Establishing Blow Fly Development and Sampling Procedures to Estimate Postmortem Intervals

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FINAL TECHNICAL REPORT

Abstract

Currently, the most reliable biological indicator of time since death in decomposition cases is blow fly development. Because insects do not maintain constant body temperatures, development is a function of time and temperature. Consequently, having accurate models for describing temperature and blow fly development is essential in producing accurate estimates of the post mortem interval (PMI). To date, data on blow fly development have been limited and comprehensive growth models lacking, our focus in this project was to develop datasets, models, and procedures to improve PMI estimates through measures of blow fly development. We used 66 sampling times in replicated laboratory studies to determine development requirements for each insect growth stage and transitions between stages. We developed improved degree day (linear) models and curvilinear models for *Lucilia sericata* and *Phormia regina*, and are working on data for additional species. We determined that stage transitions show normal distributions and can last from hours to days, depending on temperatures and stage. We noted that the third migratory stage does not show temperature-dependent development above 17.5° C in either *L. sericata* or *P. regina*. This stage also had the most variability in development time, but all stages showed substantial variability, despite having little genetic variation. Field and laboratory observations confirmed that blow fly feeding and stage transitions can occur at night and in darkness. Studies on anoxia tolerance with *Calliphora vicina*, *Cochliomya macellaria*, *P. regina*, and *L. sericata* showed limited tolerance to anoxia with LT50 times between 1-10 hours. Consequently, while larval movement in maggot masses could be associated with oxygen starvation, as LT50s are in the range of hours, rather than minutes, it is more likely that movement is associated with thermoregulation. Practical implications of these results include more precise and accurate methods for determining insect development and the associated PMI, including statistically valid confidence intervals. The recognition of the potential importance of the duration of stage transitions, normal distributions in transitions, and potential temperature-independent development of migratory third stage larvae will require changes in current recommendations and improvements in PMI estimates as changes are implemented.

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Executive Summary

Insect development data has a wide range of uses, including: agricultural pests and economic thresholds, disease vectors, medical interventions, and medicolegal interpretations. Fortunately, the relationship between temperature and its impact on insect development has been well studied. Unfortunately, the accuracy and precision of the available development data/models make it increasingly difficult to apply to areas where accuracy levels of days/weeks are inappropriate or unhelpful, such as forensic contexts.

Currently, the most common method of determining insect age is the use of thermal summation models, or degree days. Degree days are a measure of physiological growth through the interaction of time and temperature. They tend to be the most accurate over linear approximations, and tend to be the most inaccurate over the curvilinear portions of the developmental curve, where insects are experiencing higher variation due to temperature extremes. While curvilinear models would be the most comprehensive method of determining insect age at a given temperature, the data sets available for many insects do not meet validity requirements.

An area of growing interest where insect development forms the basis for all calculations is the use of insects in estimating the postmortem interval (PMI), or time since death, in death investigations. Of the available developmental data sets, most focus on blow flies (Diptera: Calliphoridae), because of their ability to quickly locate and colonize carcasses. Unfortunately, many of these data sets have methodological pitfalls or provide relatively few data points. Moreover, available developmental models with forensic insects are exclusively degree day: based on assumptions of linear relationships between temperature and insect development. Although useful, it is well known that insect development and temperature actually follow a curvilinear relationship, thus developing data sets that are more robust and sufficient to allow development of curvilinear models is of theoretical and practical importance. Additionally, with requirements for statements on variation increasingly important in federal and state courts, better developmental data will allow statistically valid expressions of variation in developmental and PMI estimates.

In pursuit of this goal of improving blow fly developmental models, we conducted various experiments to better understand blow fly developmental biology, to provide robust developmental data sets for forensically important blow flies, to allow calculations of degree day and curvilinear developmental models, and to improve sampling of forensically important blow flies. Our core research focused on *Lucilia sericata*, and *Phormia regina*, although it has been our intent to also develop datasets for *Calliphora vicina*, *Chrysomya megacephala*, and *Cochliomya macellaria* (this work is continuing). We did replicated studies (with replication by growth chamber) of development at all developmental stages with multiple samples at predicted times of stage transition. With measurements of growth for individual maggots at multiple developmental times, our datasets for *L. sericata* and *P. regina* each consist of over 30,000 data points. Besides the development data generated for these species, we examined feeding behavior in laboratory and field studies, and examined anoxia tolerance in a series of laboratory experiments. Our work on anoxia is part of our broader investigations into factors influencing maggot movement during development including movement when maggots are feeding as part of a large "maggot mass." Our on-going work, not reported here, includes validation of our developmental models with field and known time-of-death case data, development of *C. vicina*, *C. megacephala*, and *C. macellaria*, examinations of genetic variation and underlying variability in development, larval competition, detailing sampling procedures, and the role of heat shock proteins in development at high temperatures.

The development data for *Lucilia sericata* and *Phormia regina* represent the most comprehensive datasets ever obtained for forensic insects; indeed the data obtained for just one of these species

exceeds that of all published development data for all other forensically important blow flies combined. With these data it has been possible to develop a new method for calculating conventional degree days that ensures internal validity within known confidence levels. Additionally, data allowed the development of curvilinear models that avoid the intrinsic limitations of linear methods like degree days. Developing these models was the core objective of this study, and similar datasets for additional species are needed. Our stated intention was to develop such datasets for at least five blow fly species, and this remains our goal. Unfortunately, the complexity and difficulty of getting these data means that work on additional species (specifically, *Calliphora vicina*, *Chrysomya megacephala*, and *Cochliomyia macellaria*) will continue beyond the end of this project. Because *Lucilia sericata* and *Cochliomyia macellaria* thought to be the fastest developing blow fly species in North America on carrion larval development is slightly faster based on Kamal, the developmental data and model of *L. sericata* is especially valuable in putting a lower limit on potential development times of any North American species. This point is especially pertinent in cases where blow fly species identification is not possible.

Characterizing stage transition periods provides a new tool for assessing the age of sampled insects in various forensic situations. The collection of samples with only one stage or mixed stages can be used with the known transition data to reduce the uncertainty of insect ages within a stage. Practically, this technique can improve estimates by hours to days depending upon the insect stages and ambient temperatures. Additionally, the importance of stage transitions in assessing development is not currently indicated in the scientific literature, and in many instances data sets used in estimating the post mortem interval (PMI) offer no indication of transition times. Without an explicit consideration of stage transition times, it is possible to systematically over or under estimate a PMI. Obtaining detailed data on stage transitions was the most time consuming, expensive, and frustrating aspect of our research, so identifying more efficient methods would greatly accelerate research on this aspect of development.

