe was crumpled and placed in the bath cup with the respective treatment (or alone for the blank) and served as an oviposition site in the oviposition assay as flies were allowed to access this treatment. Flies trapped on the glue boards for each of the treatment sides, and those remaining in the main Plexiglas cage were killed (placed in a -20°C freezer), counted, sexed and ovarian status determined for all females. Female abdomens were dissected open, using forceps, under a Zeiss (Jena, Germany) Stemi DV4 stereo microscope to determine gravid status for the behavior

assay flies. Gravid was defined as the presence of fully developed ovaries/ovarioles. Non-gravid consequently referred to females with undeveloped ovaries.

The oviposition assay was housed in the same laboratory as the attraction assay. Approximately 100 adult *C. macellaria* flies were released in the center of the oviposition cage with both treatments contained in the white bath cups. The oviposition cage was setup to the left of the Plexiglas cage with the treatments located in the back right and front right corners of the cage closest to the halogen light above the Plexiglas cage. The same treatments tested in the behavior assay were tested simultaneously in the oviposition assay for 8 h. Number of eggs oviposited was determined gravimetrically for each treatment in the oviposition assay.

A U12-012 hobo (Onset, Bourne, MA) data logger was placed under the Plexiglas cage to take temperature, relative humidity (RH) and light intensity readings every 10 m throughout the duration of the assay. Average temperature, RH and light intensity were ~30°C ± 0.25, ~42% ± 2.81 and ~2066.67 lumens/m^2 ± 19.48 respectively.

Four replicates of each two-choice assay were conducted. To avoid bias towards one side of the cage, treatments were rotated from left to right in the Plexiglas cage and front to back in the oviposition cage. Oviposition and attraction assays were conducted concurrently for each fly cohort examined with all flies tested in the experiment having no previous oviposition experience.

Statistics: A generalized linear mixed model (GLIMMIX) was performed on the attraction data in SAS 9.2 (Carry, NC, USA) to determine the responses of the sexes

(male, non-gravid female and gravid female), adjusted for replicate to all pairwise treatment combinations. A paired t-test was run in SAS 9.2 to determine difference in mean percent oviposition between treatments. The level of significance in this experiment was held at $\alpha = 0.1$

Results

Total percent of flies to choose either treatment is presented in Figure 20. Flies released into the olfactometer were examined for the influence of sex on the probability of making a choice versus remaining in the center of the Plexiglas olfactometer (Table 12). Level of attraction to either arm (treatment 1 or treatment 2) of the olfactometer is presented in Table 13 and was calculated by taking the average of each replicates average response. The estimated probability and odds of going to treatment 1 over treatment 2 are shown in Table 14. The variance for replicate was not statistically ($P > 0.1$) different from zero and was included in the final model. Fly oviposition on either treatment is given in Figure 21 with no eggs being laid on any of the blank treatments.

***Experiment 1 (Blank v Blank*)**: Total percent of flies and percent of each sex to choose either treatment is presented in Figure 20 and broken up by treatment in Table 13. Males ($P = 0.0685$) and non-gravid females ($P = 0.0461$) had significantly greater odds of remaining in the center cage rather than make a choice (Table 12). This experiment was important as it determined if there was a bias in response to either side of the olfactometer when no treatments were presented. Response by any sex to either treatment was not significant ($P > 0.1$) and sexes did not respond significantly different from one another (Table 14). Estimated probability and odds of responding to

Table 12. Estimated probability, adjusted for replicate (N=4), with fixed variables related to *C. macellaria* response to both treatments (choice) or remaining in the center arena (no choice) for males, non-gravid and gravid females in all treatment combinations. Response was monitored for 8 h at 30°C with 42% RH and under 2066.67 lumens/m^2 light intensity.

Treatment 1 and treatment 2	***P*-value**	**Sex**	***P*-value**	**Estimated P (SE)**	**Estimated Odds (P/1-P)**
Blank v Blank	0.4431	Male	**0.0685**	0.2676 (0.07442)	0.3654
		Non-gravid Female	**0.0461**	0.2344 (0.06774)	0.3062
		Gravid Female	0.1391	0.2307 (0.1301)	0.2999
Liver v Blank	**0.0704**	Male	**0.0653**	0.3145 (0.06347)	0.4588
		Non-gravid Female	**0.0034**	0.1623 (0.05655)	0.1937
		Gravid Female	**0.0370**	0.2743 (0.05814)	0.3780
Liver with *C. macellaria* v Blank	**0.0158**	Male	**0.0312**	0.2461 (0.05964)	0.3264
		Non-gravid Female	0.3907	0.4043 (0.1035)	0.6787
		Gravid Female	0.1592	0.3604 (0.07254)	0.5635
Liver with *Ch. rufifacies* v Blank	**0.0023**	Male	**0.0130**	0.1706 (0.04719)	0.2057
		Non-gravid Female	**0.0484**	0.2794 (0.07707)	0.3877
		Gravid Female	**0.1001**	0.3205 (0.07126)	0.4717
Liver with *C. macellaria* v Liver	**<0.0001**	Male	**0.0012**	0.09178 (0.0248)	0.1011
		Non-gravid Female	**0.0063**	0.1975 (0.06274)	0.2461
		Gravid Female	**0.0828**	0.3364 (0.06363)	0.5069
Liver with *Ch. rufifacies* v Liver	0.2604	Male	**0.0461**	0.1585 (0.01616)	0.1884
		Non-gravid Female	0.1300	0.2051 (0.04572)	0.2580
		Gravid Female	**0.0615**	0.2541 (0.01979)	0.3407
Liver with *Ch. rufifacies* v Liver with *C. macellaria*	**<0.0001**	Male	**0.0134**	0.2777 (0.04501)	0.3845
		Non-gravid Female	0.2754	0.4230 (0.06443)	0.7331
		Gravid Female	**0.0570**	0.6459 (0.05068)	1.8241

** Estimated probability for each sex can be obtained from the following model: Blank v Blank (log odds (LO) = -1.1836 – 0.02067 x sex1 + 0.1769 x sex2); Liver v Blank (LO = -1.6415 + 0.6684 x sex1 + 0.8621 x sex2); Liver with C. macellaria v Blank (LO = -0.3875 – 0.1861 x sex1 – 0.7320 x sex2); Liver with Ch. rufifacies v Blank (LO = -0.9475 + 0.1959 x sex1 – 0.6342 x sex2); Liver with C. macellaria v Liver (LO = -1.4022 + 0.7230 x sex1 – 0.8899 x sex2); Liver with Ch. rufifacies v Liver (LO = -1.3545 + 0.2779 x sex1 - 0.3148 x sex2); Liver with Ch. rufifacies v Liver with C. macellaria (LO = -0.3105 + 0.9117 x sex1 - 0.6452 x sex2); where sex1 = 1 and sex2 = 0 for gravid females, sex1 = 0 and sex2 = 1 for males and sex1 = 0 and sex2 = 0 for non-gravid females.

Table 13. Mean percent response of adult *C. macellaria* separated by treatment 1*and treatment 2 for males, non-gravid, gravid females and total flies in a dual choice olfactometer for all treatment combinations. Response was monitored at the end of 8 h at 30°C with 42% RH and under 2066.67 lumens/m^2 light intensity.

		Mean % Response ± SE	
Treatment1* versus treatment 2	**Sex (N^1)**	**Treatment 1**	**Treatment 2**
Blank* v Blank	Male (184)	45.6 ± 7.1	54.4 ± 7.1
	Non-gravid Female (194)	43.2 ± 7.3	56.8 ± 7.3
	Gravid Female (3)	25.0 ± 17.7	75.0 ± 17.7
	Total (381)	43.8 ± 7.3	56.2 ± 7.3
Liver* v Blank	Male (128)	57.0 ± 4.1	43.0 ± 4.1
	Non-gravid Female (13)	44.4 ± 21.0	55.6 ± 21.0
	Gravid Female (141)	63.1 ± 6.1	36.9 ± 6.1
	Total (282)	60.1 ± 3.8	39.9 ± 3.8
Liver with *C. macellaria v Blank**	Male (103)	50.3 ± 7.2	49.7 ± 7.2
	Non-gravid Female (20)	83.3 ± 9.6	16.7 ± 9.6
	Gravid Female (155)	60.0 ± 11.3	40.0 ± 11.3
	Total (278)	56.7 ± 9.2	43.3 ± 9.2
Liver with *Ch. rufifacies v Blank**	Male (82)	48.4 ± 9.1	51.6 ± 9.1
	Non-gravid Female (30)	61.7 ± 6.5	38.3 ± 6.5
	Gravid Female (142)	77.0 ± 4.8	23.0 ± 4.8
	Total (254)	65.2 ± 6.6	34.8 ± 6.6
Liver with *C. macellaria v Liver**	Male (55)	59.6 ± 13.3	40.4 ± 13.3
	Non-gravid Female (19)	69.2 ± 10.8	30.8 ± 10.8
	Gravid Female (105)	56.8 ± 6.9	43.2 ± 6.9
	Total (179)	57.4 ± 8.4	42.6 ± 8.4
Liver with *Ch. rufifacies v Liver**	Male (81)	52.0 ± 5.0	48.0 ± 5.0
	Non-gravid Female (16)	32.7 ± 23.6	67.3 ± 23.6
	Gravid Female (123)	66.6 ± 6.6	33.4 ± 6.6
	Total (220)	58.4 ± 5.7	41.6 ± 5.7
Liver with *Ch. rufifacies v Liver with *C. macellaria***	Male (123)	64.5 ± 7.2	35.5 ± 7.2
	Non-gravid Female (62)	39.7 ± 14.1	60.3 ± 14.1
	Gravid Female (283)	62.9 ± 11.4	37.1 ± 11.4
	Total (468)	62.4 ± 7.6	37.6 ± 7.6

*Treatment 1; [1]number of lies to respond.

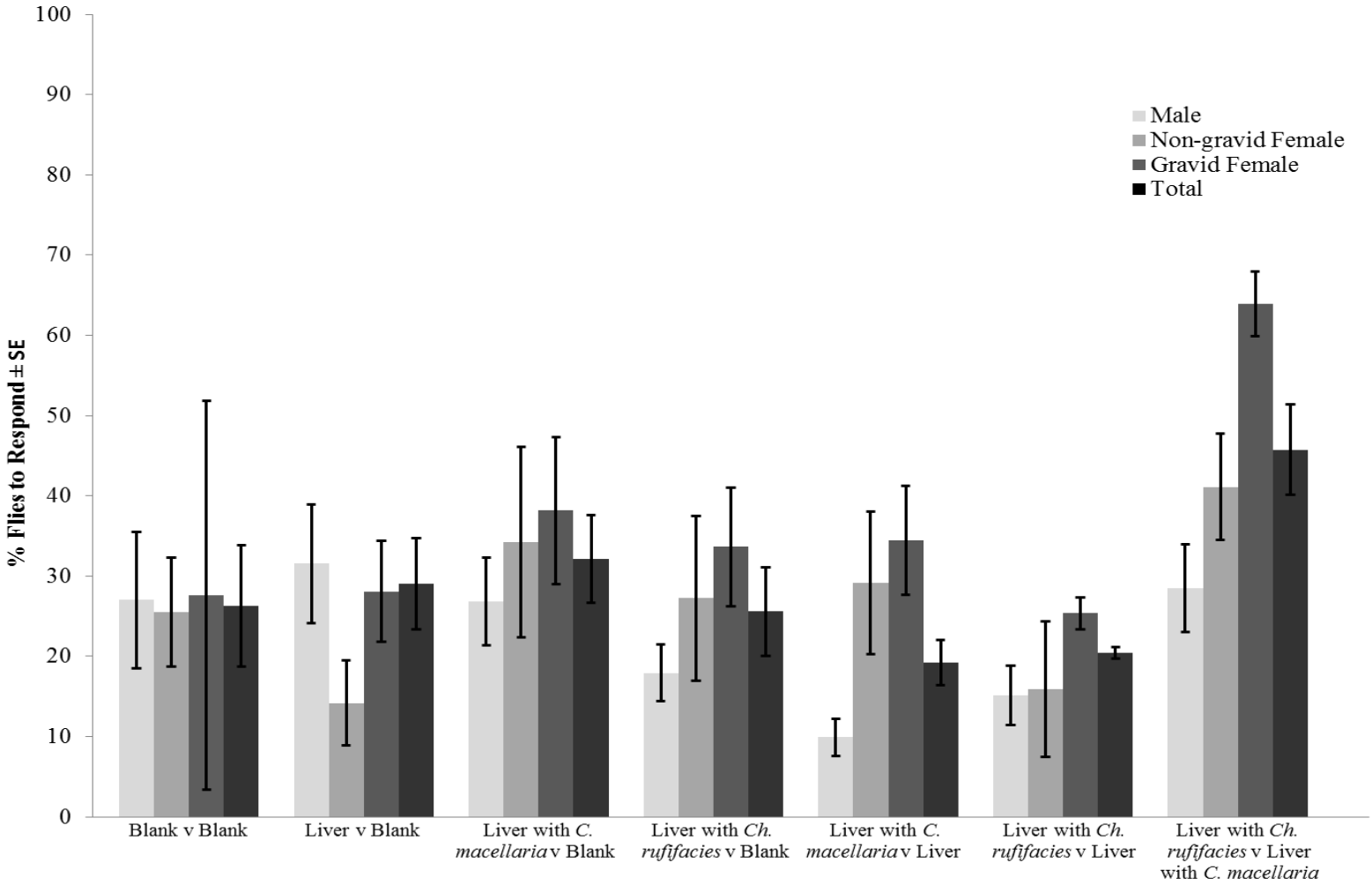

Figure 20. Mean combined (both treatments) percent response ±SE of adult *C. macellaria* for male, non-gravid female, gravid female and total flies in a dual choice olfactometer for all treatment combinations. Response was monitored for 8 h at 30°C with 42% RH and under 2066.67 lumens/m^2 light intensity.

