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Anoxia Tolerance of Forensically Important Calliphorids

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ANOXIA TOLERANCE OF FORENSICALLY IMPORTANT CALLIPHORIDS

By

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ANOXIA TOLERANCE OF FORENSICALLY IMPORTANT CALLIPHORIDS

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Forensically important blow flies, Diptera: Calliphoridae, are among the first organisms to colonize carrion. After eggs hatch, the larvae of most blow fly species feed in an aggregation or "mass." While in this mass larvae may experience periods of no oxygen (anoxia), little oxygen (hypoxia), or normal oxygen (normoxia), but the tolerance of blow fly larvae to severe hypoxic conditions is not known. I tested the anoxia tolerance of four species of calliphorids (*Calliphora vicina, Cochliomyia macellaria, Lucilia sericata, and Phormia regina*), using third stage larvae across five temperatures. Experiments were conducted by exposing larvae to pure nitrogen environments and determining mortality at set time intervals. All species show significant linear relationships between survival time and temperature under anoxic conditions. Of species tested, *C. macellaria* withstood the longest period of anoxia (LT_{50} of 9 h at 20^{o}C). In contrast, *C. vicina* was the least tolerant (LT_{50} of 2.2 h at 40^{o}C). Overall, survival of *P. regina* showed the least response and *C. macellaria* showed the greatest response to temperature. Unlike some other insects, the larvae of the calliphorids tested, which included members of three subfamilies, were not tolerant of anoxic conditions. From these findings, it seems likely that hypoxia could be a significant limitation for maggots submerged in a maggot mass, particular with high maggot mass temperature. Forensically, these data provide a limit on potential larval survival on bodies that have been submerged or otherwise experienced hypoxia before discovery.

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DEDICATION

I am honored and privileged to dedicate this thesis to my mother, Mary Breske. It was through her strength, support, and faith in me that I never gave up on myself. I am also dedicating this to the rest of my family, who stood by no matter what trial I was facing in life.

ACKNOWLEDGEMENTS

First and foremost I must thank Dr. Leon Higley. Without his faith in me and guidance I would not be the scientist I am today. I want to also thank my fellow graduate students: Amanda Fujikawa, Christian Elowsky, and Willa Torren-Senn. I want to give a special thanks to those who put time and dedication into making the lab successful: Carmen Mostek, Nathaniel Niasco, Whitney Drahota, and Liz Schweitzer. Furthermore I want to thank my graduate committee whom without this would not be possible; Dr. Wyatt Hoback, and Dr. James Brandle. Lastly I want to thank those at the University who have helped me through my trials and my successes: Dr. Karl Reinhard, Sue Voss, Danny Unruh, Alex Vogel, and Wendy Leming.

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CHAPTER1. INTRODUCTION AND LITERATURE REVIEW

Introduction

The ecology of animal decomposition is not as well understood as that of plant decomposition. Because plants, alive or dead, contain roughly ten times as much carbon as animals, it is not surprising researchers interested in nutrient cycling and carbon fate have focused on plants. Moreover, plants take much longer to decompose than animals – months to years versus days to weeks.

However, animal decomposition does create nutrient islands that may be important in defining community structure, and decomposing animals provide an essential resource for a complex of decompositional animals and microbes. Beyond these important ecological roles, understanding the ecology of human decomposition has proven essential as a technique for estimating time of death for some criminal investigations (Tibbett and Carter 2008).

In most instances, excluding winter and periods of cold temperatures, blow flies (Calliphoridae) are the first animals to use carrion (Gennard 2012). Typically, female blow flies lay eggs on carrion within the first couple hours after death. These eggs hatch into tiny maggots, which rapidly develop through two stages, and molt into a third stage where most feeding occurs. Blow fly larvae usually form an aggregation, or "mass", in which they feed. By the third stage this maggot mass can be large and substantially warmer (e.g., 10° C or more) than ambient temperatures. The maggot mass gradually skeletonizes the body (Higley and Haskell 2010).

Blow fly larvae have mouthparts consisting only of small hooks, and they secrete extra-oral enzymes to help soften food making it more easily eaten. These oral secretions also may have antibiotic properties, suggesting that maggot compete with bacteria for access to carrion (Greenberg 1991). These aspects of blow fly larval life history are important when considering why maggots form masses. One hypothesis is that the mass improves feeding efficiency, because the accumulated action of oral secretions works synergistically in breaking down tissue. Another

(non-exclusive) hypothesis is that maggot masses increase development rates by increasing temperature (Haskell and Higley 2001). Faster development may be beneficial in giving larva of different species a competitive advantage in resource use.

Often overlooked in these arguments about advantages to maggot masses are disadvantages associated with aggregation. For example, temperatures in the center of a mass can easily reach or exceed lethal limits of the species. Also, the feeding area under the mass usually becomes so liquefied that many feeding larvae are completely submerged (Higley and Haskell 2010).

A key indication that limiting factors do occur in maggot masses is the seemingly continuous movement of maggots in the mass. If conditions in a mass were ideal, presumably maggot movement would be minimal, because movement delays feeding and has a metabolic cost. But why do maggots move? Movement could be associated with thermoregulation, with the need for oxygen, or a combination of the two.

Thus, it is important to characterize blow fly tolerance to anoxia. Examining resistance to anoxia in blow fly larvae will distinguish oxygen limitation from the need to move for thermoregulation. Consequently, my research goal is to experimentally determine if there is a difference in the survival maggots when introduced to anoxia across a range of temperatures temperatures.

Literature Review

Forensic Science

In forensic science, the use of maggots helps when determining the post mortem interval (PMI). PMI is the estimation of time since death with the use of insect activity, weather, temperature, and an understanding of maggot biology (Wells and Lamotte 2010). This insect-based PMI is often the best available estimation of time since death of an individual (Higley and Haskell 2010). The PMI includes initial egg laying and time of maggot development until

maggots are collected from the body (Gennard 2012). Insects, specifically Diptera: Calliphoridae, usually feed upon carrion, which in criminal investigations is typically a human body. Female blow flies lay eggs at open orifices (eyes, ears, mouth, nose, vagina, anus, or wounds). After eggs hatch, maggots begin to feed on the carrion, eventually forming a maggot mass, and once feeding is finished (in the middle of the third larval stage) migrate off the carrion to pupate and eventually emerge as adult flies.

Determining PMI from maggot development is not a simple calculation. To get an accurate PMI a series of steps must be precisely followed. These steps start at the collection site, and end in the lab with the final classification of all species and correct analysis of the data recorded. First, when an investigator gets to the crime scene they must collect live samples and preserved larval samples.. It is not easy to classify flies, especially in the larval stage, therefore it may be necessary for samples to be taken back to the lab, transferred to a new protein source, and reared into adulthood (Byrd and Tomberlin 2010). When adults are not available larvae or pupae are identified to species. The identified insects are then compared to development growth curves using ambient temperature where the body was found and an analysis of data is performed using a degree day system (Haskell and Higley 2001). For an accurate analysis, scene temperatures must be calibrated to available recorded temperatures, potential environmental factors (e.g., rain) or conditions of a body (e.g., clothing, burning) must be considered. Adjustments for maggot mass temperatures may also be necessary, however, because the actual influence of maggot mass on development rates is unclear, incorporation of maggot mass adjustments into a PMI is controversial.

An understanding of basic maggot biology is essential for understanding properties of maggot masses. Flies go through complete metamorphosis. Therefore they start as an egg and develop into larva, pupa, and finally an adult (Byrd and Castner 2010). The development of blowflies is divided among several stages and sub-stages, specifically: 1st, 2nd, 3rd pre-migratory, 3rd migratory, and pupa. Blowflies must feed upon a protein source to be able to lay eggs and

complete their life cycle (Stevens 2003). A gravid female prefers to lay eggs on a moist warm environment. Larva feed with their mouth hooks down and their posterior ends up. The posterior ends contain spiracles, which are openings to help the larvae breathe while feeding (Service 2012). Females will usually lay 100-300 eggs, and larvae tend to feed in masses (Service 2012). As with most insects temperature is an important aspect of development of the blowfly, increasing the temperature will in turn increase the development of the larvae (Higley and Haskell 2010).