Finding that the frequency of stage transitions follows a normal distribution (for all stages and species examined) has immediate and long term implications. Many forensic entomologists have argued that samples should be made of the "largest" larvae and that developmental models should be based on modes rather than means. Neither of these points is valid, and based on the evidence here we can show that such methods will bias estimates of PMI. In developing statistically valid sampling procedures, the normal distribution of stage transitions and time in stage will make sampling procedures relatively easy, provided sites of initial oviposition are sampled.

Besides the long duration of stage transitions, a number of other findings were surprising and potentially important. Although all experiments were conducted with fly populations with little or no genetic variability, ample evidence of individual variation was seen in as aspects of development with all species examined. We have not yet looked at development of eggs from single females and confirmed lack of genetic variation through DNA testing, however, preliminary studies support the idea that blow fly development and larval behavior includes significant intrinsic variation. We see this variation manifested in stage-specific development times, larval movement, anoxia tolerance, and, particularly, migration at the L3m stage. If confirmed, this variation is itself an important phenomenon of interest from genetic, physiological, ecological, and evolutionary perspectives. Moreover, accounting for this variation will likely place an absolute limit on the potential accuracy of PMI estimates from insect development.

The confirmation from field and laboratory observations that blow fly larvae feed at night and in darkness resolves a debate that has appeared in the scientific literature. Similarly, our observations that stage transitions occurred throughout a 24-h cycle supports evidence (Nabity et al. 2007) that stage transitions in blow flies are not set by light or circadian rhythms.

Development data for *L. sericata* and *P. regina* both demonstrate that development in the migratory third stage does not increase with temperature. Instead, both species show a threshold temperature of activity (ca. 17.5° C) below which development is serious delayed. Consequently, the application of any temperature driven development model for this larval will result in errors in the resulting development time and PMI estimates. To the best of our knowledge this is the first demonstration of temperature insensitive development, and application of this information will improve PMI estimates in virtually all situations involving the L3m stage.

Regarding maggot survival in maggot masses, our experiments on anoxia tolerance indicate that blow flies have relatively low tolerance of anoxic conditions. However, the LT50's for all species tested were in the 1-6 hour range, therefore it seems more likely that larval movement within a mass is associated with temperature regulation than oxygen availability. Figure 70 shows strong linear relationships between survival times and temperature. These data) 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. 70).

Additional data analysis is being conducted on survivorship and temperature, development at high temperatures and potential involvement of heat shock proteins, and field and historical validation of development models. Most direct comparisons of our results with literature data are not possible (because investigators used different sampling procedures, reported values (like modes) that are not directly comparable, or have too few data points to allow a meaningful comparison.) Fortunately, data from Kamal (1958) do allow a comparison as shown in Fig. 71. We were able to take data from Kamal's 1958 publication, calculate means and transition times, and then calculate accumulated degree days using developmental minima we determined. We don't have data from Kamal's individual replications, so we can't do a statistical comparison, however, values are remarkably similar, and this agreement occurs in both *L. sericata* and *P. regina*. The greatest variation with the L3m stage, which isn't surprising in that L3m has the most variability of any stage. Overall, the general agreement of our data with that of Kamal has potentially important implications. Kamal's data were taken over 60 years ago and from a population (in Washington) that was over 1300 or 2000 miles distance from the blow fly populations we used to establish our colonies. These observations strongly imply that blow fly development is not as geographically variable as some workers suggest. Coupled with our data documenting the underlying variation in development within (what should be) genetically uniform individuals, we are increasingly skeptical that levels of reported geographical variation make sense. Consequently, the question of population differences in development merits a critical reexamination, with particular focus on experimental methods (given the difficulty in accurately determining developmental times for specific developmental events).

In any death investigation, the time of death is an important element. In cases where the victim's remains have decomposed, however, time of death assumes an even greater role. Without direct

observation of the crime, timing becomes critical for everything from identifying potential suspects and examining alibis to distinguishing primary and secondary crime scenes. Because blow flies are one of the key biological agents of decomposition, and because it is possible to relate their development closely to time of death, insects often are the only potential indicator of time of death.

The datasets for *Lucilia sericata* and *Phormia regina* provide the most comprehensive data on development of any forensically important insect species. Without question development calculations using these data will produce more accurate and precise PMI estimates. Additionally, these estimates can now be made with statistically valid estimates of variability. Of potentially greater importance are our findings regarding L3m temperature-insensitive development, normal distributions of larvae during stage transitions, durations of transitions, and intrinsic variability in development. We have already used these data in one on-going civil case (myiasis) and will be using data in homicide cases in the near future. The analysis of confirmed time of death cases is underway, to further validate both our data and approach.

Regarding other future research, the most obvious need is to develop comparable datasets on development of other forensically important blow flies. Our results highlight the importance of measuring stage transitions precisely, but our experience also highlights the difficulties in making such measurements. Hundreds of hours of research time taking measurements literally around the clock presents a significant barrier in obtaining data for other species. One approach we are beginning to explore is the potential use of video for observing individual maggots and stage transitions through time. While obtaining sufficient video equipment might be one problem, the current issues are having sufficient resolution to see and identify larvae, accommodating larval movement, and distinguishing individual larvae in images. One potential solution is to develop single larva experimental units, but this requires developing rearing procedures so that larval development is unaffected by larval numbers (if this is possible).

Many other research areas are indicated from our results, some of which we mentioned previously. Work on genetic variability and intrinsic variation in development, temperature and L3m development, and whether or not geographic variation in develop exists are obvious topics. As our understanding and data on temperature and insect development become more detailed, we suspect new opportunities for applications of thermal modeling (perhaps of soil or surface bacteria, or of autolytic processes) may be possible. Our results indicate that it is possible to greatly improve our precision and accuracy of insect development estimates which necessarily improves the associated PMI estimates. If we can apply some of these same procedures to other taxa associated with decomposition, we think greater improvements in PMI estimation are possible.

Main Body of the Final Technical Report

Introduction

The Problem

Homicide victims discovered in any stage of decomposition present unique challenges in an investigation. With discovery of a decomposed body the three immediate questions – identification of the victim, cause of death, and the time of death – are problematic, because the process of decomposition gradually destroys the evidence needed for answers. When a body is discovered more than a few hours to a day after death, estimating the time of death depends on the environmental factors, both biological and physical, associated with decomposition. Of these factors, it is the development of carrion-eating flies, especially blowflies that has proved most valuable.

Although the process of human decomposition and the forensic importance of blow flies in this process have been recognized for over 1000 years (T'zu 1247), the benchmark data necessary to relate insect development to the postmortem interval still does not exist. Instead, we have partial data on the development of certain insects at certain temperatures, and for some of these we also have simple linear developmental models. Despite their limitations, these partial data have been extraordinarily valuable in allowing estimates of the postmortem interval (PMI, the time between death and discovery of a body), which emphasizes the forensic importance of this approach. Because insects are one of the primary agents of animal decomposition, insects routinely provide some of the best biological information available for determining the PMI. Unfortunately, in the absence of definitive, modern developmental models for all key forensic blow flies, forensic taphonomists must specify large intervals (typically days) in their PMI estimates, they cannot specify descrete levels of probability for their PMI estimates, experts disagree on PMI estimates (because different developmental models or data are used), and PMI estimates are not possible for species having little or no developmental data.