Table 14. Estimated probability, adjusted for replicate (N=4), with fixed variables related to *C. macellaria* response to treatment 1* versus treatment 2 for males, non-gravid and gravid females in all treatment combinations. Response was monitored for 8 h at 30°C with 42% RH and under 2066.67 lumens/m^2 light intensity.

Treatment 1* versus treatment 2	***P*-value**	**Sex**	***P*-value**	**Estimated P (SE)**	**Estimated Odds (P/1-P)**
Blank* v Blank	0.5829	Male	0.7759	0.4749 (0.08199)	0.9044
		Non-gravid Female	0.4022	0.4243 (0.07865)	0.7370
		Gravid Female	0.5469	0.3096 (0.2708)	0.4484
Liver* v Blank	0.6328	Male	0.3926	0.5625 (0.04385)	1.2857
		Non-gravid Female	0.8279	0.5385 (0.1383)	1.1668
		Gravid Female	0.1999	0.6312 (0.04063)	1.7115
Liver with *C. macellaria v Blank**	0.2960	Male	0.9549	0.5063 (0.1044)	1.0255
		Non-gravid Female	0.3526	0.6498 (0.1435)	1.8555
		Gravid Female	0.3332	0.6095 (0.09509)	1.5608
Liver with *Ch. rufifacies v Blank**	**0.0066**	Male	0.9980	0.4998 (0.08946)	0.9992
		Non-gravid Female	0.6243	0.5600 (0.1168)	1.2727
		Gravid Female	**0.0157**	0.7947 (0.05734)	3.8709
Liver with *C. macellaria v Liver**	0.8136	Male	0.7047	0.5412 (0.1034)	1.1796
		Non-gravid Female	0.3992	0.6378 (0.1467)	1.7609
		Gravid Female	0.5033	0.5669 (0.08988)	1.3089
Liver with *Ch. rufifacies v Liver**	**0.0544**	Male	0.9956	0.5004 (0.07628)	1.0016
		Non-gravid Female	0.2954	0.3407 (0.1335)	0.5168
		Gravid Female	**0.0604**	0.6653 (0.06163)	1.9878
Liver with *Ch. rufifacies v Liver with *C. macellaria***	0.3841	Male	0.1424	0.6615 (0.08502)	1.9542
		Non-gravid Female	0.6573	0.5487 (0.1045)	1.2158
		Gravid Female	0.1970	0.6369 (0.08059)	1.7541

** Estimated probability for each sex can be obtained from the following model: Blank v Blank (log odds (LO) = -0.3052 – 0.3968 x sex1 + 0.2048 x sex2); Liver v Blank (LO = 0.1542 + 0.3832 x sex1 + 0.09716 x sex2); Liver with *C. macellaria* v Blank (LO = 0.6181 – 0.1730 x sex1 – 0.5931 x sex2); Liver with *Ch. rufifacies* v Blank (LO = 0.2413 + 1.1120 x sex1 – 0.2423 x sex2); Liver with *C. macellaria* v Liver (LO = 0.5657 – 0.2965 x sex1 – 0.4007 x sex2); Liver with *Ch. rufifacies* v Liver (LO = -0.6602 + 1.3474 x sex1 + 0.6619 x sex2); Liver with *Ch. rufifacies* v Liver with *C. macellaria* (LO = 0.1953 + 0.3667 x sex1 + 0.4746 x sex2); where sex1 = 1 and sex2 = 0 for gravid females, sex1 = 0 and sex2 = 1 for males and sex1 = 0 and sex2 = 0 for non-gravid females.

blank (left) over blank (right) are presented in Table 14. No eggs were laid on the blank treatments (Figure 21).

Experiment 2 (Liver v Blank): Total percent of flies and percent of each sex to choose either treatment is presented in Figure 20 and broken up by treatment in Table 13. Estimated probability and odds values for making a choice are given in Table 12 with the three sexes responding at significantly different levels ($F_2 = 3.62$, $P = 0.0704$). Gravid females ($P = 0.037$), non-gravid females ($P = 0.0034$) and males ($P = 0.0653$) had significantly greater odds of remaining in the cage rather than make a choice. Gravid females responded more than non-gravid females and males while males responded significantly ($P \leq 0.1$) more than non-gravid females. Response by any sex to either treatment was not significant ($P > 0.1$) and sexes did not respond significantly different from one another (Table 14).

Experiment 3 (Liver with* C. macellaria *v Blank): Total percent of flies and percent of each sex to choose either treatment is presented in Figure 20 and broken up by treatment in Table 13. Estimated probability and odds values for making a choice are given in Table 12 with the three sexes responding at significantly different levels ($F_2 = 6.81$, $P = 0.0158$). Males ($P = 0.0312$) had significantly greater odds of remaining in the cage rather than make a choice. Of those males to respond, they responded significantly more than non-gravid females. Response by any sex to either treatment was not significant ($P > 0.1$) and sexes did not respond significantly different from one another (Table 14).

Experiment 4 (Liver with* Ch. rufifacies *v Blank): Total percent of flies and

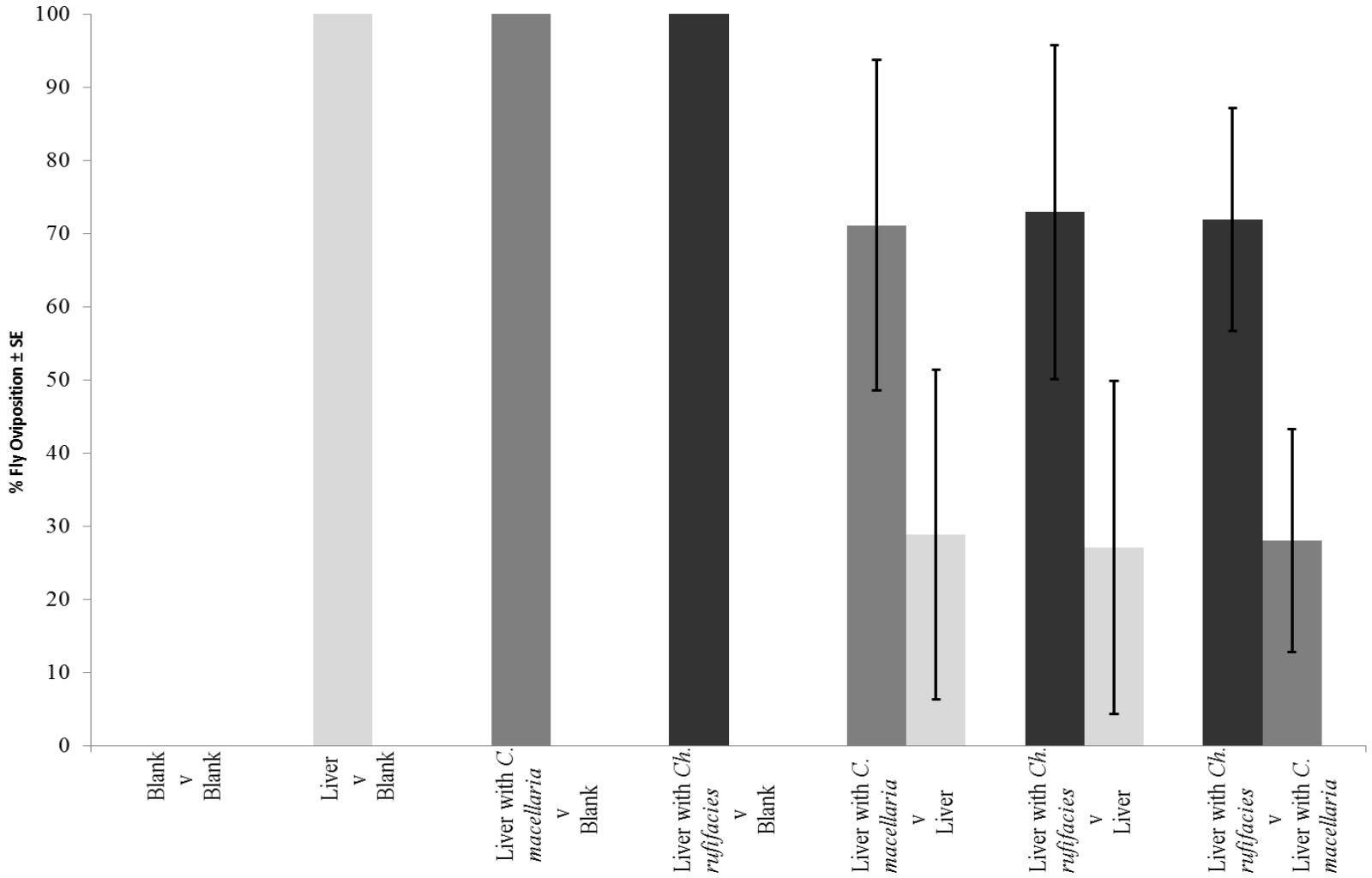

Figure 21. Mean percent oviposition of adult *C. macellaria* for each treatment in a dual choice experiment. Fly oviposition response was monitored for 8 h at 30°C with 42% RH and under 2066.67 lumens/m^2 light intensity. A paired t-test was run with no significant difference (P = > 0.1) in oviposition was observed for any treatment combination.

percent of each sex to choose either treatment is presented in Figure 20 and broken up by treatment in Table 13. Estimated probability and odds values for making a choice are given in Table 12 with the three sexes responding at significantly different levels (F_2 = 12.93, P = 0.0023). Gravid females (P = 0.1001), non-gravid females (P = 0.0484) and males (P = 0.013) had significantly greater odds of remaining in the cage rather than make a choice. Gravid females responded more than non-gravid females and males while males responded significantly more than non-gravid females.

Fly response to liver with *Ch. rufifacies* was significantly different between the three sexes (F_2 = 10.02, P = 0.0066) (Table 14). Gravid females responded with significantly greater odds (P = 0.0157) to liver with *Ch. rufifacies* than without and responded significantly more than males and non-gravid females.

Experiment 5 (Liver with* C. macellaria *v Liver): Total percent of flies and percent of each sex to choose either treatment is presented in Figure 20 and broken up by treatment in Table 13. Estimated probability and odds values for making a choice are given in Table 12 with the three sexes responding at significantly different levels (F_2 = 6.81, $P < 0.0001$). Males (P = 0.0012), gravid females (P = 0.0828) and non-gravid females (P = 0.0063) had greater odds of remaining in the cage rather than make a choice. Males responded significantly more than non-gravid females. Response by any sex to either treatment was not significant ($P > 0.1$) and sexes did not respond significantly different from one another (Table 14).

Experiment 6 (Liver with* Ch. rufifacies *v Liver): Total percent of flies and percent of each sex to choose either treatment is presented in Figure 20 and broken up by

treatment in Table 13. Estimated probability and odds values for making a choice are given in Table 12 with the three sexes not responding at significantly different levels ($P > 0.1$). Fly response towards liver with *Ch. rufifacies* was significantly different between the three sexes ($F_2 = 4.09$, $P = 0.0544$) (Table 14) with gravid females having significantly greater odds or going towards liver with *Ch. rufifacies* ($P = 0.0604$). Gravid females responded significantly more than males and non-gravid females.