Maggot Mass

Maggots feed in what has been termed a "maggot mass" (Higley and Haskell 2001). This phenomenon of larvae forming a feeding mass is not well examined in the literature despite being a relatively unique behavior among Diptera. In the maggot mass, larvae will likely experience periods of hypoxia because they are continuously feeding at the bottom of the mass, which typically is under fluids. The mass can be relatively dense and, therefore, while towards the bottom of the mass maggots will have little access to oxygen. It is also known that maggot masses cause an increase in temperature through metabolic activity (Higley and Haskell 2001). Masses not only increase in temperature, they appear to heat up into a "boil" as a constant movement of maggots is observable.

Maggot masses also presumable affect development rate. But if movement in these large masses affects larval development because they cannot feed regularly, then PMI estimates may be incorrect. Maggot masses increasing in temperature will also increase the development of these maggots (Haskell and Higley 2001). Thus development of blow fly maggots is likely influenced by access to food, temperatures in the mass, and oxygen limitations, which will affect PMI estimates.

Anoxia in Insects

The presence of oxygen in an environment is usually is defined as: normoxia – oxygen levels are not limiting; hypoxia – low oxygen present that are potential life-limiting; and anoxia – zero oxygen level that is usually lethal. How organisms cope with environments containing differing levels of oxygenation, varies among species and life stages. Aquatic insects cope with potential oxygen deficits through various adaptations for extracting oxygen from water and through adaptations for storing oxygen while submerged . Terrestrial insects typically show fewer morphological and physiological adaptations for reduced oxygen environments . As a general observation, a connection between respiration rates and metabolic activity clearly exists. Metabolic activity depends on the insect stage and species (Hoback and Stanley 2001). Some hypoxic, or oxygen-poor, environments are also often nitrogen-rich environments, and there is direct correlation between sensitivity to nitrogen and metabolic rate (Agrell 1952).

In insects anoxia and hypoxia tolerance are the result of three mechanisms (Hoback and Stanley 2001). First, reduced metabolic rates are a common mechanism for coping with transient and long term anoxia. Second, especially for species that routinely experience oxygen limitations, anaerobic metabolism, through various pathways is a common feature of anoxia tolerance, but it can be stage and species dependent. The third mechanism, oxygen storage, is less common but is found in insects that experience prolonged severe hypoxia.

Calliphorid larvae are likely to experience occasional anoxia or hypoxia. One well–studied, non-dipteran group with similar transient exposures to hypoxia, are the Coleoptera: Cicindelidae, the tiger beetles. Tiger beetle larvae live in bare soil areas that are periodically disturbed by flooding. The larval of these beetles have adaptations to survive severe hypoxic conditions. When comparing tolerance between a larva and an adult tiger beetle the results are opposite of that which has been previously discovered with dipterans. Adult tiger beetles have a lower tolerance to anoxic conditions then do the larval stages (Hoback 2009). Larval tolerance includes both reduced metabolic rates and anaerobiasis, and both show temperature sensitivity (Hoback et al. 1998).

In a contrasting example among beetles, larvae of *Tenebrio molitor* that have been reared in hypoxic conditions are found to not make it past the last larval stage. This phenomenon varies greatly depending on the degree of hypoxia. Normoxic to slight hypoxic conditions both yielded full life cycles from egg to adult. Development from egg to larval stage was also longest with hypoxic conditions as opposed to the other two conditions administered. The body mass of larvae hatched from eggs subjected to hypoxia was also significantly smaller than those with the other two conditions (Greenberg and Ar 1996).

Anoxia in Diptera

An organism's ability to regulate respiration can be defined as the relationship among the oxygen concentration and rate at which the oxygen is then consumed (Park and Buck 1960). Various amounts of oxygen concentration have been studied in the past. Many organisms from mammals to insects may be affected by hypoxia or anoxia at some point in their life cycle. Insects have been widely studied for their various encounters with hypoxic conditions, including aquatic and terrestrial insects. Ecological situations that may give rise to hypoxic conditions include, but are not limited to high altitudes, the inside of stomachs belonging to mammals, microhabitats such as; dung, carrion, under ice cover, sealed containers, and in temporarily immersed substrates (Hoback and Stanley 2001).

Among insects, as well as flies, the Chironomidae, the freshwater midges are arguably the masters at living in low oxygen environments. Some chironomid species evolved hemoglobin, which they use for storage of oxygen. Midges with hemoglobin typically occupy an ecological niche that regularly experiences hypoxic to anoxic conditions. For example, larval stages may occur in mud or shallow areas of water, and in such environments, larvae experience periods of hypoxic to anoxic conditions (Armitage et al. 1995).

Oxygen consumption can vary with stage and species. For instance, research on diapausing and non-diapausing pupae of the *Sarcophaaga crassipalpis* flesh fly shows that diapausing pupae were able to withstand a longer exposure to anoxia than non-diapausing pupae. In particular, the

non-diapausing pupae did not emerge as adults. Ultimately anoxia was so detrimental to the further development of pupal stage in non-diapausing pupae that pupae couldn't recover, even when reintroduced to oxygen (Kukal et al 1990).

Most insects are well adapted to recover from time spent in anoxic conditions. This process occurs when the insects switch to and from aerobic to anaerobic pathways (Wegner 1993). The recovery processes in holometablous (complete metamorphous) insects are stage specific (Hoback and Stanley, 2001). The literature also shows that not only is there a wide range in tolerance among species, but also that this range in tolerance can be very different between closely related species (Keister and Buck 1961). Much of the literature focuses on this phenomenon in adult insects, with less emphasis on other stages. However, in their review of insects and hypoxia, Hoback and Stanley (2001) state that "At 25°, larvae of three common carrion-dwelling fly species survived less than 4 hours in anoxic chambers (von Brand 1946)". Studies also show that the pupal stage, which has substantial metabolic activity, suffers the most from anoxic conditions (Hoback and Stanley 2001).

Anoxia and Calliphorids

Calliphorid larvae are often found in a microhabitat in which they have little competition and gain protection from predators while feeding. Such habitats can be hypoxic, so adaptations to low oxygen might be expected among calliphorids . In support of this notion, *Phormia regina* larvae show great respiratory resistance to changes in oxygen concentrations in contrast to the adult stages where little to no respiratory changes were noticed until severe oxygen depletion (Keister and Buck 1961). Additionally, P. *regina*, were found to be oxygen dependent at oxygen concentrations levels of 0-21%, in the larval stage. Metabolically active stages have a lower tolerance to hypoxic conditions, in the case of *P. regina*, these stages are the larval stages specifically well into the third stage of feeding (Hoback and Stanley 2001, Keister and Buck 1961). Temperature is also a main factor in determining development in blow flies, as it is in all insects. A correlation between temperature and anoxia tolerance has been found (Keister and

Buck 1961). However, *P. regina* interestingly enough have a higher tolerance to higher temperatures than to *Calliphora* larvae. Overall, temperature plays a great role in the tolerance for varying oxygen concentrations (Keister and Buck 1961).

PROJECT OBJECTIVES

The objective for this thesis is to quantify survival by third stages of four calliphorid species: *Phormia regina*, *Lucilia sericata*, *Calliphora vicina*, and *Cochliomyia macellaria* at five temperatures 20^{o} C, 25^{o} C, 30^{o} C, 35^{o} C, and 40^{o} C.

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CHAPTER 2. ANOXIA TOLERANCE IN FOUR FORENSICALLY IMPORTANT CALLIPHORID SPECIES

Abstract

Forensically important blow flies, Diptera: Calliphoridae, are among the first organisms to colonize carrion. After eggs hatch, the larvae of most blow fly species feed in an aggregation or "mass." While in this mass larvae may experience periods of little to no oxygen, but the tolerance of blow fly larvae to anoxic conditions is not known. I tested the anoxia tolerance of four species of calliphorids (*Calliphora vicina, Cochliomyia macellaria, Lucilia sericata, and Phormia regina*), by examining third stage larvae across five temperatures. Experiments were conducted by exposing larvae to pure nitrogen environments and determining mortality at set time intervals. All species show significant linear relationships between survival time and temperature under anoxic conditions. Of species tested, *C. macellaria* had the greatest tolerance to anoxia (LT_{50} of 9 h at 20^{o}C). In contrast, *C. vicina* was the least tolerant (LT_{50} of 2.2 h at 40^{o}C). With all species survivorship decreased with increasing temperature. Overall, survival of *P. regina* showed the least response and *C. macellaria* showed the greatest response to temperature. Unlike many other insects, tested in severe hypoxia the larvae of the calliphorids tested (which included members of three subfamilies) were not tolerant of anoxic conditions. From these findings, it seems likely that hypoxia could be a significant limitation for maggots submerged in a maggot mass, particular with high maggot mass temperature. Forensically, these data provide a limit on potential larval survival on bodies that have been submerged or otherwise experience hypoxia before discovery.