More alarmingly, the scientific basis for developmental estimates involving forensically important blow flies are at least 30 years behind the standard approaches used in agricultural entomology and insect ecology, which involve the calculation of curvilinear developmental models (for example, see . Additionally, the current methods recommended for collecting blow flies larvae (maggots) from bodies are not scientifically justified based on research to guarantee that samples reflect the larval population age structure within a specified error rate.

These observations are not esoteric points: we believe a legal argument against the current use of insect-based PMI could be made relative to the Daubert evidentiary standard and scientific standards suggested in the 2009 NRC report. Specifically, it could be argued that (1) insect-based PMIs do not represent a scientific consensus (as indicated by expert disagreements in court and disagreements in the scientific literature), (2) methods used do not accurately reflect established biological reality (the curvilinear nature of insect development-temperature relationships), and (3) current methods do not provide a statistically based measure of variability relative to insect sampling or PMI estimates. Consequently, our intent in this proposal is for research to directly address these critical needs associated with maintaining and improving the use of insect-based development data to estimate PMIs.

Review of Relevant Literature

There are many current reviews on the use of insect development data in forensic science, including recent chapters by Wells and LaMotte (2010) and Higley and Haskell (2009), so we will not repeat that information here. Among the most pertinent information relative to the objectives of this proposal are the comparisons of existing developmental data provided by Nabity et al. (2006) for *P. regina* and by Higley and Haskell (2009) for *C. vicina*, *L. sericata*, and *P. regina*. These summaries of existing developmental data document the limited temperature ranges in most existing data set and disagreements in published studies.

Undoubtedly, many of these differences among studies stem from differences in experimental methods and limits in the temperatures considered. If development rates are not determined with temperature treatments representing equal intervals, the calculated linear regression may not reflect underlying biological relationships (Arnold 1960). Similarly, failing to use the mathematically appropriate base temperature will skew development estimates (Arnold 1960, Nabity et al. 2006, Higley and Haskell 2009). Additionally, many studies have been conducted under continuous light, but our recent research (Nabity et al. 2007, Fisher et al. *submitted*) shows that blow fly development rates are significantly changed under variable light-dark cycles (particularly at low temperatures). Also, treatments reported in the literature vary with some studies conducted under constant temperature and others under fluctuating temperatures (e.g., Byrd and Allen 2001, Byrd and Butler 1996, 1997, Nabity et al. 2006). Similarly, some authors used time of first emergence for determining the length of a stage, others use the mean or median stage transition time.

Beyond issues associated with existing data, the methods used for describing the development of forensically important insects are currently limited to degree-days, a linear estimate of the development rate-temperature relationship. In turn, these degree day estimates are used to calculate the postmortem interval (PMI). Estimates of the PMI are based primarily on the knowledge that blow fly larvae develop at predictable rates at known temperatures. These rate vs. temperature relationships can be expressed as a number of accumulated degree-hours or degree-days (Higley and Haskell 2001). Because different blow fly species have different thermal developmental requirements, accurate species level identification is essential for calculating the correct PMI. Knowledge of a species' habits and ecology are also necessary when estimating the PMI (Wells and LaMotte 2001).

The concept of the combining time and temperature into a single measure of "physiological time" has a long history. The idea was first proposed by Reamur in 1735 and was extensively developed fifty years ago (Arnold 1960, Higley et al. 1984, Wagner 1984). At the core of the procedure is the requirement that the relationship between development rate and temperature be approximated by a straight line. However, experimental data demonstrates that the actual relationship is curvilinear (Figure 1), and using a linear approximation only applies for a portion of the development curve (Higley et al. 1984, Higley and Haskell 2009, Wagner 1984).

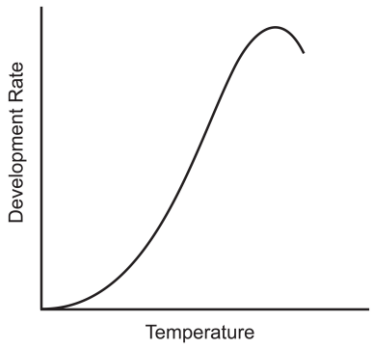


Figure 1. The relationship between insect development rates and temperature (after Higley and Haskell 2009).

An additional issue with the degree-day approach is that measures of variation in development estimates are different at high and low temperature, a phenomenon that has prompted the construction of modified calculation methods (Ikemoto and Takai 2000).

Given that the actual relationship is curvilinear, why have linear (i.e., degree-day) methods persisted? Most likely the core reason is that data have not been available to construct more accurate models. Also, linear approximations are adequate if the environmental temperatures occur in the linear range. Unfortunately, other than our calculations reported in Higley and Haskell (2009), we are unaware of any effort to define these limits of linearity for existing developmental data on forensically important blow flies. Also, because of the mathematical peculiarities of the degree day model, statistical estimates of variability are largely unavailable.

There are two potential approaches to resolving this problem, and both depend on obtaining data sets with multiple points sufficient to mathematically describe the development rate vs. temperature curve (Figure 1). The first is the use of a "biophysical model" based on proposed reaction kinetics of rate-limiting steps in development (Sharpe and DeMichele 1977, Wagner et al. 1984). This method has been extremely influential, however, many authors (including us) have challenged the underlying assumption of these models, namely that development is regulated by rate limiting enzymes. Indeed, evidence exists demonstrating that this assumption clearly is not met at low temperatures (see Higley and Haskell 2009 for a brief summary of this debate). Alternatively, the curvilinear model can be determined through multiple regression anlysis and curve fitting. This approach assumes no underlying biophysical mechanism and has the added advantages of requiring fewer data points that the biophysical model and allows the calculation of statistically valid measures of variation.

In applying insect develop data to determination of the PMI, there are three key areas of biological variation: (1) time of oviposition, which requires a consideration of factors delaying oviposition; (2) development time in relationship to temperature, which requires data relating temperature to development time, and (3) sampling and aging maggots from a body, which requires an accurate sample of maggots reflecting the underlying age structure of the population and the accurate determination of maggot age (within available limits).