Experiment 7 (Liver with* Ch. rufifacies *v Liver with* C. macellaria*): Total percent of flies and percent of each sex to choose either treatment is presented in Figure 20 and broken up by treatment in Table 13. Estimated probability and odds values for making a choice are given in Table 12 with the three sexes responding at significantly different levels ($F_2 = 56.81$, $P < 0.0001$). Males ($P = 0.0134$) and gravid females ($P = 0.057$) had greater odds of remaining in the cage rather than make a choice. Response by any sex to either treatment was not significant ($P > 0.1$) and sexes did not respond significantly different from one another (Table 14).

Discussion

Behavioral assays with olfactometers are often used to decipher the chemical cues utilized by insects to locate resources necessary for their survival (Callahan 1970). Results from this work have led to the development of novel methods for trapping pest species as well as monitoring their populations to determine appropriate timing for initiating control measures (Ashworth and Wall 1994). However, information produced from such research is also essential for providing an ecological context to the behavior the animal exhibits in the field.

Sex was not a significant indicator of response except in the experiments examining liver with *Ch. rufifacies* v blank experiment and the liver with *Ch. rufifacies* v liver experiment. Stoffolano et al. (1990) documented few male *Phormia regina* Meigen (Diptera: Calliphoridae) trapped at blow fly resources (rat carcasses) as compared to blow fly protein sources (cat feces) (Stoffolano et al. 1990). Males responded to liver and liver with larvae but were never the majority sex observed responding to the treatments. Sexes did respond differently to treatments and the greatest response was observed in the liver with *Ch. rufifacies* v liver with *C. macellaria* experiment. The gravid females in this previously mentioned treatment responded at levels (~65%, Figure 10) similar to those seen in Tomberlin et al. (2012) for *Lucilia sericata* (Meigen) (~68-75%) olfactory responses using the same apparatus (Tomberlin et al. 2012). Likewise, gravid females had greater odds of making a choice (responding to either arm containing at least one treatment) versus the baseline data of making a choice towards the blank arms.

When gravid female flies were allowed to make a choice between liver with conspecifics or heterospecifics, contrary to the *a priori* outcome predicted, they responded to liver with heterospecific larvae indicating that the presence of conspecific larvae were far less attractive than the other choice. This response could partially be explained by kin selection, an animals' ability to distinguish between kin, where attraction to conspecifics is dose dependent, becoming repellent at greater levels (Reeve 1989).

This phenomenon has been demonstrated with the house fly, *Musca domestica*

Linnaeus (Diptera: Muscidae) (Lam et al. 2007). Lam et al. (2007) demonstrated the attraction and eventual repellence of adult house flies to *Klebsiella oxytoca*, deposited by house fly females, in the aggregation of conspecific eggs depending on their age (Lam et al. 2007). Fresh eggs (~3 h) had oviposition occur while eggs aged to 24 h or inoculated with *K. oxytoca* levels consistent with 24 h levels, inhibited oviposition. Additionally adult fly attraction to bait placed in the environment containing fresh resource, aged (4 d) resource, nutrient depleted resource (4 d with feeding larvae) and extract resource (resource treated with feeding extracts or larvae) has been previously examined (George et al. 2012). The authors observed more attraction to fresh, extract, aged and nutrient depleted resources respectively. Flies preferred resources with no larvae (fresh, aged or extract) even when those resources contained chemical cues or heterospecific larvae (extract). However when larvae were allowed to feed on the resource and "deplete" the resource, flies were less attracted to that treatment.

Additionally, *C. macellaria* selectively oviposited on resources, and given the choice between conspecific and heterospecific larvae, more eggs were laid on liver with heterospecific larvae. Ovipositional responses to resources with or without predator and prey larvae have been previously studied (Giao and Godoy 2007, Yang and Shiao 2012). The former study (Giao and Godoy 2007) observed the ovipositional responses of the predatory blow fly *Ch. albiceps* and prey blow flies *Ch. megacephala* and *L. eximia,* finding no preference for liver with predator or prey larvae. Eggs were laid with predator and prey larvae, with prey blow flies laying the least amount of eggs with predatory larvae present. The ovipositional preference of predator (*Ch. rufifacies*) and prey (*Ch.*

megacephala) blow flies to a resource with conspecific and heterospecific larvae was observed (Yang and Shiao 2012). Prey blow flies preferred resources with conspecific larvae or even heterospecific larvae of a non-predatory blow fly *Hemipyrellia ligurriens* (Wiedemann). However, the predatory blow fly preferentially laid eggs on resources with both heterospecific and conspecific larvae. I expected to observe *C. macellaria* (prey) avoid oviposition on resources with predatory larvae as the *Ch. megacephala* flies did however, these results were not observed. An explanation for *Ch. megacephala* prey flies to avoid predatory larvae could be that *Ch. rufifacies* and *Ch. megacephala* are native competitors while *C. macellaria* has only interacted with the predatory *Ch. rufifacies* since its introduction to the Americas in 1978 (Jiron 1979) and to the United States in 1983 (Richard and Ahrens 1983).

Gravid female attraction and oviposition to a resource containing a predator seems detrimental to the survival of corresponding offspring. As has been shown previously, arriving first to a resource can restrict additional colonization by subsequent species or if colonization does occur, fitness effects (e.g., size, survival and development time) are observed (Shorrocks and Bingley 1994). However, widening the temporal gap in arrival, fugitive effects may allow for coexistence between species (Hanski 1983). Evidence of priority effects and fugitive effects leading to coexistence between *C. macellaria* and *Ch. rufifacies* has been demonstrated in the laboratory (Brundage 2012).

Cues necessary for *C. macellaria* to detect *Ch. rufifacies* could be stage specific. *Chrysomya rufifacies* is known to be predaceous in the 3rd instar. However, the temporal range within the 3rd instar when this behavior is exhibited is not known. In the case of

my research, *C. macellaria* adults responded more towards the liver with *Ch. rufifacies* larvae. Thus, *Ch. rufifacies* larvae used in the experiment might not have been within the age range when predation is demonstrated. Furthermore, *Ch. rufifacies* larvae could have conditioned the resource rendering it suitable for subsequent larval feeding (Fuller 1934). For instance, house fly larvae arriving late in succession to a resource containing fungi will not allow for larval survival (Lam et al. 2009). However large numbers of flies arriving in sequence can curtail fungi from overtaking a resource and make it suitable for subsequent species to colonize. The presence of larvae already on a resource (conspecific or heterospecific) might convey the message that a resource is suitable for colonization as fungi and bacteria may already be suppressed. *Cochliomyia macellaria* may even distinguish the quality of the resource as nutrient depleted resources were unfavorable to fly attraction (George et al. 2012) (see above).

Data generated from these experiments provide insight to the biological factors regulating *C. macellaria* attraction and oviposition. By understanding the biology of these blow flies one can better explain the mechanisms governing arthropod succession on vertebrate carrion (Tomberlin et al. 2011). Successional patterns of arthropods on vertebrate remains have been used traditionally in estimating TOC (Higley and Haskell 2009). *Cochliomyia macellaria* is observed to readily oviposit on a resource containing *Ch. rufifacies* larvae; however when life stages are closer in age, perhaps variation in *C. macellaria* succession may be observed. When both species are observed on remains methods should be developed to accurately age the larvae in order to determine which species arrived first and is therefore the oldest for use in TOC estimates. Generalized

additive model estimates (chapter 3) may be one way to help age larvae collected from death scenes.

Studies on the coexistence of *Ch. rufifacies* and *C. macellaria* should be continued. Previous research indicates the invasive predator blow fly species have decimated native blow fly populations in other regions of the world (Baumgartner and Greenberg 1984, Wells and Greenberg 1994a). When these flies are forced to interact under laboratory conditions, the NCE of *Ch. rufifacies* on *C. macellaria* adult behavior (i.e., resource attraction and oviposition) are observed. Should *C. macellaria* colonize a resource *Ch. rufifacies* what would the NCE on *C. macellaria* growth and development demonstrate? This hypothesis will be tested next and the consequences of such studies could be beneficial in studying areas such as arthropod and microbial diversity, food web dynamics invasive species biology, and associated nutrient recycling.

CHAPTER VI

NON-CONSUMPTIVE EFFECTS OF PREDATORY *CHRYSOMYA RUFIFACIES* LARVAL CUES ON LARVAL *COCHLIOMYIA MACELLARIA* GROWTH AND DEVELOPMENT

Introduction

Blow fly competition on ephemeral resources is based on density dependent and independent factors and usually takes place in the larval stage as adults are not dependent on the carrion for sustenance (Beaver 1977, Abrams 2009). One factor limiting carrion species abundance is suspected to be carrion availability along with the resulting intra- and interspecific competition (Fuller 1934). Oscillations in adult numbers within a contained population resulted in variation in larval density and resulting survivorship and size (Nicholson 1957). During high population numbers, larval mortality increased and adults produced were small. Inversely, low numbers of adults resulted in reduced larval numbers and mortality while resulting adult size increased.

Chrysomya rufifacies (Macquart) (Diptera: Calliphoridae) is an invasive blow fly species first introduced to Central America in 1978 and is now found throughout North and South America (Baumgartner 1993). *Chrysomya rufifacies* larvae, unlike all other blow fly species found on carrion in North America, predate on larvae of other Diptera (Fuller 1934, Baumgartner 1993). Laboratory studies have been conducted on the consumptive effects of flies belonging to the *Chrysomya* genus on other blow flies (Goodbrod and Goff 1990, Faria et al. 2004). Faria et al. (2004) observed the predacious

habits of *Chrysomya albiceps* (Wiedemann) (Diptera: Calliphoridae) when one larva was presented with one prey larvae (*Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) or *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae)) at different instars (Faria et al. 2004). They demonstrated that *Ch. albiceps* had a higher predation rate (100%) on *Ch. megacephala* in the 2nd instar and 97.5% on *C. macellaria* in the 3rd instar. Goodbrod and Goff (1990) reared larvae of *Ch. rufifacies* and *Ch. megacephala* in pure and mixed cultures at 4, 10 and 20 larvae per gram of beef liver (Goodbrod and Goff 1990). When in mixed culture, they observed an increase in larval mortality for *Ch. megacephala* and the inverse for *Ch. rufifacies* which also resulted in greater pupal weight.

Wells and Greenberg (1992 a, b, c) studied the interaction between *Ch. rufifacies* and *C. macellaria* in the field and the laboratory (Wells and Greenberg 1992c, b, a). They observed predation rates, emergence rates and effects on time to development under these conditions. Wells and Greenberg (1992c) determined that predation by *Ch. rufifacies* in the lab occurred in 17 out of the 20 petri dishes between when both species were 3rd instars and only in 7 out of 20 petri dishes when *Ch. rufifacies* was 3rd instar and *C. macellaria* 2nd instar (Wells and Greenberg 1992a). *Ch. rufifacies* larvae in the 2nd instar were predaceous on *C. macellaria* (0 out of 20 petri dishes) which has been speculated previously (James 1947, Goodbrod and Goff 1990, Baumgartner 1993). Size of the 3rd instar *Ch. rufifacies* did not relate to the act of predation. Small (approx. 10.5 mm) and large (approx. 16.2 mm) third instar *Ch. rufifacies* equally predated upon mid-size (approx. 12.5 mm) *C. macellaria* (Wells and Greenberg 1992a). Wells and

Greenberg (1992c) demonstrated that the presence of *Ch. rufifacies* on rabbit carcasses was correlated with a reduction of resulting *C. macellaria* (Wells and Greenberg 1992c). However, when *Ch. rufifacies* were removed from the rabbit carcasses, approximately 2,000 adult *C. macellaria* emerged from the rabbit carcass which was four times greater than when *Ch. rufifacies* was allowed to remain on the carcasses.

Wells and Greenberg (1992b) conducted a follow up study and examining the influence that density had between mixed and pure culture rearing of *Ch. rufifacies* and *C. macellaria* at 22 and 29°C (Wells and Greenberg 1992b). They recorded survivorship, sex ratio and development rate of both species. Survivorship was not affected by increasing density in pure but was significantly lowered in *C. macellaria* with increasing *Ch. rufifacies* density in mixed cultures. However, when combined with temperature, survivorship shifted being approximately 100% and 25% greater for *C. macellaria* and *Ch. rufifacies* respectively. Sex ratio was unaffected by density or temperature.