Introduction

The ecology of animal decomposition is not as well understood as that of plant decomposition. Plants take much longer to decompose than animals – months to years versus days to weeks. However, animal decomposition creates nutrient islands that serve as ecological niches for the organisms and microbes working to break them down. Beyond these important ecological roles, understanding the ecology of human decomposition has proven essential as a technique for estimating time of death in some criminal investigations (Tibbett and Carter 2008).

In most instances, excluding winter and periods of cold temperatures, blowflies are the first animals to use carrion (Gennard 2012). Typically, female calliphorids, blow flies, lay eggs on carrion within the first couple hours after death. These eggs hatch into tiny maggots, which rapidly develop through two stages, and molt into a third stage where most feeding occurs. Blow fly larvae usually form an aggregation, or "mass", in which they feed. By the third stage this maggot mass can be large and substantially warmer than ambient temperatures (e.g., 10° C or more). The maggot mass gradually skeletonizes the carrion (Higley and Haskell 2001).

Because blow fly larvae have mouthparts consisting only of small hooks, they secrete extra-oral enzymes to help soften food making it more easily eaten. These oral secretions also may have antibiotic properties, suggesting that maggots compete with bacteria for access to carrion (Greenberg 1991). These aspects of blow fly larval life history are important when considering why maggots form masses. One hypothesis is that the mass improves feeding efficiency, because the accumulated action of oral secretions works synergistically in breaking down tissue. Another (non-exclusive) hypothesis is that maggot masses increase development rates by increasing temperature. Faster development may be beneficial in giving larva of different species a competitive advantage in resource use.

Often overlooked in these arguments about advantages to maggot masses are disadvantages associated with aggregation. For example, temperatures in the center of a mass can

easily reach or exceed lethal limits of the species. Also, the feeding area under the mass usually becomes so liquefied that many feeding larvae are completely submerged.

A key indication that limiting factors do occur in maggot masses is the seemingly continuous movement of individual maggots in the mass. If conditions in a mass were ideal, presumably maggot movement would be minimal, because it delays feeding and has a metabolic cost. But why do maggots move? Movement could be associated with thermoregulation, or to obtain oxygen, or a combination of the two.

Thus it is important to characterize blowfly tolerance to anoxia. Oxygenation of a biological environment usually is defined by three terms: normoxia – oxygen levels are not limiting; hypoxia – low oxygen levels that are potential life-limiting; and anoxia – zero oxygen level that is usually lethal. Larvae in the Calliphoridae experience anoxic conditions in a maggot mass, which may limit survival in the mass and affect overall developmental rate. However, there is little information on the biological and physiological factors associated with maggot masses in the literature.

Hoback and Stanley (2001) reviewed anoxia tolerance among insects. Although some flies show considerable adaptation to low oxygen, relatively little is known about anoxia tolerance in the Calliphoridae. *Phormia regina* larvae show great respiratory resistance to changes in oxygen concentrations in contrast to the adult stages where little to no respiratory changes were noticed until severe oxygen depletion (Keister and Buck 1961). Additionally, P. *regina*, were found to be oxygen dependent at oxygen concentrations between 0-21% of the atmosphere, in the larval stage. Metabolically active stages have a lower tolerance to hypoxic conditions. In the case of *P. regina*, the third stage is most sensitive (Hoback and Stanley 2001, Keister and Buck 1961). Temperature is also a main factor in determining development in blow flies, as it is in all insects. As with other insects, a correlation between temperature and survival of anoxia has been found to be directly correlated (Keister and Buck 1961). However, *P. regina* have a higher tolerance to higher temperatures than to *Calliphora* larvae (Keister and Buck 1961).

Forensically the use of maggots helps when determining the post mortem interval (PMI). PMI is the estimation of time since death with the use of insect activity, weather, temperature, and an understanding of maggot biology (Wells and Lamotte 2009). This insect-based PMI is often the best available estimation of time since death of an individual. The PMI includes initial egg laying and time of maggot development until maggots are collected at the crime scene (Gennard 2012). Blow fly maggots usually feed upon carrion, which in criminal investigations is typically a human body. Because they have mouthparts (mouthhooks) that cannot easily tear undamaged tissue, maggots congregate at open orifices (eyes, ears, mouth, nose, vagina, anus, or wounds), and female flies start by laying their eggs in these openings. After eggs hatch, maggots begin to feed on the carrion, eventually forming a maggot mass, and once feeding is finished (in the middle of the third larval stage) usually migrate off the carrion to pupate and eventually emerge as adult flies.

Determining the PMI is not a simple calculation. For an accurate analysis, scene temperatures must be calibrated to available recorded temperatures, potential environmental factors (e.g., rain) or conditions of a body (e.g., clothing, burning) must be considered. It is also known that maggot masses cause an increase in temperature. Maggot masses increase in temperature (Haskell and Higley 2001). Therefore, adjustments for maggot mass temperatures may also be necessary, however, because the actual influence of maggot mass on development rates is unclear, how to incorporate maggot mass adjustments into a PMI remains controversial.

Maggot mass temperatures lead to questions about maggot development rate. Is this movement in these large masses affecting their development because they cannot feed regularly? Maggot masses increasing in temperature will also affect the development of these maggots, as it has been shown that an increase in the temperature is directly related to the increase in development. Furthermore, researchers have not examined oxygen concentrations of these masses and how this lack of oxygen affects the maggots in the mass. It is possible that the competition to feed may be affected by both the temperature and the oxygen levels of the mass.

Consequently, my research goal is to experimentally determine if there is a difference in the survival of maggots when introduced to severe hypoxia at five different temperatures.

Materials and Methods

Flies and Rearing Conditions

Four species of calliphorids were used in experiments: *Calliphora vicina*, *Cochliomyia macellaria*, *Lucilia sericata*, and *Phormia regina*. These species are among the most common and forensically important blow flies in North America (Haskell and Williams 2008), and all four species routinely produce maggot masses on carrion.

All flies used in the experiments were from colonies maintained in the laboratory. These colonies were established and have been maintained to minimize genetic variation within the colony. Our purpose in this effort is to obtain genetic homogeneity among test subjects, so we can get an indication of physiological variation in response without confounding from population variation. Thus, results here are intended as a baseline against which potential variation among populations can be tested. The chief danger in using such inbred lines experimentally is the potential for inadvertent selection. With insects, inadvertent selection in colonies most frequently occurs in oviposition behavior and in reduced fecundity, however, no indications of change in either of these factors were observed in any of our colonies over many generations.

The *C. vicina* colony was established in October 2012 from a single field-collected female from Lincoln, NE. At the time of these experiments the colony had been maintained through a minimum of 20 generations. The *C. macelleria* colony was established in August 2011 from a single female, collected from the field in Lincoln, NE. At the time of experiments the colony had been maintained through a minimum of 75 generations. The *L. sericata* colony was established from insects provided by Dr. Jeff Wells (at West Virginia University) in Oct. 2010, and this colony was established with field-collected insects from near Morgantown, West Virginia. At the time of experiments our colony had been maintained through a minimum of 100

generations. The *P. regina* colony was established in Aug. 2011 from a single female field-collected from Lincoln, NE. At the time of experiments the colony had been maintained through a minimum of 75 generations.

Adult flies were maintained in cages in a rearing room with temperature maintained at 27.5° C (± 3° C), with a 16:8 light: dark cycle. Multiple generations were maintained in a single cage, and ca. 1000 adult flies were introduced every 1-2wk (adult flies in colony live approximately one month adult lifespan). Adults were provided sugar water as a carbohydrate source, and raw beef liver for protein and as an ovipositional substrate. After egg laying, eggs and liver were maintained in 1.7 l plastic boxes in I30-BLL Percival biological incubators (Percival Scientific, Inc., Perry, IA) set at 26° C (which was ± 1.5° C of this set temperature based on internal temperature measurements). Within the plastic box, liver and feeding maggots were placed in a smaller 0.8 l plastic cup, which rested on pine shavings. The pine shavings provided an area for larval migration at the end of the third larval stage and as a substrate for pupation (larvae bury themselves within the pine shavings after migratory movement).

Experimental Design and Conditions

A series of preliminary experiments were conducted to determine the potential range of survival times, best method for producing anoxic conditions, and methods to minimize mortality in controls. From these trials, final protocols were established.