Of these three sources of variation, the second is clearly represents the largest potential source of error. Indeed, this is the reason establishing comprehensive, curvilinear development models for forensically important blow flies is so needed. For the first source of variation, oviposition issues typically involve delays of 12 to 48 hours, and are largely specific to species and unique situations (e.g., burning, wrapping bodies, indoor vs. outdoor scenes). Given the array of potential situations associated with ovipositional delays, this question falls outside the scope of our proposed research. However, the third source of potential variation, sampling error, more directly relates to our research goals because the calculation of a PMI estimate from insect evidence will only be valid if the initial insect identification and aging are accurate. Indeed, the need for improved sampling procedures has been an area of some research focus (e.g., Schoenly et al. 1991, Schoenly et al. 2005). To date, however, formal sampling procedures for blow fly larvae, which would include statistically valid measures of variation, have not been developed.

Purpose

To develop curvilinear temperature models for key forensic blow fly species to allow estimation of a PMI with a statistically sound measure of variability and to develop blow fly larval sampling procedures for use with specified levels of accuracy and precision.

Goals

Broadly, our goal is to provide comprehensive developmental models for key forensic blow fly species of the United States of America, and to define methods that can be used for other blow fly species. Additionally, we will implement these models through spreadsheets and other software to allow determining a PMI from maggot development with a calculated level of variation. Finally, we will develop maggot sampling procedures for crime scene investigators that ensure a specified level of statistical accuracy.

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 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 our experiments were from colonies maintained in our 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 (and in our ecophysiological studies) 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 was observed in any of our colonies over many generations.

Regarding specifics of colony establishment, the *C.vicina* colony was established in October 2012 from a single field-collected female from Lincoln, NE. At the time of 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 his 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 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 had a ca. 1 month adult lifespan). Cages themselves were 0.61 x 0.61 x 0.61m in size (collapsible cage #1450D, Bioquip Products,

Inc., Rancho Dominguez, CA, www.bioquip.com) to avoid any potential selection on adult size (as has been reported for blow flies rearing in small cages (Kamal 1955). 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 plastic boxes (15x10x5 cm) in a Percival growth chamber set at 26° C (which was ± 1.5° C of this set temperature based on internal temperature measurements). Within the plastic container, liver and feeding maggots were placed on a plate, which rested on pine shavings. The pine shavings provided an area for larval migration at the end of the third larval stage and a substrate for pupation (larvae bury themselves within the pine shavings after migratory movement). Because larvae of *P. regina* are more likely to move off the substrate as compared to other species, moist sand was used instead of pine shavings to avoid desiccation as larvae move on and off the liver, as well as providing an acceptable pupation medium.

Incubators

Incubators were customized model SMY04-1 DigiTherm® CirKinetics Incubators (TriTech Research, Inc., Los Angeles, 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). 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. Although growth chambers have been shown to display substantial differences between programmed temperatures and actual internal temperatures (Nabity et al. 2007), incubators tested with internal thermocouples in a replicated study, showed 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. 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.

Experimental Design

Development Studies. Each development study comprised examined eleven temperatures (7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5, 30, and 32.5 °C) with a light:dark cycle of 16:8. Twenty eggs (collected within 30 minutes of oviposition) were counted onto a moist black filter paper triangle and placed in direct contact with 10 g of beef liver in a 29.5 mL plastic cup. The cup was placed in a 7 cm x 7 cm x 10 cm plastic container that had 2.5 cm of wood shavings in the bottom. The container was then placed randomly in an incubator. Each life stage (egg-1st stage, 1st-2nd stage, 2nd-3rd stage, 3rd-3rd migratory, 3rd migratory-pupation, pupation-adult) was calculated using Kamal's (1958) data which was converted to accumulated degree hours (ADH) and divided equally into five sampling times (Table 1). Each sample was replicated four times, for a total of 20 samples per life stage. During each sample time, the container was pulled from the incubator, larvae were killed (by being placed in preservative) and the stage of each maggot was documented morphologically using the posterior spiracular slits and cephalopharyngeal skeleton.

During egg hatch, a larva was recorded as 1st stage if they had broken the egg chorion and were actively emerging. Pharate larvae (larvae that have undergone apolysis but not ecdysis) were recorded as the earlier stage (e.g. 3rd stage spiracular slits can be seen beneath the current spiracular slits would be recorded as 2nd stage), since they had not yet molted. Pupariation started when the larva had a shortened body length and no longer projected its mouth hooks when put in KAAD. There were times when a larva appeared to be entering the puparium stage but would extend its body length and begin crawling if disturbed or placed in KAAD. These larvae were recorded as 3rd

migratory. All life stages were preserved in 70% ethyl alcohol. Third and 3rd-migratory stages were fixed in KAAD for 48 hours and transferred to 70% ethyl alcohol.

Anoxia Studies. 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 a third-stage larvae. 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 needed 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 to displace any oxygen. Then the maggot was placed in the vial while it was still upside down and finally 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 helped minimize any desiccation of the maggots.

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 our 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 pulled 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 by then full of oxygen) and capped and pulled 24 hours later (Burst and Hoback 2009). After the 24 hours the maggot was then pulled from the vial and checked for movement. A score of no movement for the initial removal and the 24 hour removal would result in a response of dead. As well, if the maggot was scored as movement for both times or for the 24 hour check, it was recorded as n live response. Samples were continued until all maggots were recorded as a no movement in the initial test.

Field Studies. For observational studies on maggot feeding behavior, 4 euthanized pigs (ca. 45 kg.) were obtained from the University of Nebraska-Lincoln Swine Management Unit. Pigs were euthanized within 1 hour prior to their use in experiments. Carcasses were placed in the field at the Agricultural Research and Development Center near Mead, Nebraska (ca. 40 miles north of the UNL campus). Temperatures on pigs were monitored, and once maggots reached the third instar maggot feed was observed over night with infrared goggles. Observations from the field were repeated in the laboratory.

Analysis

In all previous work on the development of blow flies (in so far as we are aware), the transition from one stage to another has been treated as (essentially) a single time. For example, methods in a development experiment might specify checking stages every 24h, which implies transitions are complete within a 24 h period. However, the actual duration of stage transitions for blow fly species may exceed 24 hours and will vary with temperature. Because hours matter in a forensic context, determining the relationship between temperature and stage duration is an important, but largely ignored issue. Moreover, to describe how stage transitions occur through time, determining the appropriate mathematical distribution of a stage (i.e., frequency of occurrence of a stage through time) is needed. Consequently, a goal of our development experiments was to determine the distribution of stage transitions by temperature for each of six transitions. With 11 temperatures and 6 transitions we needed to model 66 relationships. We used two regression procedures. First, to determine the appropriate transition distributions, we used TableCurve 2d , version 5.01 (SYSTAT Software Inc. , San Jose, CA http://www.sigmaplot.com/products/tablecurve2d/tablecurve2d.php), and Prism, version 6.02 (GraphPad Software, Inc., La Jolla, CA, http://www.graphpad.com/scientific-software/prism/). Here, we fit one of four functions (specifically, a regressed proportion (percentage) in stage versus time, at each temperature tested). The equations used were:

1. A Gaussian equation (a standard normal curve):

$$y = a \exp\left[-\frac{1}{2}\left(\frac{x-b}{c}\right)^2\right]$$

2. A modified Gaussian equation (a form of Gaussian curve with a plateau at 100%):

$$y = a \exp\left[-\frac{1}{2}\left(\frac{|x-b|}{c}\right)^d\right]$$

3. A cumulative Gaussian equation (a form of the Gaussian curve used for adults, to model a sigmoidal increase to a plateau)

$$y = \frac{a}{2}\left[1 + erf\left(\frac{x-b}{\sqrt{2}c}\right)\right]$$

4. A reversed cumulative Gaussian equation (a form of the cumulative Gaussian equation used for eggs, to model a sigmoidal decrease from a plateau)

$$y = \frac{a}{2}\left[1 - erf\left(\frac{x-b}{\sqrt{2}c}\right)\right]$$

Cumulative forms of the equations were needed to model the transitions from egg or to adult. For the larval and pupal stages, the distinction between fitting a Gaussian or modified Gaussian equation usually depended on length of time in stage. Because longer lasting stages often had a plateau between transitions, the modified Gaussian relationship was more appropriate. Fitting these

relationships provided evidence for the mathematical distribution of individuals during stage transitions.

A different regression procedure was needed to determine the duration of individual stages. Various approaches could be used, for example, determining the time from peak of one stage to peak of the next. However, we used the time between 50% transition into a stage to 50% transition into the next stage. We made this choice because we can determine a standard error in the 50% transition point, which is not always possible with determining peaks. Determining the 50% transition point itself is straightforward through the use of a probit model, with the probit choices of being in the first stage or the next. Through probit modelling it is possible to determine any desired % transition and the associated variation. Probit models were constructed with Prism 6.02. For all regression analyses, the data were examined closely to determine their propriety for inclusion in analysis. In a few instances, individuals were sampled with extraordinarily extended durations. These were treated as outliers and excluded from analysis.

Detailed statistical results (F values, r^2 values, equations for regression lines, and similar details) are not provided in the results (for brevity, given the large number of analyses conducted) but these are available.

Results

Statement of Results

Development Modeling: *Lucilia sericata*. For *Lucilia sericata*, no development was observed at 7.5° C and only partial development at 10° C. Consequently, data were analyzed from 10-32.5° C. Initial studies indicated that transition times between stages were substantially longer than indicated in the literature. Consequently, it was necessary to characterize distribution patterns for stage transitions and model these mathematically. With *L. sericata*, results of modeling stage transition distributions by temperature are shown in Figs. 1-10, and data were fit with either a standard Gaussian (=normal) curve or a modified Gaussian equation (to provide a plateau which was necessary for development within some stages). A comprehensive picture of stage durations and transitions is provided in Figs. 11-20. To provide a statistically valid, repeatable, indicator of time to stage and of stage duration, we decided to use the time between 50% transition into a stage and 50% transition out of a stage to indicate the length of a stage. For the egg stage (E), the duration was from oviposition to 50% transition to the first larval stage (L1). By treating proportion of stage transition like a dose response, we were able to use a standard dose response analysis to determine the 50% transition points with associated confidence limits. Figures 21-30 show these dose response curves and confidence limits. Where "curves" are incomplete or are represented by a vertical line, our transition data did not encompass sufficient points to produce a curve (usually because the transition was faster than predicted with our sampling program). Also, certain stage transitions (particularly those involving the third migratory stage (L3m)) were highly variable.

Once 50% transition points were identified, it was possible to calculate degree days. Data used are shown in Table 1, and regression relationships are illustrated in Figs. 31 and 32. Regression results and degree day requirements are indicated in Table 2. We tested the validity of initial results and iteratively modified these by calculating accumulated degree days for each stage-temperature combination and regressing these against temperature (Fig. 33 and Table 3). To be valid the resulting line should have a slope of zero. Where this was not the case, we identified data points contributing to slope, eliminated these and recalculated the 1/days regression. This procedure ensures that the calculated degree day accumulations and developmental minima are valid across the temperature ranges indicated. Although other workers have identified points on a temperature

development curve that are non-linear, and excluded these in calculating degree days (e.g., Richards et al. 2009)

Finally, curvilinear models for development were determined by simple non-linear regression. Based on initial data, an exponential decay function was found to provide the best fit to the data. The results are indicated in Fig. 34. One surprising observation is that the migratory third stage shows little association between temperature and development. As indicated in Fig. 34, it appears that development of the L3m stage requires a threshold temperature (ca. 17.5° C), after which development proceeds independent of temperature (lasting ca. 50 hours).

Development Modeling: *Phomia regina*. Procedures and results for *Phormia regina* mirror those with *Lucilia sericata*. Stage transition distributions are shown in Figs. 35-45, and complete time durations of stages from egg to adult are indicated in Fig. 45-53. Dose response analysis to determine 50% transition points are shown in Figs. 54-65, and data resulting from this analysis is listed in Table 4. Regressions of 1/days versus temperature are show in Fig. 66 and 67, and Table 5 indicates the results of these analyses. To adjust and validate the degree day model, data are show in Fig. 68 and Table 6 illustrating the range of data points for which the resulting model is valid. Finally, curvilinear models for development of *Phormia regina* are shown in Fig. 69. Note that as with *Lucilia sericata*, the migratory third stage larvae of *P. regina* show a threshold temperature response and insensitivity to temperature increases above 17.5° C.

Results of Observational Studies. Briefly, field and laboratory observations demonstrated that larvae of *L. sericata* and *P. regina* maintain their feeding behaviors during day and night, and under lighted and unlit conditions. Larvae of both species exhibit movement off and on their food sources, and, in particular, *P. regina* has considerable movement independent of feeding requirements or other obvious stimuli. 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).

Anoxia Experiments. 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, species generally had similar relationships (Fig. 70). 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.

Tables

Table 1. Time (hours) to stage transitions and hours in stage for *Lucilia sericata* determined from experimental data.