Development rate was influenced significantly by temperature with those exposed to 29°C taking less time to go from oviposition to adult for both species. Development of *Ch. rufifacies* was reduced significantly (1d on average) with increasing intraspecific (between *Ch. rufifacies*) density for 2 of the 3 treatments. Across all three interspecific (between *C. macellaria* and *Ch. rufifacies*) densities, *Ch. rufifacies* development was lengthened significantly (3d on average) from the egg to the adult. *C. macellaria* development was not influenced by inter- or intraspecific egg combinations. This study determined a negative impact of *Ch. rufifacies* on *C. macellaria* in the laboratory.

Field and laboratory studies have demonstrated interspecific interactions resulting in decreased fly abundance (Fuller 1934, Wells and Greenberg 1992c). Furthermore, the effects of introduced *Chrysomya* blow flies on native blow flies in North and South America indicate in some instances they has decimated native blow fly populations (Baumgartner and Greenberg 1984, Wells and Greenberg 1994a). For instance, *Ch. albiceps* is thought to be responsible for the eradication of *Lucilia caesar* (Linnaeus) in Madeira (Hanski 1977). In the laboratory *Ch. albiceps* reduced abundance of *Phaenicia* (=*Lucilia*) *sericata* larvae by nearly 100% in all treatments (Ullyett 1950). *Chrysomya putoria* (Wiedemann) has almost completely replaced *C. macellaria* in Goiania and Campinas, Brazil (Guimaraes et al. 1979), perhaps because *Ch. putoria* arrives, and develops, at a carcass faster than *C. macellaria* (Baumgartner and Greenberg 1984). In Guam *Ch. megacephala* larvae were observed to grow faster and crowd out competitors (Baumgartner and Greenberg 1984). In Peru Baumgartner and Greenberg (1984) determined that through trapping over several years, *C. macellaria* was the most abundant blow fly trapped (89%) but 18 months later had become replaced by *Ch. putoria* and *Ch. albiceps* with *C. macellaria* making up only 0.19% (Baumgartner and Greenberg 1984). In Australia *Ch. rufifacies* outcompetes flies from the genera *Phaenicia* (=*Lucilia*) and *Calliphora* Robineau-Desvoidy (Norris 1959). In contrast, and quite interesting, *C. macellaria* populations appear to remain well established in regions where *Ch. rufifacies* has become established in North America.

One explanation for co-occurrence of these two species could be that they respond different to important abiotic conditions related to their ecology as the

evolutionary pressures in which they have developed are different (West-Eberhard 1989). *Ch. rufifacies* larval development is sensitive to tissue type more than temperature (chapter 3). However, *C. macellaria* populations in central Texas are reversed with larvae being more sensitive to temperature rather than tissue type (Boatright and Tomberlin 2010). Temperature may be one of the primary abiotic factors which could explain why *C. macellaria* has not experienced population declines as populations in other regions of the world where temperature is more stable, such as the tropics (South America and the native habitats of *Ch. rufifacies*), (Baumgartner and Greenberg 1984). Although lowering of native blow fly numbers has not been observed in North America, the question still remains if *Ch. rufifacies* will affect the growth and development of *C. macellaria*? Likewise, *C. macellaria* development can also be influenced by biotic factors as well (e.g., predation) but it appears from the laboratory research such a biotic factor may not be as influential as temperature in the establishment of co-existing populations.

Most research examining predator-prey interactions in the blow fly literature focus on consumptive effects (CE) (Goodbrod and Goff 1990, Wells and Greenberg 1992a). However, the non-consumptive effects (NCE) are now known to play a significant role in such interactions as well (Peckarsky et al. 2008). Peckarsky et al. (2008) list a number of studies evaluating cascading effects of predators in habitats including north temperate lakes (piscivore–planktivore-photoplankton-zooplankton), Isle Royal ecosystems (wolves *Canis lupus* Linnaeus-moose *Alces alces* (L.)-balsam fir *Abies balsamea* L.), and kelp forests (killer whales-sea otters-sea urchins-kelp)

(Peckarsky et al. 2008). However, while CE studies are common in literature examining vertebrate decomposition ecology (Wells and Greenberg 1992a, Rosa et al. 2006), NCE studies are lacking.

The strength of the NCE can be dependent on the predator and the effectiveness of the foraging strategy it implements (e.g., sit and wait or active searching) (Preisser et al. 2007). In the case of the carrion system, larval olfaction for detecting has been previously studied (Byrd 1998). *Cochliomyia macellaria* larvae are able to detect and locate porcine tissue, conspecific larvae and conspecific larval trails (Byrd 1998). This behavior is useful for finding an alternate resource in the event the previous resource is depleted or removed from the environment. Larval olfaction may aid in the sensing of conspecific or heterospecific larvae which could be indicative of competition.

Excretion/secretions (ES) produced by larvae could contain cues utilized by conspecifics and predators alike. ES has been well studied in blow flies as these materials have known antibiotic properties (van der Plas et al. 2008). The impact of ES from three insects, *Calliphora vicina* (Robineau-Desvoidy) (Diptera: Calliphoridae), *Dermestes maculatus* (DeGeer) (Coleoptera: Dermestidae) and *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae), were compared to the ES of *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) on five species of bacteria, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Proteus mirabilis* (Barnes et al. 2010). Calliphorids, *C. vicina* and *L. sericata* ES, were able to reduce *E. coli* (63% and 84% respectively) while the ES from the beetle species actually promoted *E. coli* growth by an increase of 10^2 in the bacterial population over the control. During the first 4 h of

incubation of *B. cereus,* both calliphorids ES had 100% reduction in growth while *T. molitor* ES inhibited growth in the first 4 h and *D. maculatus* ES did not. *Dermestes maculatus* and *T. molitor* ES allowed growth of *P. aeruginosa* throughout the 24 h period. *Calliphora vicina* ES reduced *P. aeruginosa* by 85% within the first 4 h while *L. sericata* ES reduced it by 58%. However, *L. sericata* ES maintained its bactericidal effects throughout the next observations (55%) while *C. vicina* ES allowed re-growth. For the final bacterial test (*Pr. mirabilis*) the blow flies ES were unable to inhibit bacterial growth. *Tenebrio molitor* ES was the most effective of the two beetles reducing *Pr. mirabilis* by 66% within the first 4 h and then by 77% during the final observations. *Dermestes maculatus* ES reduced bacterial counts by 60% in the first 4 h and then allowed re-growth.

The objective of this experiment was to examine the NCE of *Ch. rufifacies* ES (potential predatory cues) on *C. macellaria* larval development and growth via predatory ES cues.

Materials and Methods

Fly source: *Chrysomya rufifacies* and *C. macellaria* larvae (> 500 individuals) were collected from decomposing animal remains located in College Station, Texas, USA. Larvae were brought to the Texas A&M University Forensic Laboratory for the Investigation of Entomological Sciences (FLIES Facility) to initiate colonies. Resulting adult flies were held in multiple 30 cm^3 BioQuip® (Rancho Dominguez, CA, USA) lumite screen collapsible cages in the FLIES Facility (~24.4°C, 50% RH and 14:10 L:D). Adult flies were provided deionized water (dH_2O) in 250 ml Erlenmeyer flasks

(VWR, Radnor, PA, USA) with paper towels as wicks and table sugar *ad libitum*. Adult flies were provided approximately 30 g beef liver as a protein source changed every 2 d for the first 8 d of life.

Larval Excretions/Secretions Collection: Methods for ES collection were modified from previous studies (Bexfield et al. 2004, Cazander et al. 2009b). Fifty 3rd instar larvae of *Ch. rufifacies* were placed in an eight dram screw cap vial (BioQuip®, Rancho Dominguez, CA) with 10 ml of dH_2O in a VWR gravity convection oven (Radnor, PA) for 1 h at 35°C in complete darkness. The vial containing the larvae and the dH_2O was placed at a ~45 degree angle to prevent larvae from drowning. Controls were treated the same by placing the same amount of dH_2O in an eight dram vial and placing it in complete darkness at an angle in the oven. Fresh ES (<3 h old) was pipetted from the vial when used in an experiment. All vials used for ES or controls were cleaned with liqui-nox (White Plains, NY, USA) liquid detergent, rinsed with water (three times), rinsed with dH_2O (three times), rinsed with acetone (three times) and baked in an oven (~176°C) for 1 h to remove any odors or residues.

For the second experiment (see below), larval ES were filtered by taking the fresh ES and vacuuming it through a sterile Nalgene bottle top filter with a 0.2 µm filter. ES for all treatments was combined in a 250 ml sterile bottle and then measured out for treatment of the jars.

Experiment 1, 2 and 3 Fresh ES Methods: Mason jars (79 mm x 178 mm; 946 ml, Ball Inc., Daleville, IN, USA), were used in this study containing coarse vermiculite (Sungro Agriculture, Agawam, MA) as a pupation medium. One hundred *C. macellaria*

eggs, determined gravimetrically, were obtained by placing a 50 g piece of beef liver in a *C. macellaria* colony cage. The eggs were weighed out to 150 eggs and placed on 100 g of beef liver. Beef liver was cubed to increase the surface area for larvae to feed on. Ten mason jars: 4 controls (dH_2O), 4 treatments (*Ch. rufifacies* larval ES), one undisturbed treatment and one undisturbed control will be made for each treatment. A total of three treatments (run simultaneously) 1st, 2nd and 3rd instar *C. macellaria* treated with fresh 3rd instar *Ch. rufifacies* larval ES or dH_2O were run. Two replicates of all three treatments were run within three months of each other. The treatments were arbitrarily placed in a Percival environmental chamber (136LLVL Percival® Percival Scientific Inc., Perry, IA, USA) at 27°C with a 14:10 (L:D), 75-80% RH and a hobo (Onset, Bourne, MA) data logger (U12-006) with three probes placed on each level of the chamber to record temperature once every hour. Observations of the eggs were made every hour until hatch and then observations switched to every six hours. Every six hours three larvae were collected, hot water killed (HWK) and length, weight and instar measured. Treatment of the 1st instar was when the eggs hatched and for the 2nd and 3rd instars at known hours for larval instar transitions (determined in preliminary work). Fresh ES or dH_2O was made before each treatment time and never stored longer than 3 h. Fresh ES or control dH_2O was placed directly on the beef liver/feeding *C. macellaria* larvae at the appropriate stage transition time. The controls were sampled as well to compare to the treatments while the undisturbed treated and control jars were not sampled to note the effect of handling the jars on time to pupation and adult emergence. The undisturbed jars were observed every 6 h for the presence of pupae. All flies used in the experiments

were between F_2-F_5 generations.

Experiment 4 Filtered ES Methods: For the filtered ES experiment, the setup was the same as the fresh ES experiment. All 5 treatment and control jars were sampled. In the first replicate 10 jars (dH_2O) and 20 jars (filtered ES) were run. In the second replicate 10 jars (dH_2O), 10 jars (filtered ES) and 10 jars (fresh unfiltered ES) were run to compare results across treatments. Additionally, the results for the 20 jars with filtered ES in the first trial were compared to those from the 10 jars filtered ES in the second trial. They were not determined to be significantly different and were combined to increase power in the overall analysis described below.

***Statistics*:** A repeated measures analysis of covariance (ANCOVA) was run to examine the change in length and weight over time between all treatments in SAS (Carry, NC, USA). Time was cubed to better fit the data and taking the quartic of time did not improve upon the R^2 value, 0.35 for time^3 and 0.36 for time^4. Least squares means, with a Tukey-Kramer adjustment, were used for between treatment comparisons for the filtered ES experiment with significance observed at $\alpha = 0.1$.

Results

Experiment 1 Unfiltered ES Treated* C. macellaria *(1st Instar): Neither weight (Figure 22) or length (Figure 23) gain over time was significantly different ($F_1 = 0.81$, $P = 0.3679$ and $F_1 = 0.11$, $P = 0.7361$ respectively) for larvae treated with dH_2O or unfiltered ES. Minimum time needed to finish 1st, 2nd and 3rd instar stages, pupal stage, total time to pupariation and total time to adult emergence, after treatment with ES or dH_2O were not significantly ($P > 0.1$) different between treatment or control (Table 15).