All experiments were conducted with feeding, third-stage larvae (typically collected from colonies 3-5 d after molting). The experimental unit was a vial with one third-stage larva. The experimental design was a factorial arrangement of oxygen environment x temperature, with five replications. Oxygen treatments were with (normoxic, the controls) and without oxygen (anoxic, nitrogen gas only). Temperature treatments were 20, 25, 30, 35, and 40° C. Treatments were evaluated by sampling at set (1 h) intervals, so a complete set of experimental units (2 treatments x 5 replications) were used for each sampling period. In principle, a total of 10 sampling periods

were anticipated, requiring 50 experimental units per species-temperature combination. In practice, 100% mortality usually occurred well before 10 h, and experiments were terminated when 100% mortality in the anoxia treatments occurred.

The treatment (anoxic) vials contained N_2(g). The N_2(g) was placed into the vials by first submerging the vial completely in water and then steaming the N_2(g) into the vial while it was upside down until all the air was displaced. Then the maggot was placed in the vial, while it was still upside down, under the water, and the vial was capped. The control (normoxic) vials were submerged under water as well, lifted out to remove the water, the maggot was placed in, and then the vial was capped. The water that was left in both the control and treated vials (<2 ml) helped minimize any desiccation of the maggots.

Temperature treatments were established in incubators. Our incubators were customized model SMY04-1 DigiTherm® CirKinetics Incubators (TriTech Research, Inc., Los Angles, CA). The DigiTherm® CirKinetics Incubator have microprocessor controlled temperature regulation, internal lighting, recirculating air system (to help maintain humidity), and use a thermoelectric heat pump (rather than coolant and condenser as is typical with larger incubators and growth chambers). Our customizations included addition of a data port, vertical lighting (so all shelves were illuminated), and an additional internal fan. The manufacturer's specifications indicate an operational range of 10-60° C ±0.1 °C. It is worth noting that a range of ±0.1 °C is an order of magnitude more precise than is possible in conventional growth chambers.

Often anoxia measurements are conducted in water baths to ensure constant temperature, and in our initial trials we compared water baths to our incubators. The great advantage with incubators was that we could examine all treatments for a given species-temperature combination simultaneously (in that we needed as many as 50 experimental units for a single species-temperature experiment). Although growth chambers have been shown to display substantial differences between programmed temperatures and actual internal temperatures (Nabity et al. 2007), Fujikawa (of our group) tested the incubators with internal thermocouples in a replicated

study, and determined that internal temperatures on all shelves within incubators never varied more than 0.1° C from the programmed temperature, in agreement with the manufacturer's specifications (Fujikawa personal communication). Given the high level of measured accuracy with programmed temperatures, we were able to use incubators for temperature treatments, which improved our experimental efficiency and helped reduce experimental error

Each vial contained one larva. One larva was used because the metabolic activity is then only concentrated to one maggot, and individual variation in response could be measured. Treatments were in incubators in a completely randomized order. Each hour a subset was removed with the five replications. The maggot was removed from the vial and checked for movement. As in previous studies, because several other insects can recover from anoxia, the maggot was placed back into the vial (which was left open to the air) and capped and rechecked 24 hours later (Burst and Hoback 2009). A score of no movement for the initial removal and the 24 hour removal resulted in a response of dead, and if the maggot had movement for both times or for the 24 hour check, it was recorded as a live response. Sampling was continued until all maggots were recorded as no movement in the initial test. No maggots recorded as dead initially recovered after 24 hours.

Analysis

Although our experimental design follows a factorial treatment arrangement (oxygenation x temperature) which might imply use of analysis of variance, the idea that anoxia response differs with temperature was not central to the experiments. Instead, the key questions were how survivorship differed at different temperatures, and what was the mathematical relationship between anoxia survivorship and temperature.

To address the question of survivorship, we used Kaplan-Meier survivorship analysis through the Life module of the XLSTAT (Addinsoft, Inc., Paris, France) plugin to Microsoft Excel 2010 (Microsoft, Inc. Redmond, WA). As part of the Kaplan-Meier analysis XLSTAT provides comparisons of survivorship distribution functions through three different tests: Log-

rank, Wilcoxon, and Tarone-Ware tests. The appropriateness of these comparisons depends upon when most mortality occurs, and as we had no prior indication of the nature of our survivorship curves, we decided to use all three tests in evaluating differences in distribution functions. Because the control would necessarily be different from treatments (i.e., the control survivorship "curve" should be a flat line at 1.0), control data were excluded from comparisons of survivorship distribution functions.

For regression analyses, we chose the LT50 and LT75 as the most appropriate response variables (in the literature the LT50 is most commonly used), and we conducted regression analyses with Prism 6.0 software (Graphpad Software, San Diego, CA). In these analyses we used runs testing to identify potential departures from linearity.

Results

Proportional survival in anoxia for larvae of all four calliphorid species were determined at five temperatures, 20-40° C (Fig. 1-4). Control treatments showed essentially no mortality over the period of the experiments (Fig. 1f, 2f, 3f, and 4f). Consequently, no corrections were necessary in analyzing anoxia treatments.

As expected, survival was substantially greater at lower temperatures than higher temperatures. The range of survival times across species and temperature were a minimum of 2.2 h at 40° C for *C. vicina* (Fig. 1e) and a maximum of 9.0 h at 20° C for *C. macellaria* (Fig. 2a). Most survivorship distributions differed with temperature (Fig. 1-3f), however, with *P. regina* survivorship distributions were more similar than those with other species, and the end point (0% survivorship) occurred at ca. 6 h, irrespective of temperature (Fig. 4f).

Tests of differences among survival distributions within species are indicated in Table 1. Because identifying differences between survival distributions depends upon assumptions regarding when deaths occur, different hypothesis testing procedures are used for different assumptions. To avoid potential bias, we used a range of non-parametric tests to examine potential differences, specifically log-rank, Wilcoxon, and Tarone-Ware test. As Table 1 indicates,

differences in survival distributions (excluding the control) were observed with all three tests for *C. vicina*, *C. macellaria*, and *L. sericata* ($P<0.0001$). In contrast, *P. regina* showed no differences in survival distribution functions, indicating that the pattern of survival did not differ with temperature.

Table 2 shows LT25, LT50, and LT75 values for each species, where LT refers to the lethal time to a given % mortality). A significant linear relationship between LT50 and temperature was observed in all four species (Fig. 5), with R^2 varying between 0.98 and 0.83 among species. Very similar linear relationships were observed for LT75 (Fig. 6). For both LT50 and LT75 runs testing was used to determine if significant non-linearity occurred; these tests were not significant for any species. Table 3 reports mean survival times, however, given the substantial variation in these times across temperatures, the means are much less informative than LT50 and LT75 data.

Discussion

Experiments demonstrated that the species tested showed relatively limited abilities to tolerate anoxia. At temperatures associated with maggot masses (typically in excess of 30° C), none of the species tolerated anoxia longer than 6.5 h. Moreover, at higher temperatures, survival times were much more limited (ca. 2-3 h).

Although some differences may exist in anoxia tolerance among species (e.g., Fig. 5 and 6, slopes of LT50 and LT75 versus temperature of *C. macellaria* versus slopes for other species), species generally had similar relationships. The species used represent various subfamilies of the Calliphorid; specifically, Calliphorinae (*C. vicina*), Chrysomyinae (*C. macellaria* and *P. regina*), and Luciliinae (*L. sericata*). Because the variation within the Chrysomyinae (slopes of *C. macellaria* versus *P. regina*) is greater than that observed between subfamilies, it seem likely that the responses observed here may be broadly characteristic of the Calliphoridae.

Certainly, the relatively low tolerance to anoxia seen here could contribute to the need for larval movement in maggot masses. However, our tests represent extreme conditions. In an actual

mass, the larvae likely experience hypoxia rather than anoxia, and would, therefore, have greater tolerance (Hoback and Stanley 2001). The anoxia tolerances observed here set one limit to the ability of larvae to remain submerged while in a mass. Additionally, maggot masses can reach temperatures exceeding 45° C, and at these temperatures maggots clearly have limited ability to withstand prolonged anoxia. Maggot mass temperatures at or above 45° C do represent near lethal limits for many species, however, it is noteworthy that in our experiments, we saw virtually no mortality in control treatments even at temperatures of 40° C. Like anoxia tolerance, temperature tolerance is a function of time of exposure, and the environmental cues for maggot movement could represent a combination of hypoxia and temperature.