		Transitions at 50% by Dose Analysis											
Temp		Hours to Stage Transition						Hours in Stage					
Mean	SE	E-L1	E-L2	E-L3f	E-L3m	E-P	E-A	Egg	L1	L2	L3f	L3m	P
10.4	0.51	155.0	317.3	451.5	683.8	1380.2	3798.4	155.0	162.3	134.2	232.3	696.3	2418.3
12.7	0.18	32.3	136.1	230.5	431.3	1970.6	2891.0	32.3	103.7	94.4	200.8	1539.3	920.4
15.1	0.14	51.7	137.6	179.1	313.8	754.0	1708.5	51.7	85.9	41.5	134.7	440.2	954.5
17.5	0.12	45.6	85.4	136.6	237.2	326.4	760.8	45.6	39.8	51.2	100.6	89.2	434.4
20.1	0.09	28.0	57.9	92.1	189.3	217.0	553.4	28.0	29.9	34.2	97.2	27.7	336.4
22.5	0.10	22.1	45.7	77.5	141.1	202.4	424.6	22.1	23.6	31.8	63.6	61.3	222.2
25.0	0.06	16.9	38.4	62.3	111.3	167.8	370.2	16.9	21.5	23.9	49.0	56.4	202.4
27.5	0.06	11.8	30.9	46.5	124.3	157.6	344.5	11.8	19.0	15.6	77.8	33.3	186.9
30.0	0.05	11.4	24.6	41.6	86.7	139.3	297.2	11.4	13.2	17.0	45.1	52.6	157.9
32.5	0.02	9.7	19.2	35.6	84.5	156.8	308.4	9.7	9.5	16.4	48.9	72.3	151.6

Table 2. Linear regression (1/days development vs. temperature) and resulting developmental minimum and accumulated degree day (ADD) requirements for *Lucilia sericata.*

	Linear Regression Results (from Graph Pad Prism)											
	1/Days to Stage Transition						1/Days in Stage					
	E-L1	E-L2	E-L3f	E-L3m	E-P	E-A	Egg	L1	L2	L3f	L3m	P
Dev Min (x-intercept):	12.6	10.8	10.5	8.8	10.3	10.7	9.5	10.9	9.3	6.6	11.5	10.4
ADD (1/slope):	8.2	21.3	35.2	82.5	107.5	230.2	10.3	11.7	14.3	47.2	29.8	127.9
Range min:	15.0	15.0	15.0	15.0	17.5	17.5	15.0	15.0	15.0	17.5	17.5	17.5
Range max:	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0

Table 3. Confirmation of accumulated degree day (ADD) requirements based on an iterative process of regressing ADD by temperature, and then identifying points that produced slopes greater than zero. Data points contributing to non-zero slopes indicate that the points lie outside of the linear portion of the development curve (thereby violating the assumption of linearity required with the use of degree days.

	Transition ADD by 1/Day						Stage ADD by 1/Days					
	E-L1	E-L2	E-L3f	E-L3m	E-P	E-A	Egg	L1	L2	L3f	L3m	P
10.4												
12.7												
15.1	5.3	24.4	34.4	82.4			12.0	14.9	9.9			
17.5	9.4	24.0	40.3	86.7	97.8	218.2	15.3	11.0	17.5	45.8	22.3	129.8
20.1	8.7	22.4	36.9	89.3	88.0	217.3	12.4	11.5	15.3	54.5		136.2
22.5	9.2	22.4	39.0	81.1	102.9	210.3	12.1	11.5	17.5	42.2	28.1	112.7
25.0	8.8	22.7	37.7	75.3	102.4	221.2	10.9	12.6	15.6	37.5	31.6	123.4
27.5	7.4	21.5	33.1	97.2	112.8	242.1	8.9	13.2	11.8	67.7	22.2	133.6
30.0	8.3	19.7	33.9	76.8	114.1	239.7	9.8	10.5	14.6	44.0	40.5	129.3
32.5												
mean	8.2	22.5	36.5	84.1	103.0	224.8	11.6	12.2	14.6	48.6	28.9	127.5
SE	1.3	1.5	2.6	7.1	8.9	11.9	1.9	1.4	2.6	9.9	6.8	7.7
n	7	7	7	7	6	6	7	7	7	6	5	6
Regression ADD	8	21	35	83	108	230	10	12	14	47	30	128
% deviation	-0.8%	5.4%	3.5%	1.9%	-4.2%	-2.3%	13.3%	4.1%	2.4%	3.1%	-3.0%	-0.3%
ADD Range min:	15.0	15.0	15.0	15.0	17.5	17.5	15.0	15.0	15.0	17.5	17.5	17.5
ADD Range max:	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30

Table 4. Time (hours) to stage transitions and hours in stage for *Phormia regina* determined from experimental data.

		Transitions at 50% by Dose Analysis											
Temp		Hours to Stage Transition						Hours in Stage					
Mean	SE	E-L1	E-L2	E-L3f	E-L3m	E-P	E-A	Egg	L1	L2	L3f	L3m	P
10.2	0.16												
12.5	0.13	70.2	354.3	564.3	612.5	843.8	1451.0	70.2	284.1	210.0	48.2	231.4	607.1
15.0	0.15	61.4	154.1	230.7	445.9	468.6	965.9	61.4	92.7	76.6	215.2	22.7	497.3
17.5	0.09	40.0	126.9	217.6	277.4	318.6	622.5	40.0	86.9	90.7	59.8	41.2	303.9
20.0	0.1	24.3	74.6	122.7	202.4	244.8	447.2	24.3	50.4	48.1	79.7	42.4	202.4
22.4	0.90	15.5	49.8	91.6	134.0	178.3	356.1	15.5	34.3	41.8	42.4	44.3	177.8
25.0	0.08	15.2	43.3	71.3	123.2	154.8	283.4	15.2	28.2	28.0	51.9	31.6	128.6
27.5	0.07	13.8	33.9	56.6	95.3	122.1	239.2	13.8	20.0	22.7	38.7	26.7	117.2
30.0	0.23	10.3	27.3	46.6	75.6	106.7	217.8	10.3	17.1	19.3	28.9	31.1	111.1
32.5	0.07	10.1	25.1	42.0	78.2	105.9	205.6	10.1	15.1	16.9	36.2	27.7	99.7

Table 2. Linear regression (1/days development vs. temperature) and resulting developmental minimum and accumulated degree day (ADD) requirements for *Phormia regina*.

Linear Regression Results (from Graph Pad Prism)												
	1/Days to Stage Transition						1/Days in Stage					
	E-L1	E-L2	E-L3f	E-L3m	E-P	E-A	Egg	L1	L2	L3f	L3m	P
Dev Min (x-intercept):	11.5	12.9	12.8	11.9	10.5	10.2	11.5	13.5	12.5	8.2	2.3	10.5
ADD (1/slope):	8.4	20.4	34.7	62.9	91.4	181.9	8.4	12.1	14.4	29.3	30.2	84.9
Range min:	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	17.5	17.5	15.0
Range max:	32.5	32.5	32.5	32.5	32.5	32.5	32.5	32.5	32.5	30.0	27.5	32.5

Table 3. Confirmation of accumulated degree day (ADD) requirements based on an iterative process of regressing ADD by temperature, and then identifying points that produced slopes greater than zero. Data points contributing to non-zero slopes indicate that the points lie outside of the linear portion of the development curve (thereby violating the assumption of linearity required with the use of degree days.