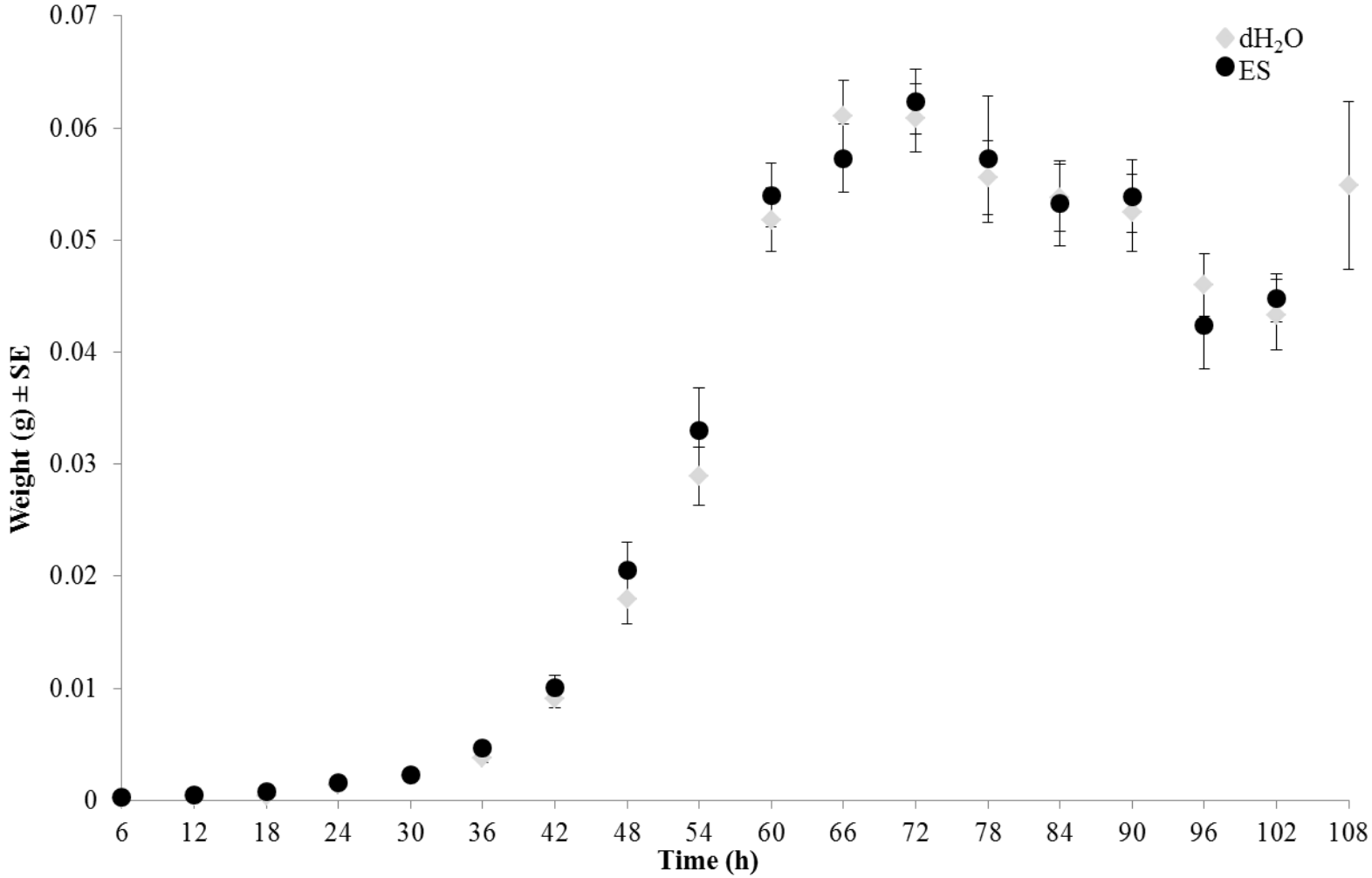

Figure 22. Weight of combined 1st instar treated larvae of *C. macellaria* treated with deionized water (dH_2O) and 3rd instar larval *Ch. rufifacies* excretions/secretions (ES). Flies were reared at 27 °C 75-80% relative humidity and a 14:10 (L:D).

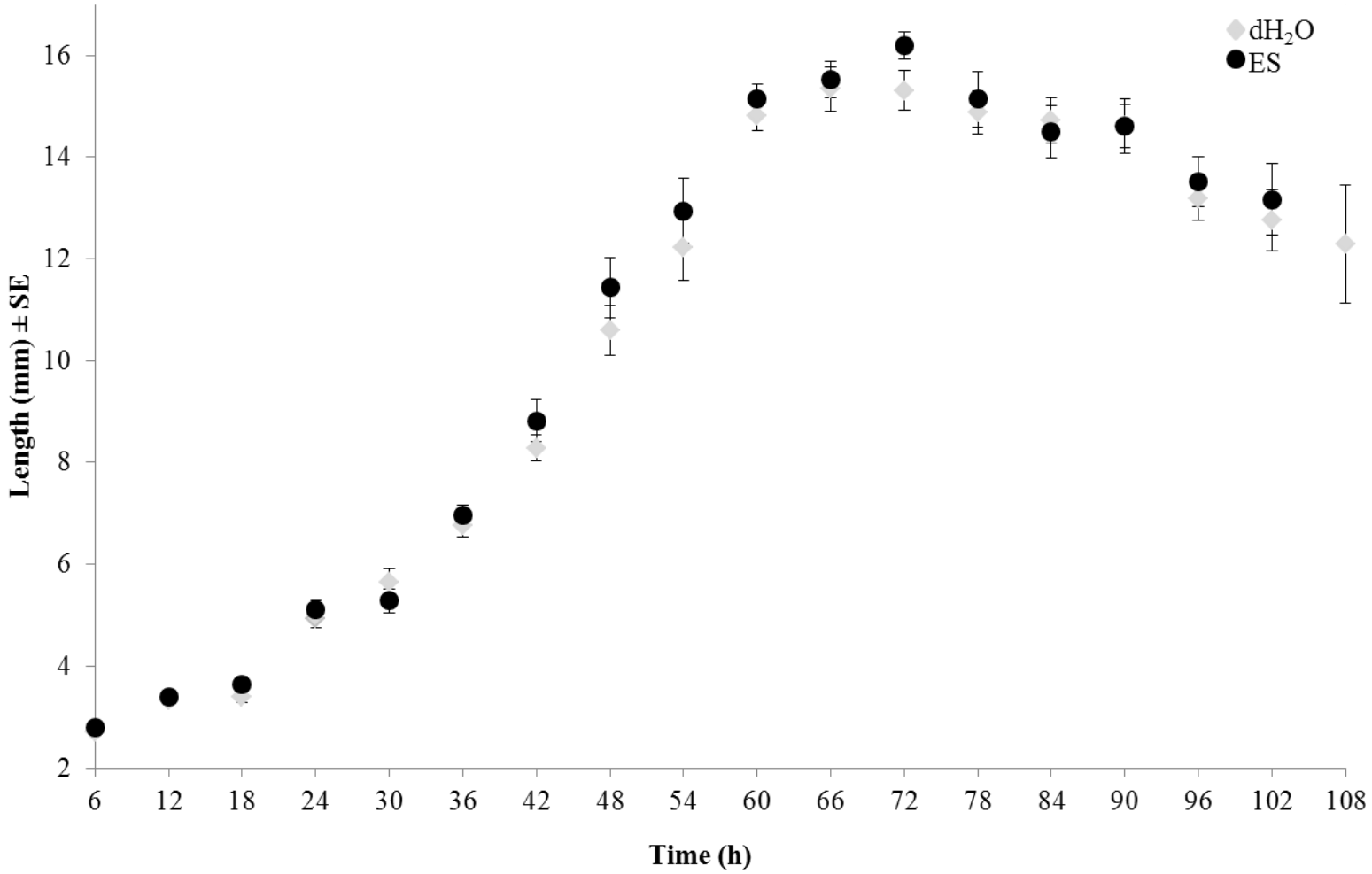

Figure 23. Length of combined 1st instar treated larvae of *C. macellaria* treated with deionized water (dH_2O) and 3rd instar larval *Ch. rufifacies* excretions/secretions (ES). Flies were reared at 27 °C 75-80% relative humidity and a 14:10 (L:D).

Table 15. Minimum stage development time (h) ± SE for 1st, 2nd and 3rd instar treated *C. macellaria* treated with excretion/secretion (ES) or deionized water (dH_2O) at 27 °C, 75-80% RH and 14:10 (L:D).

Instar	Treatment	First (h) ± SE	Second (h) ± SE	Third (h) ± SE	Pupa (h) ± SE	Time to pupariation (h) ± SE	Time to adult (h) ± SE
1st instar	ES	24.0 ± 0.0 A	30.0 ± 1.1 A	65.3 ± 2.4 A	111.0 ± 1.6 A	118.3 ± 2.5 A,B	229.3 ± 1.8 A
	dH_2O	24.0 ± 0.0 A	27.8 ± 1.1 A	65.3 ± 2.1 A	111.8 ± 1.1 A	116.5 ± 2.1 A	228.3 ± 2.1 A
2nd instar	ES		29.3 ± 1.8 A	65.3 ± 2.1 A	113.3 ± 3.5 A	122.8 ± 2.0 B,C	236.0 ± 3.6 B
	dH_2O		28.5 ± 1.9 A	69.0 ± 1.1 A	114.0 ± 2.0 A,B	123.8 ± 1.1 B,C	238.0 ± 3.0 B,D
3rd instar	ES			59.3 ± 0.8 B	116.3 ± 3.0 A,B	126.8 ± 0.7 C	243.3 ± 3.5 C,D
	dH_2O			60.8 ± 2.6 B	119.3 ± 3.3 B	128.3 ± 2.7 C	249.0 ± 3.3 C

Uppercase letters indicate significant ($P < 0.1$) difference in recorded development times for larvae treated with dH_2O or ES at different instars.

Experiment 2 Unfiltered ES Treated* C. macellaria *(2nd Instar): Neither weight (Figure 24) or length (Figure 25) gain over time were significantly different ($F_1 = 0.17$, $P = 0.6813$ and $F_1 = 0.02$, $P = 0.8854$ respectively) between ES and dH_2O water treated larvae. Minimum time needed to finish 1st, 2nd and 3rd instar stages, pupal stage, total time to pupariation and total time to adult emergence, after treatment with ES or dH_2O were not significantly ($P > 0.1$) different between treatment or control (Table 15).

Experiment 3 Unfiltered ES Treated* C. macellaria *(3rd Instar): Rate of weight (Figure 26) and length (Figure 27) gain over time for treatment and controls was significantly different for weight ($F_1 = 1.22$, $P = 0.2691$) but not for length ($F_1 = 7.22$, $P = 0.0075$). Minimum time needed to finish 1st, 2nd and 3rd instar stages, pupal stage, total time to pupariation and total time to adult emergence, after treatment with ES or dH_2O were not significantly ($P > 0.1$) different between treatments or controls (Table 15).

Experiment 4 Filtered ES Treated* C. macellaria *(3rd Instar): Rate of weight (Figure 28) and length (Figure 29) gain over time for treatment and controls were significant for weight ($P = 0.0532$) and length ($P = 0.0538$). Least square means demonstrated significant differences in mean lengths between dH_2O/unfiltered ES ($P = <0.0001$), dH_2O/filtered ES ($P \leq 0.0001$) and unfiltered ES/filtered ES ($P = 0.0044$) comparisons. Also, significant differences were observed between mean weights for dH_2O/unfiltered ES ($P = <0.0001$), dH_2O/filtered ES ($P \leq 0.0001$) and unfiltered ES/filtered ES ($P \leq 0.0001$) comparisons.