Figures 5 and 6 show strong linear relationships between survival times and temperature. These data, as well as Figs. 1-4) also illustrate variation in responses. Because we deliberately tested flies with very uniform genetic backgrounds, the variation we observe is not attributable to underlying genetic differences. Instead, this variation represents the intrinsic physiological variation associated with anoxia tolerance. Naturally, we would expect greater variation to be observed were we to conduct the same tests with wild flies (i.e., flies with greater genetic variability). However, we would not expect the underlying linear relationship to be appreciably different (given that linear relationships were observed in all species, across subfamilies of Calliphoridae).

One possible forensic application of these findings pertains to bodies with maggots found in conditions where anoxia or hypoxia is expected. For example, if a submerged body is found with live maggots, based on survival time-temperature relationships determined here, we could calculate a limit on the time of submergence (given the temperature of the water in which the body was found). As a rule of thumb, we would not expect to find live maggots on bodies that had been submerged longer than 10 hours, even at temperatures below 20° C (based on extrapolations of the linear models in Fig. 5 and 6).

Regarding the central question of the role of anoxia tolerance in behavior of larvae in maggot masses, a number of conclusions emerge from this study. First, anoxia tolerance among the blow fly species tested is not so great that a maggot could remain in anoxic or hypoxic conditions for extended periods (more than a few hours). Therefore, access to oxygen is necessary while feeding, and some movement by maggots in a mass is likely associated with oxygen access (assuming hypoxic conditions exist in maggot masses, which needs to be established).

Second, in comparing the relative important and potential interaction of oxygen access and thermoregulation as factors in maggot behavior while in a maggot mass, these results show that the influence of oxygen deficiency occurs over a time frame of only a few hours. If thermoregulatory responses occur at a similar time frame, then it seems likely these factors interact to influence larval movement. In contrast, if thermoregulatory responses occur at a shorter time frame (minutes rather than hours), then temperature would be the key influence on larval movement in a maggot mass. This later explanation seems most likely, because behavioral responses of insects to temperature typically occur rapidly (minutes), especially at higher temperatures.

Third, the variation in survival times we observed within species and experiments were unexpected given the lack of genetic variation in our experimental fly populations. Because oxygen use is directly tied to metabolism, the simplest explanation for our observations is that there are metabolic differences among individuals of the same species and with similar genetics. Presumably these differences arise from individual differences in feeding (specifically, variation in how much food has been consumed). Although blow flies are among the first insects to arrive at carrion and seem optimized for rapid development (because they are feeding on what is a transient resource), our results suggest feeding behavior is not optimized – all maggots may not feed as fast as they could. If this conclusion regarding variability in feeding rates proves accurate, then interesting possibilities emerge: is variation in larval feeding rates a reflection of intraspecific competition, is variation a consequence of differences in thermogulatory movement

(perhaps maggots close to the center of a mass feed less and move more because they experience higher temperatures), or is variation in feeding somehow evolutionarily advantageous, perhaps as a form of spreading the risk to avoid developmental synchrony that might benefit parasites or predators?

References for Chapter Two

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List of Tables

Table 1. Tests of equality of survival distribution functions by Log-rank, Wilcoxon, and Tarone-Ware tests. Survival distribution functions from Kaplan-Meier survivorship analysis of anoxia survivorship by time at different temperatures, for 3rd-stage larvae of each of four species: *Calliphora vicina*, *Cochliomyia macelleria*, *Lucilia sericata*, and *Phormia regina*.

Statistic	Observed value	Critical value	p-value
Calliphora vicina			
Log-rank	48.656	9.488	< 0.0001
Wilcoxon	38.487	9.488	< 0.0001
Tarone-Ware	43.249	9.488	< 0.0001
Cochliomyia macellaria			
Log-rank	156.311	9.488	< 0.0001
Wilcoxon	131.133	9.488	< 0.0001
Tarone-Ware	142.963	9.488	< 0.0001
Lucillia sericata			
Log-rank	74.768	9.488	< 0.0001
Wilcoxon	48.944	9.488	< 0.0001
Tarone-Ware	60.742	9.488	< 0.0001
Phormia regina			
Log-rank	1.509	9.488	0.825
Wilcoxon	6.412	9.488	0.170
Tarone-Ware	3.529	9.488	0.473

Table 2. Estimates of LT50 and LT75 (lethal time to 50% or 75% survivorship) and 95% confidence limits, at 5 temperatures for 3rd-stage larvae under anoxia for each of 4 species: *Calliphora vicina*, *Cochliomyia macelleria*, *Lucilia sericata*, and *Phormia regina*.

Temp	Quantile	Estimate	Lower bound (95%)	Upper bound (95%)
	Calliphora vicina			
20	75%	4.100		4.100
	50%	4.100	3.267	4.100
	25%	3.267	2.217	4.100
25	75%	3.783		3.783
	50%	3.783	2.983	3.783
	25%	2.983	1.917	3.783
30	75%	3.033	2.033	3.033
	50%	3.033	2.033	3.033
	25%	2.033	2.033	3.033
35	75%	2.533	1.467	2.533
	50%	2.533	1.467	2.533
	25%	1.467	1.467	2.533
40	75%	2.167	1.100	2.167
	50%	2.167	1.100	2.167
	25%	1.100	1.100	2.167
	Cochliomyia macellaria			
20	75%	9.000	7.850	9.000
	50%	7.850	6.667	9.000
	25%	6.667	5.800	7.850
25	75%	5.250		5.250
	50%	5.250	4.300	5.250
	25%	4.300	4.300	5.250
30	75%	5.917	5.050	5.917
	50%	5.050	3.217	5.050
	25%	3.217	3.100	5.050
35	75%	3.917	3.017	3.917
	50%	3.017	3.017	3.917
	25%	3.017	1.933	3.017
40	75%	2.317	1.333	2.317
	50%	2.317	1.333	2.317
	25%	1.333	1.333	2.317

Table 2. Estimates of LT50 and LT75 (lethal time to 50% or 75% survivorship) and 95% confidence limits, at 5 temperatures for 3rd-stage larvae under anoxia for each of 4 species: *Calliphora vicina*, *Cochliomyia macellaria*, *Lucilia sericata*, and *Phormia regina*, continued.

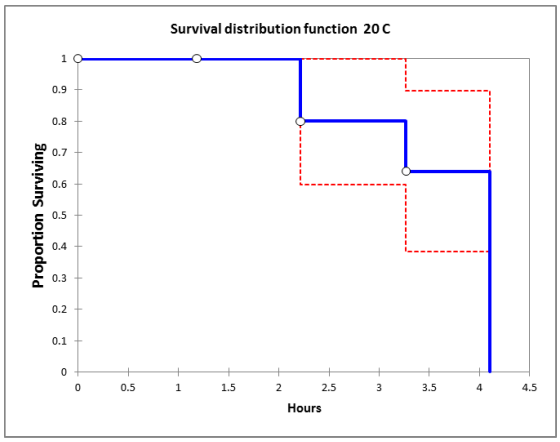
Temp	Quantile	Estimate	Lower bound (95%)	Upper bound (95%)
	Lucilia sericata			
20	75%	7.500	6.633	7.500
	50%	6.633	6.633	7.500
	25%	6.633	6.633	7.500
25	75%	6.300	5.200	6.300
	50%	5.200	3.967	6.300
	25%	3.967	3.067	5.200
30	75%	5.133	4.117	6.050
	50%	4.117	3.150	5.133
	25%	3.150	2.000	4.117
35	75%	5.250	4.333	6.317
	50%	4.333	3.300	5.250
	25%	2.483	2.483	3.300
40	75%	3.050	2.183	3.050
	50%	2.183	2.183	3.050
	25%	2.183	2.183	3.050
	Phormia regina			
20	75%	6.167		6.167
	50%	6.167	5.050	6.167
	25%	5.050	4.100	6.167
25	75%	6.167	5.000	6.167
	50%	5.000	5.000	6.167
	25%	5.000	4.017	5.000
30	75%	6.050	5.133	6.050
	50%	5.133	5.133	6.050
	25%	5.133	4.117	5.133
35	75%	5.250	4.333	6.317
	50%	4.333	3.300	5.250
	25%	3.300	2.483	4.333
40	75%	5.250	4.333	6.317
	50%	4.333	3.300	5.250
	25%	3.300	2.483	4.333

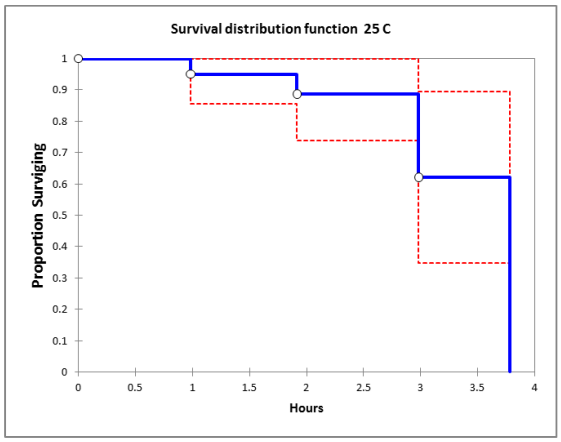
Table 3. Estimates of mean survival times, standard deviation, and 95% confidence limits, at 5 temperatures for 3rd-stage larvae under anoxia for each of 4 species: *Calliphora vicina*, *Cochliomyia macelleria*, *Lucilia sericata*, and *Phormia regina*.