	Transition ADD by 1/Day						Stage ADD by 1/Days					
Temp (mean)	E-L1	E-L2	E-L3f	E-L3m	E-P	E-A	Egg	L1	L2	L3f	L3m	P
10.2												
12.5												
15.0	8.9	13.7	21.3	58.8			8.9	6.0	7.9			
17.5	10.0	24.6	42.8	65.6	94.0	191.2	10.0	14.7	18.9	23.3	26.2	89.2
20.0	8.6	22.2	37.0	69.0	97.8	184.1	8.6	13.8	15.0	39.3	31.4	80.5
22.4	7.0	19.7	36.6	58.8	88.7	181.5	7.0	12.7	17.2	25.1	37.1	88.2
25.0	8.5	21.9	36.2	67.5	93.9	175.3	8.5	13.5	14.5	36.4	29.9	77.8
27.5	9.2	20.6	34.6	62.1	86.7	172.8	9.2	11.7	14.2	31.2	28.1	83.0
30.0	7.9	19.5	33.4	57.1	86.8	179.9	7.9	11.7	14.0	26.3	35.9	90.2
32.5	8.8	20.5	34.5	67.3			8.8	11.9	14.1			91.5
mean	8.6	20.3	34.6	63.3	91.3	180.8	8.6	12.0	14.5	30.2	31.4	85.8
SE	0.8	2.9	5.7	4.4	4.2	6.0	0.8	2.5	3.0	5.9	4.0	4.9
n	8	8	8	8	6	6	8	8	8	6	6	7
Regression ADD	8	20	35	63	91	182	8	12	14	29	30	85
% deviation	2.1%	-0.5%	-0.4%	0.6%	-0.1%	-0.6%	2.1%	-1.0%	0.7%	3.1%	4.2%	1.0%
ADD Range min:	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	17.5	20.0	15.0
ADD Range max:	32.5	32.5	32.5	32.5	32.5	32.5	32.5	32.5	32.5	32.5	32.5	32.5

Figures

Fig. 1. Stage distributions *Lucilia sericata*, 10.0° C

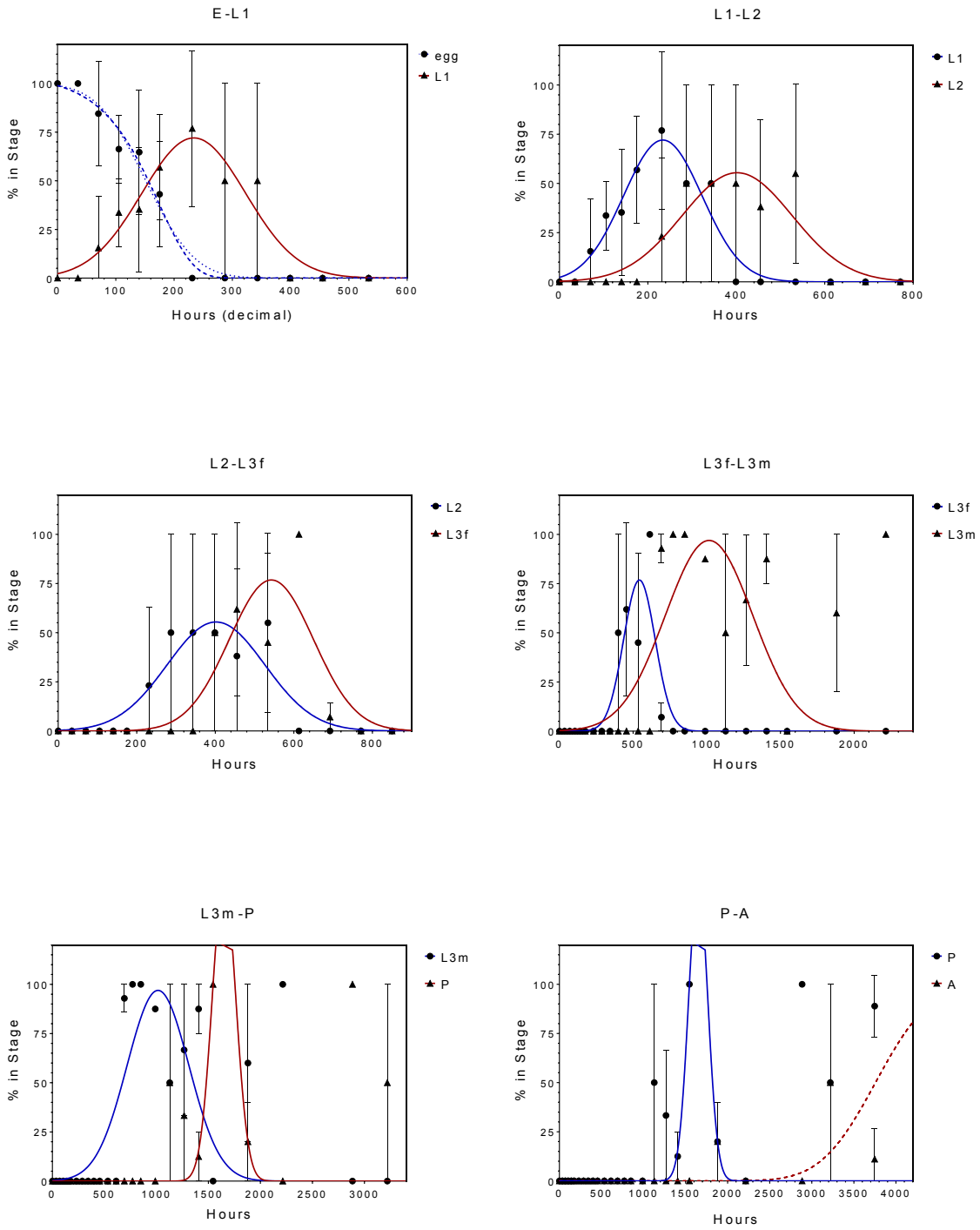


Fig. 2. Stage distributions *Lucilia sericata*, 12.5° C.