Minimum time needed to finish the 3rd instar stage for larvae treated with ES was significantly shorter than that for larvae treated with dH_2O ($P = 0.0159$) or Filtered ES

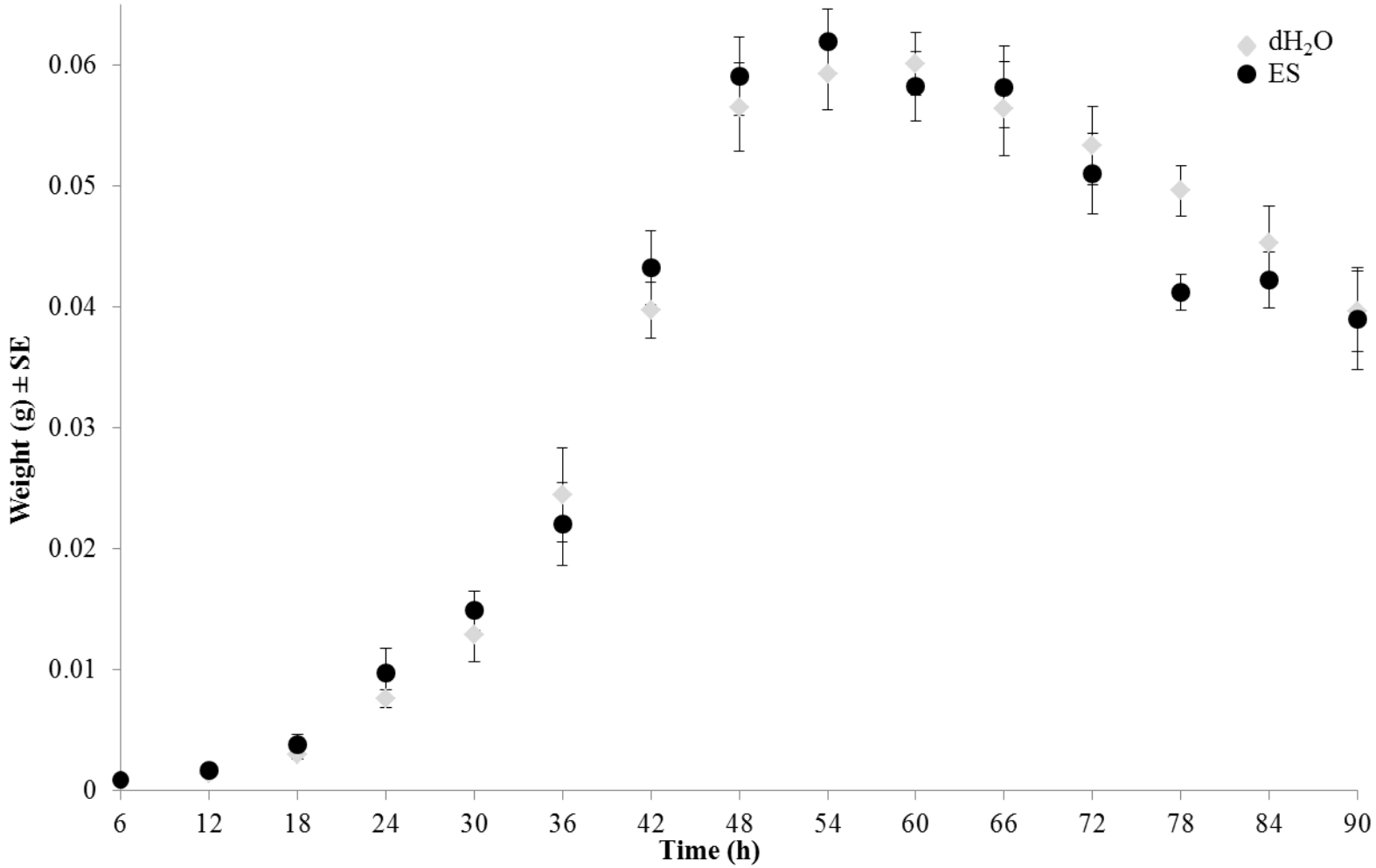

Figure 24. Weight of combined 2nd instar treated larvae of *C. macellaria* treated with deionized water (dH_2O) and 3rd instar larval *Ch. rufifacies* excretions/secretions (ES). Flies were reared at 27 °C 75-80% relative humidity and a 14:10 (L:D).

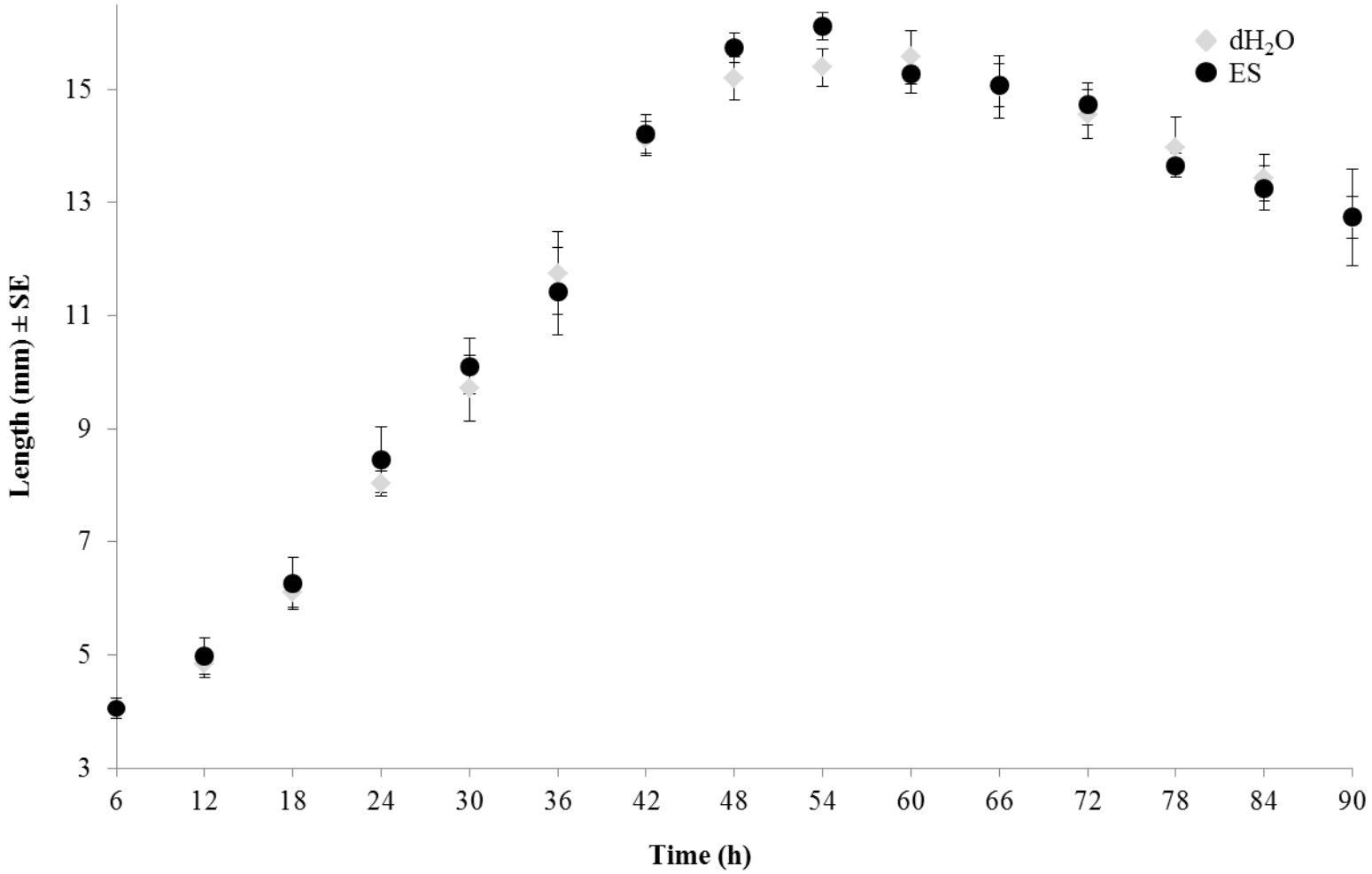

Figure 25. Length of combined 2nd instar treated larvae of *C. macellaria* treated with deionized water (dH_2O) and 3rd instar larval *Ch. rufifacies* excretions/secretions (ES). Flies were reared at 27 °C 75-80% relative humidity and a 14:10 (L:D).

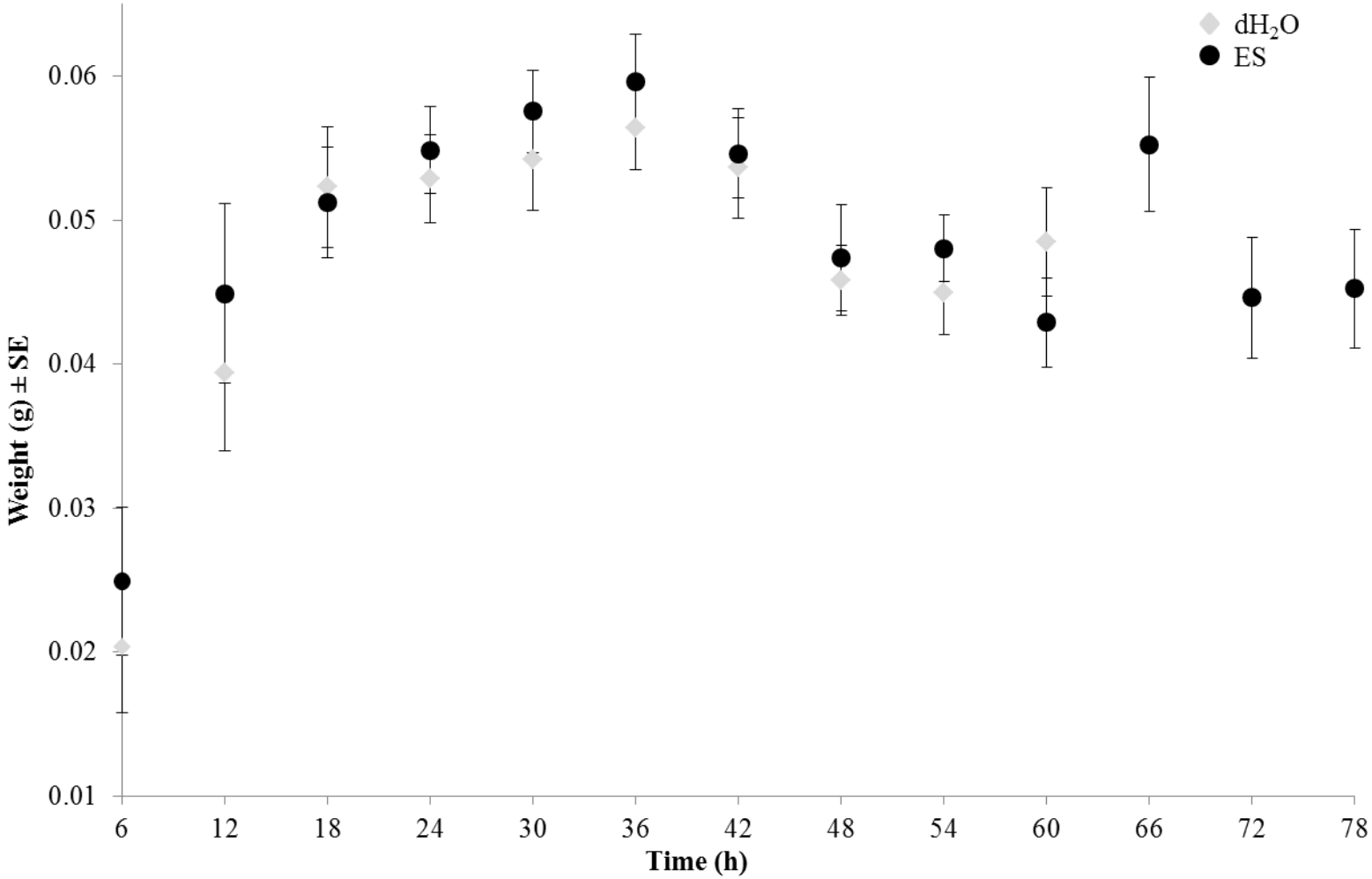

Figure 26. Weight of combined 3rd instar treated larvae of *C. macellaria* treated with deionized water (dH_2O) and 3rd instar larval *Ch. rufifacies* excretions/secretions (ES). Flies were reared at 27 °C 75-80% relative humidity and a 14:10 (L:D).