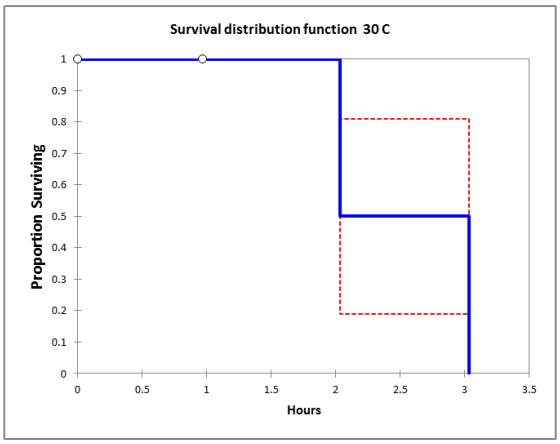
Temp	Mean survival time (h)	Standard deviation	Lower bound (95%)	Upper bound (95%)
Calliphora vicina				
20	3.590	0.207	3.184	3.996
25	3.312	0.196	2.927	3.697
30	2.533	0.167	2.207	2.860
35	2.000	0.178	1.652	2.348
40	1.633	0.178	1.285	1.982
Cochliiomyia macellaria				
20	7.413	0.318	6.790	8.036
25	4.432	0.285	3.873	4.990
30	0.967	0.000	0.967	0.967
35	3.160	0.203	2.762	3.558
40	1.825	0.164	1.504	2.146
Lucilia sericata				
20	6.882	0.230	6.431	7.333
25	4.884	0.309	4.279	5.489
30	3.978	0.306	3.378	4.578
35	4.011	0.309	3.407	4.616
40	2.509	0.173	2.170	2.848
Phormia regina				
20	3.973	0.093	3.792	4.155
25	5.270	0.228	4.823	5.717
30	5.283	0.212	4.868	5.698
35	4.407	0.287	3.845	4.970
40	4.407	0.287	3.845	4.970

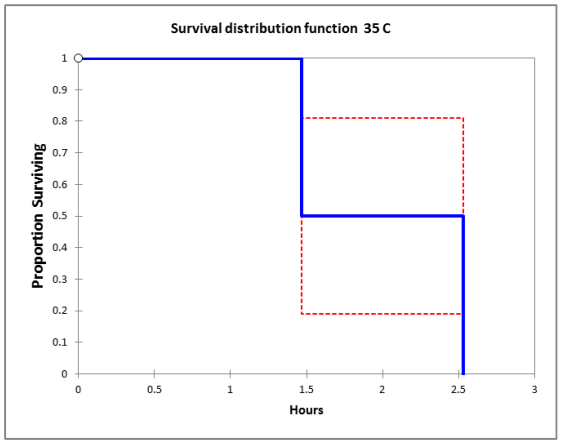
List of Figures

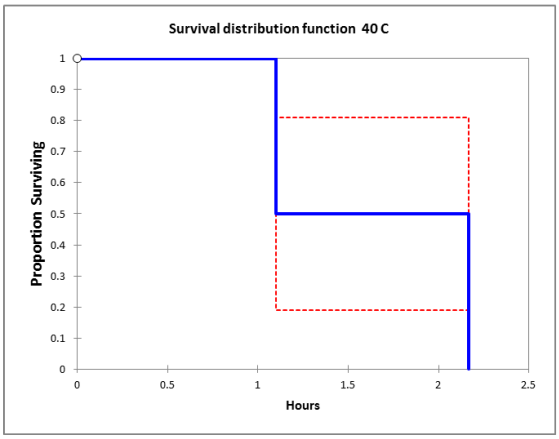
Figure 1. Survival distribution functions (proportion survival versus time) of 3rd-stage *Calliphora vicina* larvae at each of 5 temperatures (Fig. 1a-e), and combined (Fig. 1f) from Kaplan-Meier survivorship analysis. Dotted lines indicate 95% confidence limits.











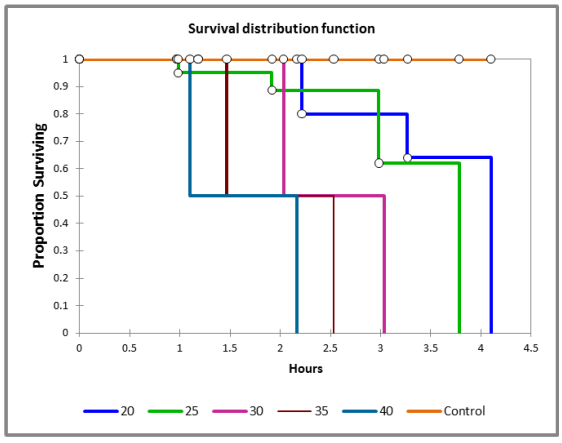
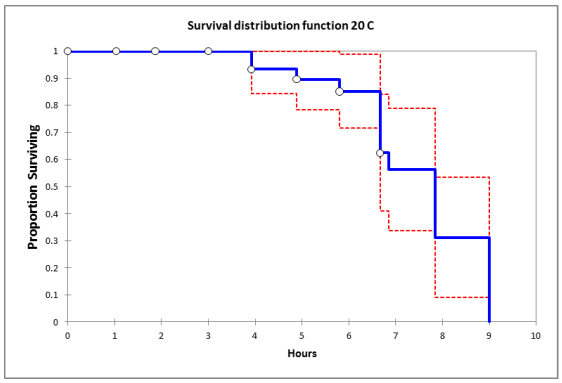
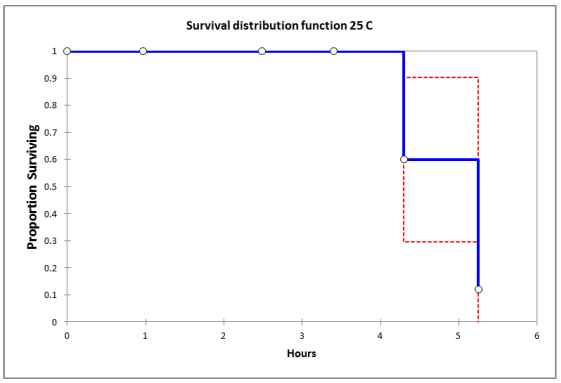
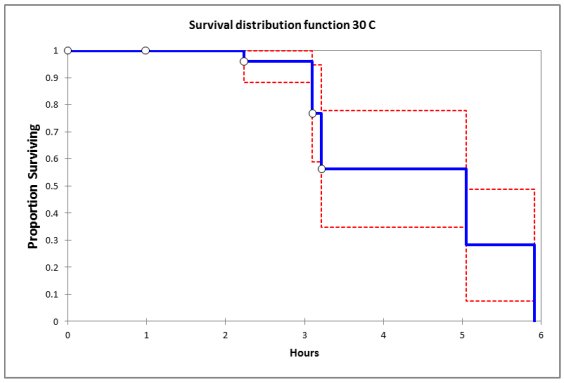
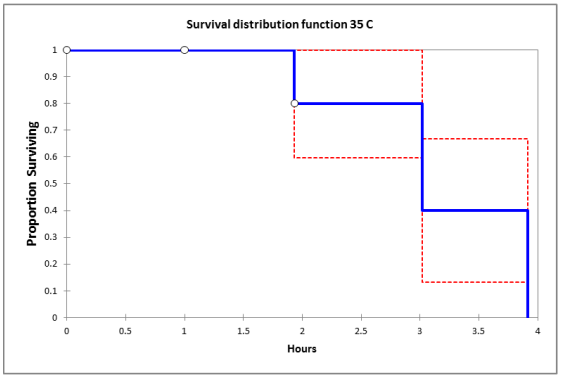