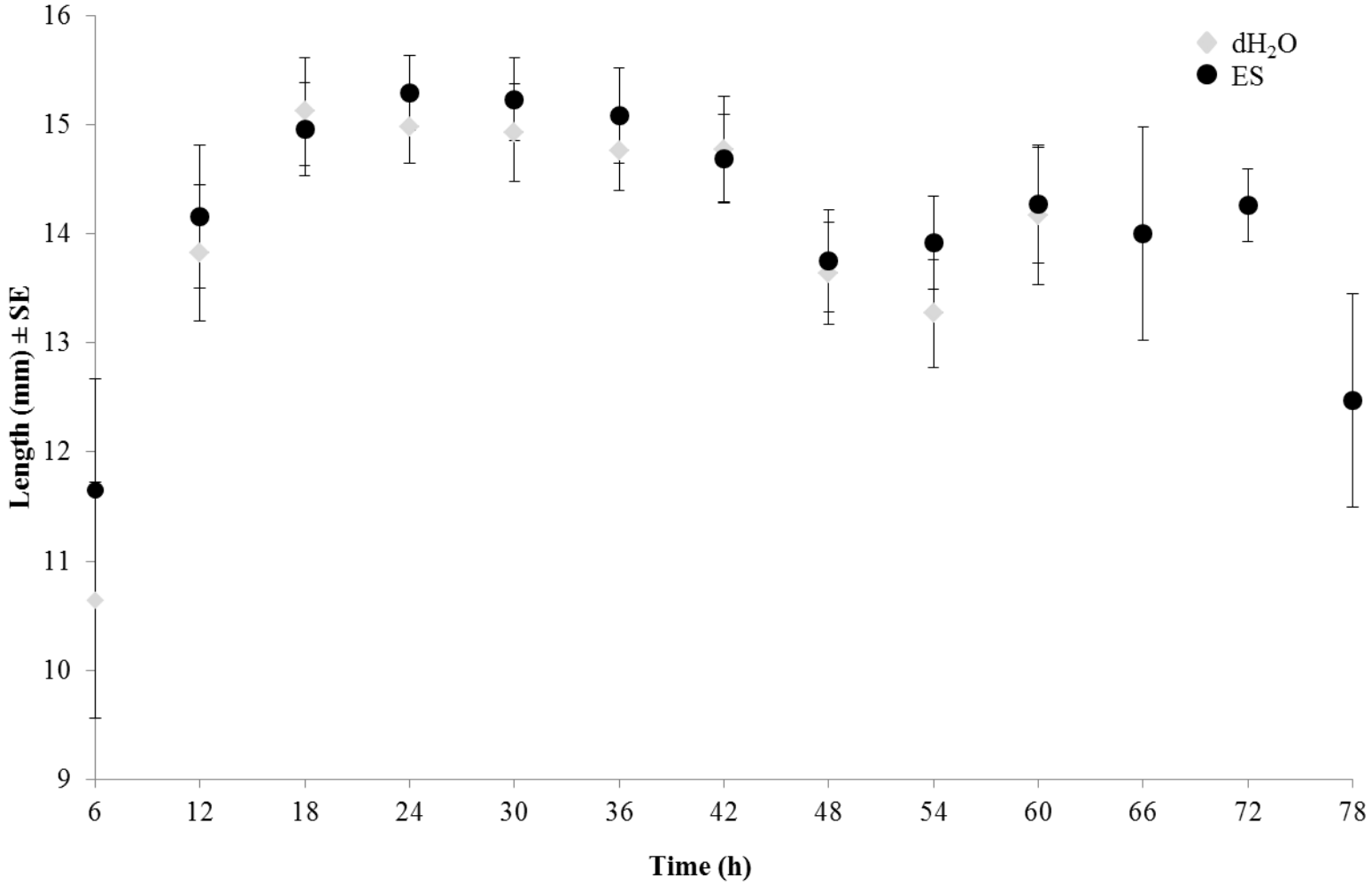

Figure 27. Length of combined 3rd instar treated larvae of *C. macellaria* treated with deionized water (dH_2O) and 3rd instar larval *Ch. rufifacies* excretions/secretions (ES). Flies were reared at 27 °C 75-80% relative humidity and a 14:10 (L:D).

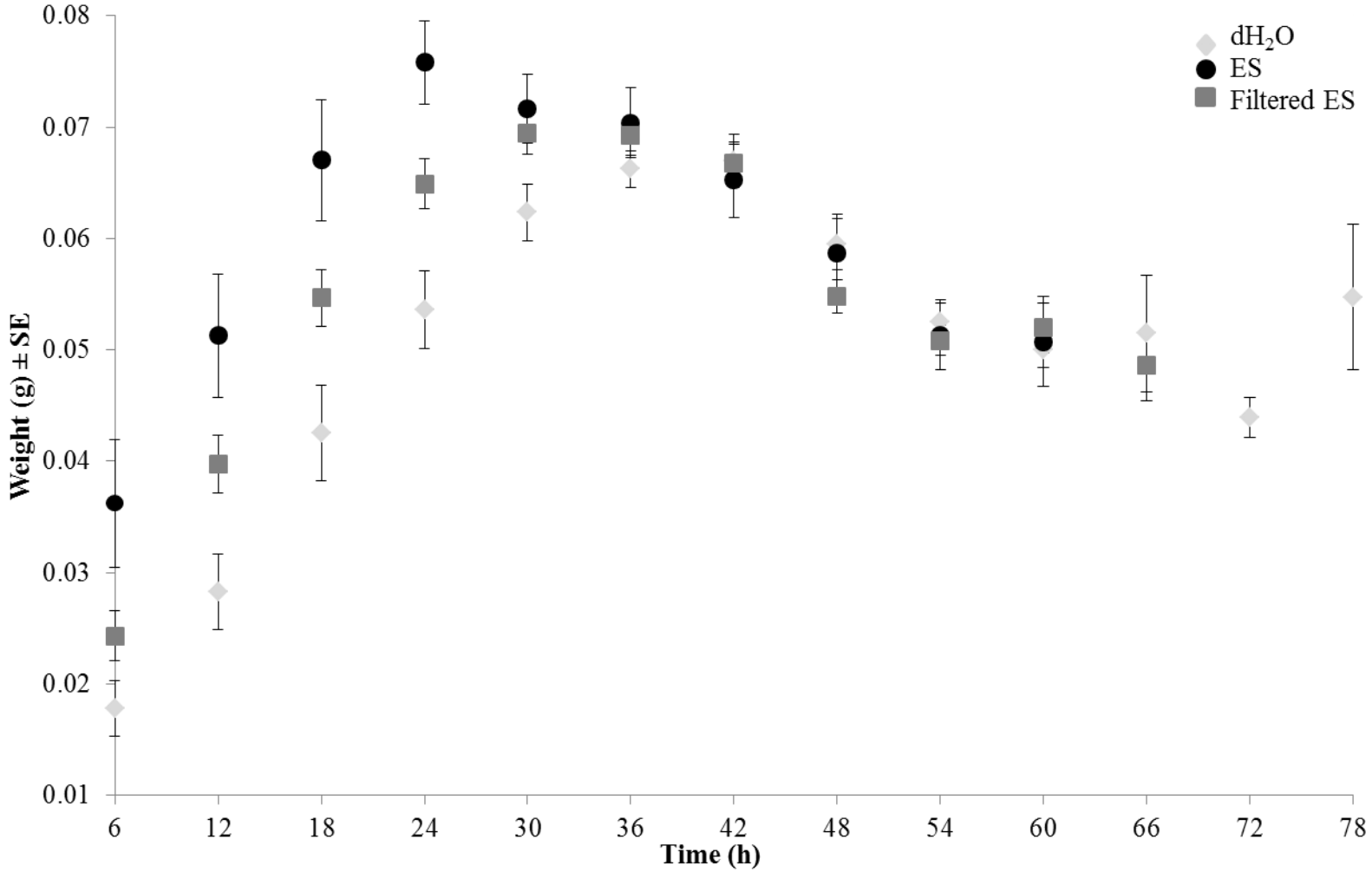

Figure 28. Weight of combined 3rd instar treated larvae of *C. macellaria* treated with deionized water (dH_2O), 3rd instar larval *Ch. rufifacies* unfiltered excretions/secretions (ES) or 3rd instar larval *Ch. rufifacies* 0.2 µm filtered excretions/secretions (Filtered ES). Flies were reared at 27 °C 75-80% relative humidity and a 14:10 (L:D).

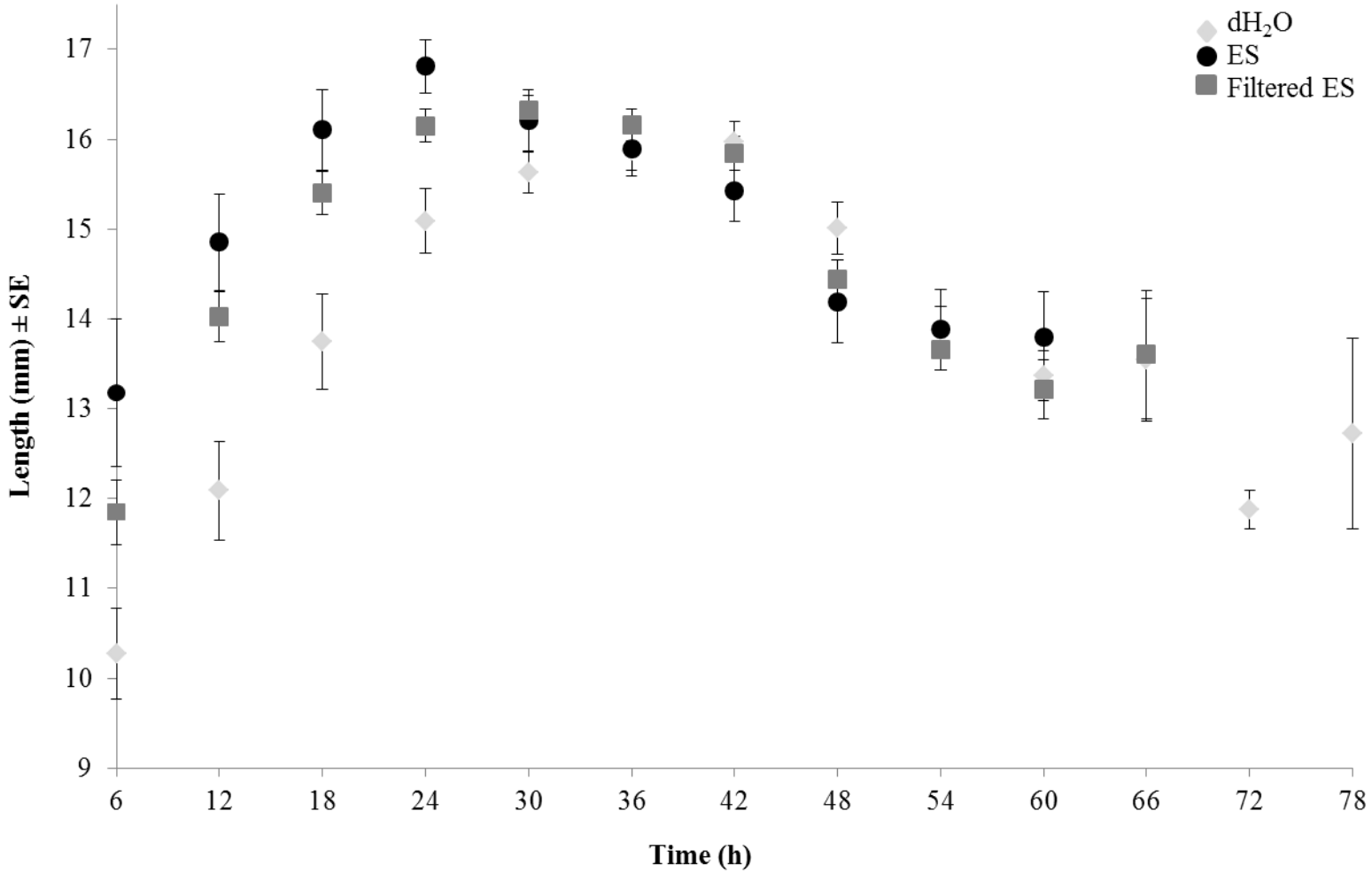

Figure 29. Length of combined 3rd instar treated larvae of *C. macellaria* treated with deionized water (dH_2O), 3rd instar larval *Ch. rufifacies* unfiltered excretions/secretions (ES) or 3rd instar larval *Ch. rufifacies* 0.2 µm filtered excretions/secretions (Filtered ES). Flies were reared at 27 °C 75-80% relative humidity and a 14:10 (L:D).

($P = 0.053$) with no difference in time between dH_2O and Filtered ES treated larvae ($P = 0.4081$) (Table 16). No difference in time spent in the pupal stage was observed for any treatment; however, time from oviposition to pupariation was fastest for the ES treatment compared to dH_2O ($P = 0.0004$) and Filtered ES ($P = 0.069$). Likewise time for filtered ES treated larvae to reach pupariation was faster than time for dH_2O larvae to reach pupariation ($P = 0.0122$). Larvae treated with ES ($P = 0.0244$) or filtered ES ($P = 0.0237$) took less time from oviposition to emerge as adults than for larvae treated with dH_2O.

Discussion

Larval ES have been examined in a number of studies for their bactericidal effects and use in biodebridement (Bexfield et al. 2004, Bexfield et al. 2008, van der Plas et al. 2008, Cazander et al. 2009a). However, it appears no studies have examined ES as a cue used by blow fly larvae to detect the presence of predatory larvae. Significant differences in 3rd instar ES, filtered ES and dH_2O treated larvae in time from oviposition to adult emergence were observed. Wells and Greenberg (1992c) reared *Ch. rufifacies* larvae in mixed culture with larvae of *C. macellaria* and observed no difference in time to adult emergence for *C. macellaria* (Wells and Greenberg 1992b). However, they only made observations daily and determined time to adult emergence. More frequent observations as in this study (every 6 h) yielded greater temporal resolution and detected differences measuring as much as a 15 h reduction in larval development, which could have easily been missed in once daily observations. Additionally, they did not make any morphometric measures of the larvae.

Table 16. Minimum stage development time (h) ± SE for 3rd instar treated *C. macellaria* treated with deionized water (dH_2O), unfiltered excretion/secretion (ES) or filtered ES at 27 °C, 75-80% RH and 14:10 (L:D).

Instar	Treatment	Third (h) ± SE	Pupa (h) ± SE	Time to pupariation (h) ± SE	Time to adult (h) ± SE
3rd instar	Filtered ES	55.8 ± 0.9 A	114.6 ± 0.8 A	124.4 ± 1.0 A	238.5 ± 1.0 A
	ES	52.2 ± 0.9 B	114.6 ± 0.6 A	120.9 ± 1.0 B	237.3 ± 0.7 A
	dH_2O	57.0 ± 1.3 A	113.7 ± 1.1 A	128.3 ± 1.4 C	242.0 ± 1.8 B

Uppercase letters indicate significant ($P < 0.1$) difference in recorded development times for larvae treated with dH_2O or ES at different instars.

These NCE could have ecological ramifications for *C. macellaria*. Size in blow flies impacts flight ability, consequently the ability to find an oviposition resource in females, and decreased fecundity from smaller individuals (Williams and Richardson 1983, Honěk 1993). By limiting the amount of food for the blow flies, *Lucilia cuprina* (Wiedemann), *Calliphora hilli* Patton, *Ca. stygia* (F.) and *Ca. vicina* the authors showed a 12% decrease in pupal size and a decrease in ovariole numbers. ES treated larvae in this study had a higher mean length. Likewise, 3rd instar *C. macellaria* treated with dH_2O, filtered ES and unfiltered ES were observed to have significantly different rates of growth for length and weight with dH_2O larvae being the smallest, followed by filtered ES and lastly unfiltered ES with the largest larvae. Predatory cues, whether free of bacteria (filtered ES) or not (ES) led to larger larvae. Perhaps larger larvae, as affected by their environment, have greater fitness traits (e.g., successful pupation/adult emergence, flight reserves) (Robertson 1957). Growing larger but in about the same amount of time as control larvae may suggest "stressed" larvae become more efficient in utilizing a resource by increasing growth during the log phase on the growth curve (Levot et al. 1979). Consequently, increasing growth may have negative effects on traits previously listed in order to utilize fat body reserves. Future work to determine the effects of predatory cues on larval size in relation to fitness costs (i.e., oviposition capabilities, adult longevity or flight parameters etc.) should be conducted. Thus, understanding the factors regulating blow fly development and potentially dispersal as related to their locating and colonizing remains could lead to greater refinements associated with estimating the pre-colonization interval resulting in more accurate

postmortem interval estimates in forensic investigations.

From a forensic entomology perspective, early arrival and colonization of remains will result in estimates of the minimum postmortem interval (mPMI) to more align with the actual time of death (Tomberlin et al. 2011). Ecologically speaking, such behavior will aid in the priority effects associated with early arrival (Shorrocks and Bingley 1994). Dispersal ability, along with population abundance in the environment may be factors affecting colonization times and the variation associated with them. It is known that dispersing to find a resource is energetically costly and can ultimately affect the number of eggs a fly can lay (Roff 1977). Additionally, estimating the age of blow fly larvae associated with decomposing remains can be based on their length, weight and/or stage of development (Wells and LaMotte 1995, Byrd and Butler 1996, 1997, Byrd and Allen 2001, Grassberger and Reiter 2001). If the primary colonizer is a prey species and its size in increased in the presence of a predator the age of those individuals could be overestimated thus yielding an error in the mPMI.

Competition by blow flies typically occurs between the larval stages. For *C. macellaria* and *Ch. rufifacies,* predation usually occurs when both are 3rd instars (Nicholson 1957, Wells and Greenberg 1992a) which probably explains why no differences in larval growth or development were observed for 1st and 2nd instar *C. macellaria* in this study. If the larvae can associate the larval cues (ES) to a predator, in particularly a 3rd instar predator, the overlap of the predator-prey interaction window will never be experienced and prey larvae would escape CE of predator larvae in time (Atkinson and Shorrocks 1977). Faster development in the presence of a predator would

also minimize the overlap in the necessary predator-prey interaction life stages.

Blow fly growth has been studied by many authors (Kamal 1958, Greenberg 1991, Byrd and Butler 1996, 1997, Anderson 2000). In most instances, these studies were conducted due to their application in areas such as forensic entomology. However, none of these studies examined the growth and development of species in mixed cultures (Wells and Greenberg 1992a) and never before examining NCE. From the more recent studies of *C. macellaria* (Boatright and Tomberlin 2010) and *Ch. rufifacies* (chapter 2) growth it was determined that both species respond differently to both tissue type and temperature. Although it may be so that these species are ecological counterparts as has been suggested in the literature (Wells and Kurahashi 1997) if the conditions do not favor the predatory *Ch. rufifacies*, prey species may be able to avoid competition and thus local extinction. It is evident from previous studies (Wells and Greenberg 1992b, a) as well as this one that *Ch. rufifacies* is capable of outcompeting and lowering abundance of *C. macellaria* but it is unlikely that these conditions are met everywhere that these species coexist.

I found that "stressed" larvae were larger but grew at the same rates as controls and could therefore the adults could have a competitive ability over intra- and interspecific Diptera seeking out remains. However, research presented represents only the beginning in understanding the evolutionary implications of the NCE effects on blow fly development and vertebrate carrion ecology. Multispecies overlaps on carrion could have wide reaching applications for postmortem interval estimations and growth and development in competitive interactions and should be evaluated beyond just the CE.

CHAPTER VII

CONCLUSIONS

Third instar larvae of the blow fly *Chrysomya rufifacies* (Macquart) are predacious on other 3rd instar blow fly (Diptera: Calliphoridae) larvae present on carrion (Baumgartner 1993). This behavior has significant ramifications for the ecology of the predator and prey, as well as the application of this information in areas such as forensic entomology. Such interactions hold the potential to disrupt succession patterns of arthropods and their associated development rates which are traits used to estimate the time of colonization (TOC), or minimum postmortem interval (mPMI), of human remains. However, studies examining the interactions between predator and prey species often focus on the consumption of the prey by the predator (Wells and Greenberg 1992b, a, Wells and Kurahashi 1997, Faria 1999, Peckarsky et al. 2008).

Cochliomyia macellaria (Fabricius) is a primary colonizer of decomposing remains in the southern United States and is used in forensic entomology to establish a mPMI (Byrd and Butler 1996). Factors affecting adult attraction to remains have been studied in the context of developing trapping methods for nuisance flies (Mackerras et al. 1936) but within the context of vertebrate carrion ecology, little work has focused on the attraction, or avoidance of remains in the presence of conspecific and heterospecific (predatory in our case) flies (Wells and Greenberg 1992c, Brundage 2012). I determined that adult *C. macellaria* odds of attraction were greater towards liver with *Ch. rufifacies* larvae rather than liver with conspecifics which was contrary to what was expected. This

attraction was also observed with adult *C. macellaria* attraction to *Ch. rufifacies* eggs (Brundage personal communication). Work with these flies should examine the attraction of the predacious *Ch. rufifacies* to larvae of *C. macellaria*. Will variations in adult sex's response be different as observed in this work?

Non-consumptive effects (NCE) studies have demonstrated predator effects on prey development based on the predators presence in the environment (Abrams 1984). These are the results I expected to see; however, one explanation for these findings could be that eggs and early instar larvae are not known to be prey of *Ch. rufifacies,* and adult *C. macellaria* may not be responding to cues of 3rd instar larvae as they will not present a threat. Furthermore, these data may indicate that intraspecific competition is greater than interspecific competition within the 3rd instar with *C. macellaria* larvae consuming targeted resources that differ from those consumed by *Ch. rufifacies*. By manipulating the overlap in predatory cues with varying age larvae of prey species one can observe more closely the effects of NCE on the prey. I would predict that by exposing 1st instar larvae to 1st instar predatory cues or 2nd instar larvae to 2nd instar predatory cues one would observe similar effects to those seen with the overlapping 3rd instar larvae and 3rd instar predatory cues.

I also determined *Ch. rufifacies* development is more sensitive to resource type than temperature. This is contrary to its hypothesized ecological counterpart *C. macellaria* which exhibits a reversed phenotype comparatively speaking (Boatright and Tomberlin 2010). These differences between species could be due to variations in phenotypic plasticity across the species. *Cochliomyia macellaria* is from the temperate

regions of the world and consequently experiences more variation in temperature. In contrast, *Ch. rufifacies* is from the tropics. Consequently, greater selective pressure exists on *C. macellaria* to exhibit flexibility in development as a response to temperature than its competing counterpart. The differences in species response to tissues and temperatures are useful in pest management as such information can be used to refine trapping systems that are specific for each fly type. Traps are useful for species monitoring or even as methods of actively lowering numbers of flies in the environment to decrease their chances of coming into contact with unwanted conditions (i.e., livestock or crops). Understanding the species you are trapping can lead to better trap catches through modifying of traps by attraction source or time of year for trapping.

Estimating the age of blow fly larvae associated with decomposing remains can be based on their length, weight and/or stage of development (Wells and LaMotte 1995, Byrd and Butler 1996, 1997, Byrd and Allen 2001, Grassberger and Reiter 2001). These estimates rely on quantifying heat accumulation over time (i.e., accumulation degree hour (ADH) degree day (ADD)) which accounts for temperatures experienced by organisms (i.e., insects) above lower developmental thresholds (Arnold 1959, 1960). Such an approach allows for a more accurate estimate of how long the immature fly was potentially associated with the remains in question, and for a unit of comparison between expected development times under laboratory conditions and expected TOC under field conditions at the site of the collection of the flies (Higley and Haskell 2009). Laboratory data can only benefit from the validation of the data under field conditions. Further evaluation of the conservative estimations using time to begin and end an instar should

be evaluated in all validation studies as this method I believe will almost always encompass or be near the TOC of the remains. These data will prove useful for forensic entomologist working on death investigations in Texas, USA to make TOC estimates. Statistical methods have been put forth that can strengthen the analysis and evaluation of laboratory data (Baqué and Amendt 2013).

I determined the size variation of larvae in a given sample is highly variable. And with the utilization of larval distributions for TOC estimates, understanding this variation is critical to reducing error with such estimates. Currently, no set protocols exist on how many larvae should be collected from a scene in order to give the highest resolution estimate of larval age and thus, a mPMI. Whether to use the largest larvae collected from a scene or some other qualitative measurement of size (e.g., mode, mean, minimum or maximum) needs more studying to help minimize errors and best represent variation in continuous growth variables.

Not only does my work have great implications in the field of forensic entomology but also in the fields of ecology (e.g., ephemeral resource, conservation, and invasive species) and veterinary and medical entomology. From studying these two species of blow flies I was able to evaluate both behavioral and ecological traits associated with this predator-prey interaction. The negative population effects by *Ch. rufifacies* on *C. macellaria* have yet to be observed in the continental United States but are far ranging in South America (Baumgartner and Greenberg 1984, Wells and Greenberg 1994a). The novel selection pressures of *C. macellaria* co-evolving with an invasive predator could have consequences that have yet to be seen. Resource partitions

may shift for *C. macellaria* to aid in their survival in the presence of a consumptive and NCE (Denno and Cothran 1975, 1976). Conversely the effects of naïve prey species on *Ch. rufifacies* are still unknown and how they will affect the colonization and resource use patterns of this fly are still to be determined.

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APPENDIX A

CONTROLLED FIELD VALIDATION TEMPERATURE DATA