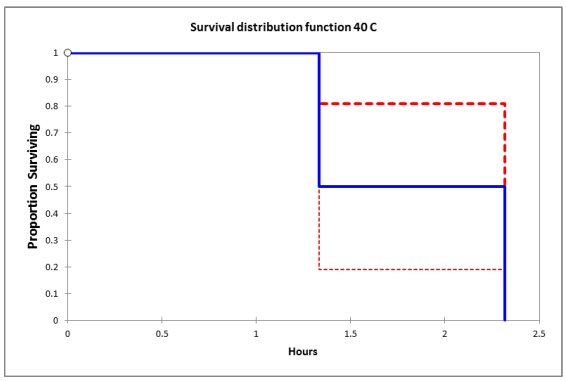
Figure 2. Survival distribution functions (proportion survival versus time) of 3rd-stage *Cochliomyia macellaria* larvae at each of 5 temperatures (Fig. 2a-e), and combined (Fig. 2f) from Kaplan-Meier survivorship analysis. Dotted lines indicate 95% confidence limits.











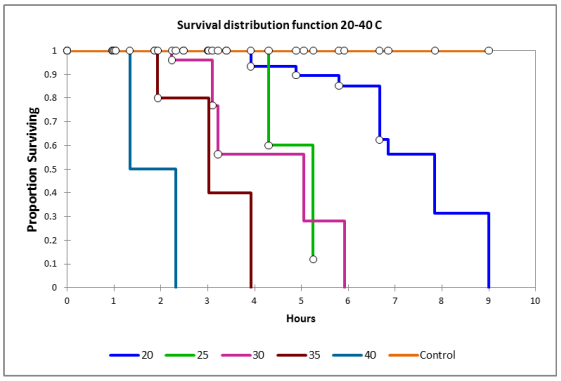
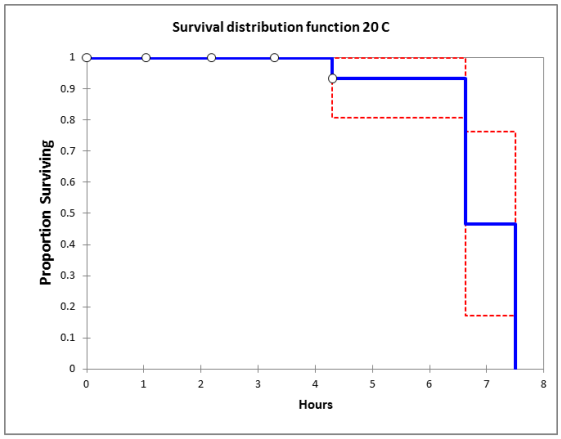
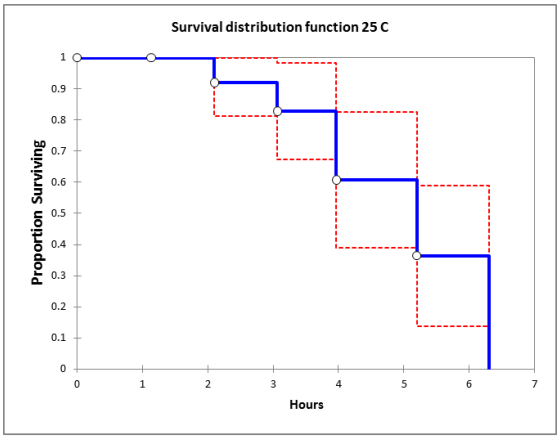
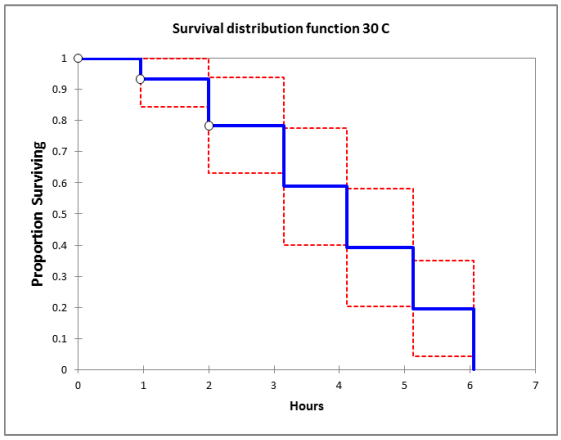
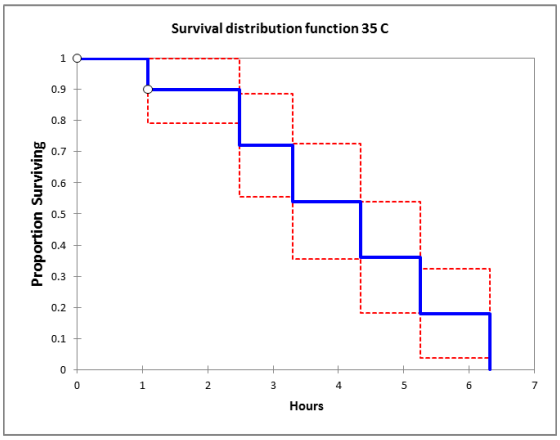


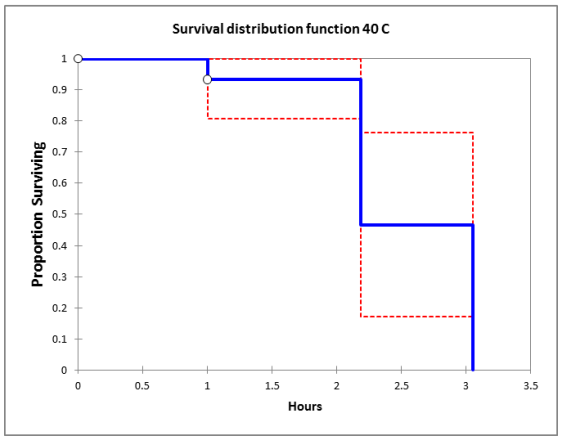
Figure 3. Survival distribution functions (proportion survival versus time) of 3rd-stage *Lucilia sericata* larvae at each of 5 temperatures (Fig. 3a-e), and combined (Fig. 3f) from Kaplan-Meier survivorship analysis. Dotted lines indicate 95% confidence limits.











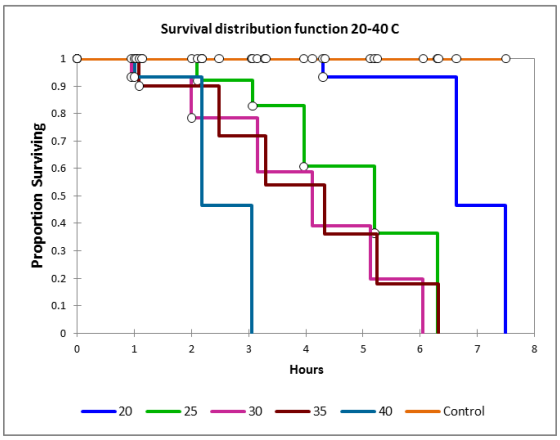
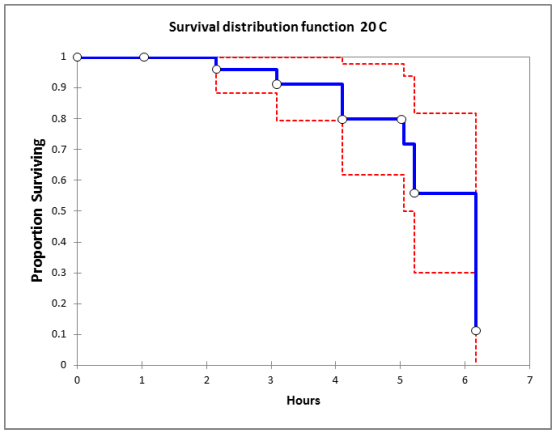
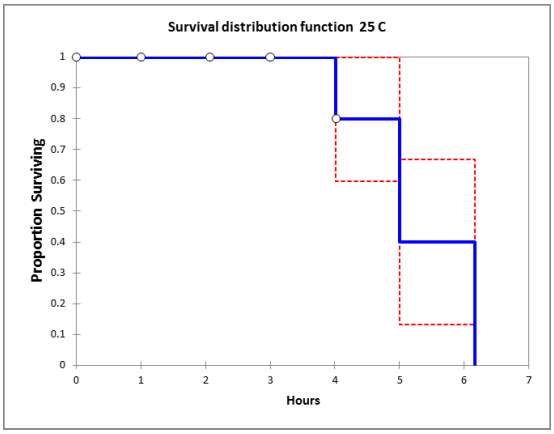
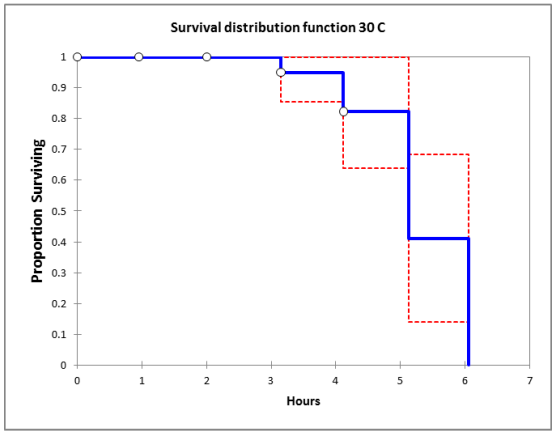
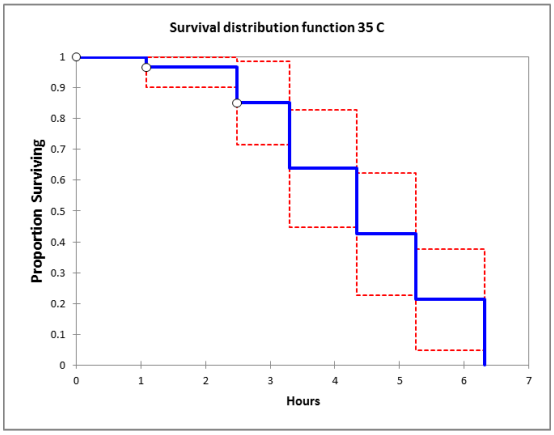


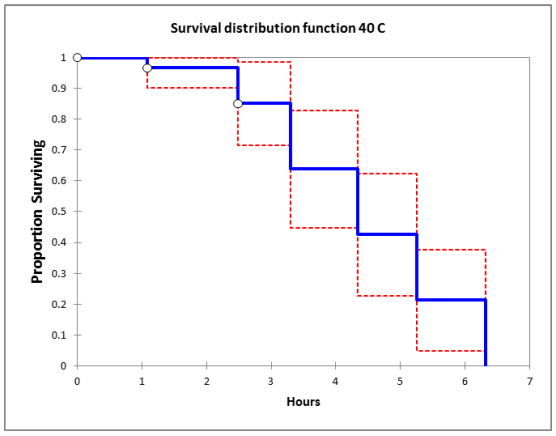
Figure 4. Survival distribution functions (proportion survival versus time) of 3rd-stage *Phormia regina* larvae at each of 5 temperatures (Fig. 4a-e), and combined (Fig. 4f) from Kaplan-Meier survivorship analysis. Dotted lines indicate 95% confidence limits.











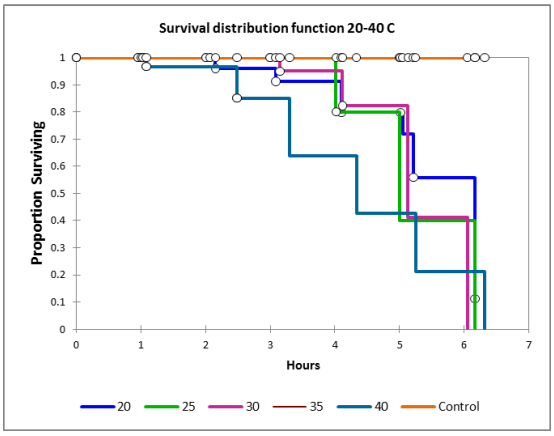


Figure 5. Relationships between LT50 (lethal time to 50% survivorship) and temperature (°C) for four species of calliphorid: a) *Calliphora vicina*, b) *Cochliomyia macellaria*, c) *Lucilia sericata*, and d) *Phormia regina*. Dotted lines indicate 95% confidence limits.

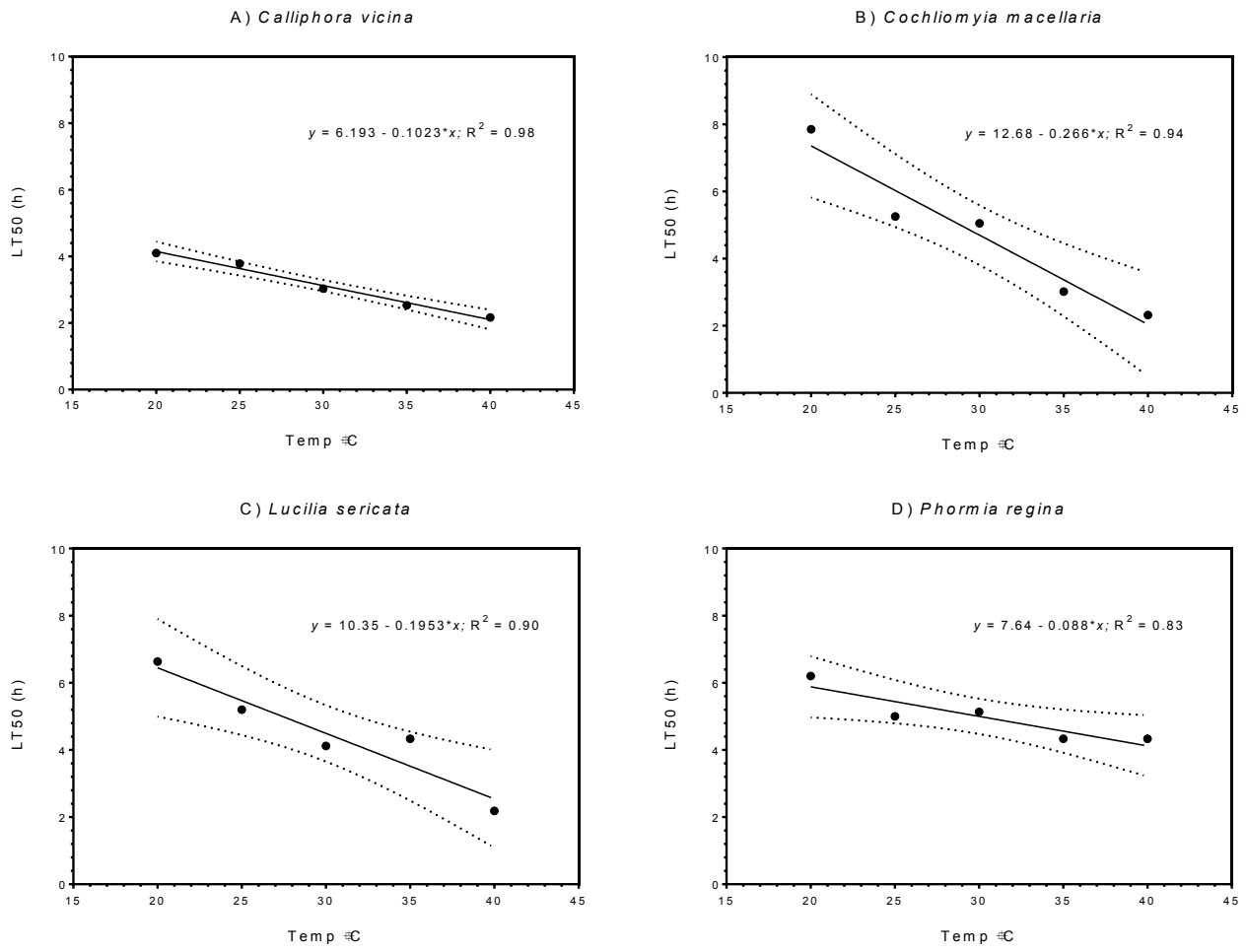


Figure 6. Relationships between LT75 (lethal time to 75% survivorship) and temperature (°C) for 3rd-stage larvae of four species of calliphorid: a) *Calliphora vicina*, b) *Cochliomyia macelleria*, c) *Lucilia sericata*, and d) *Phormia regina*. Dotted lines indicate 95% confidence