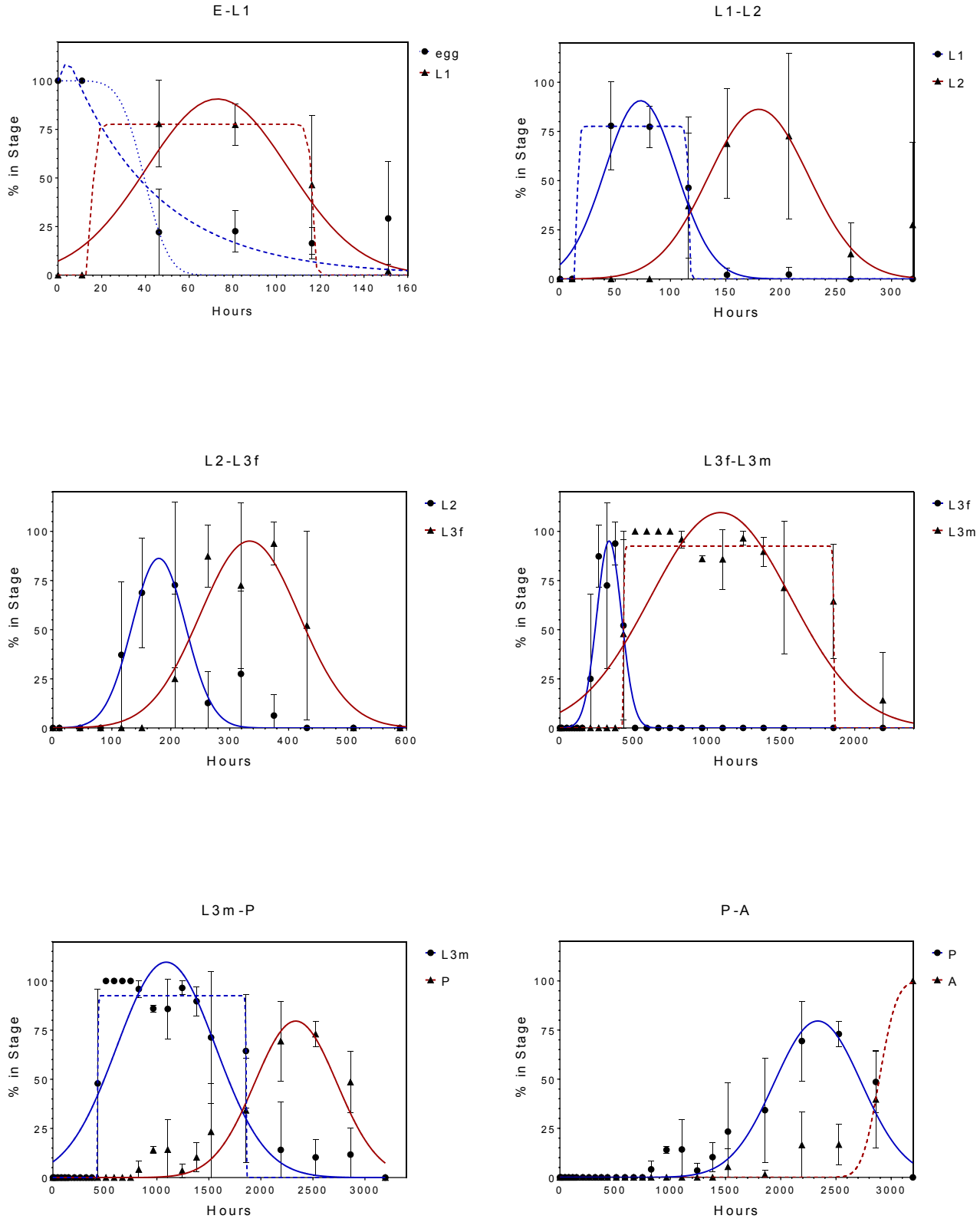


Fig. 3. Stage distributions *Lucilia sericata*, 15.0° C.

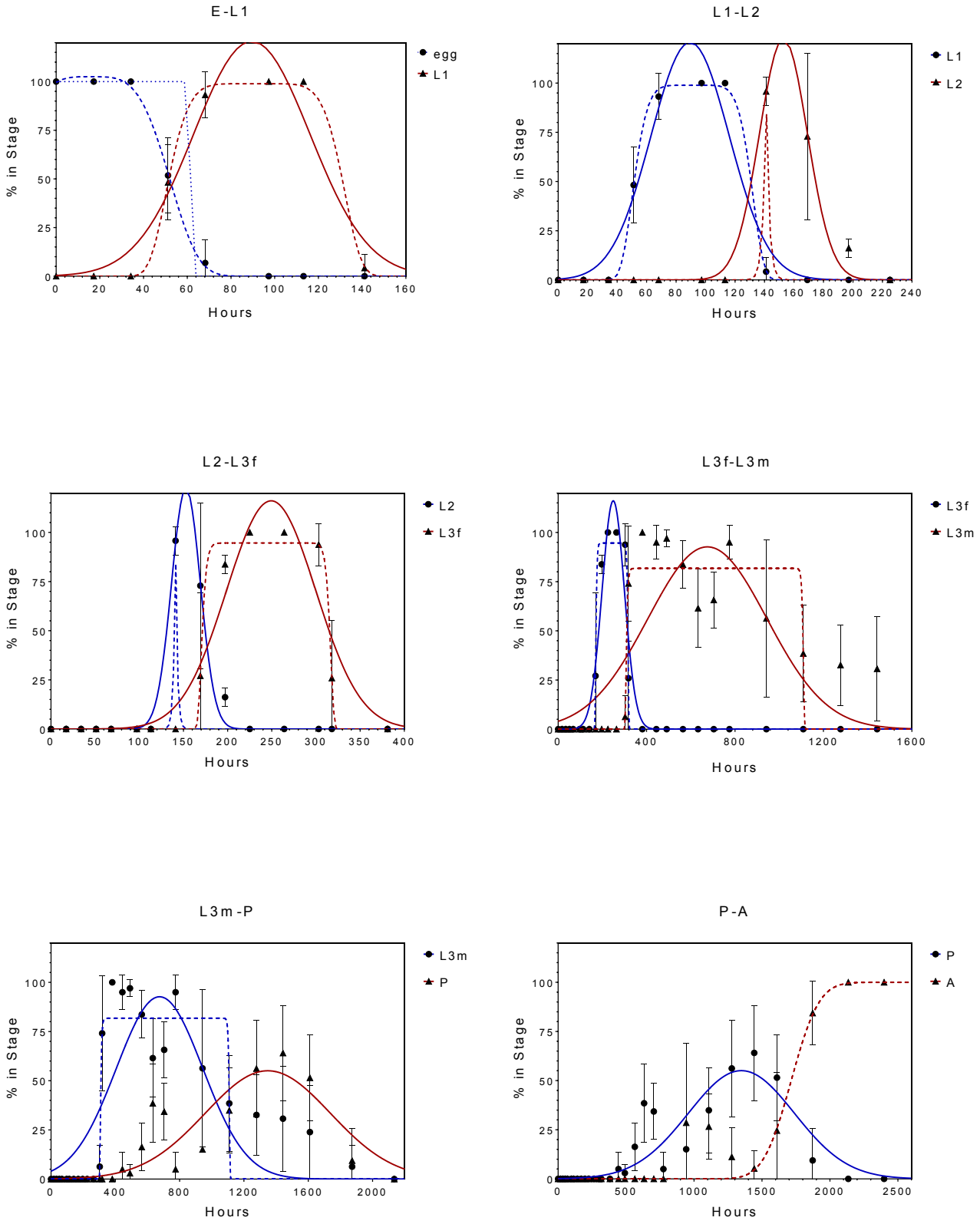
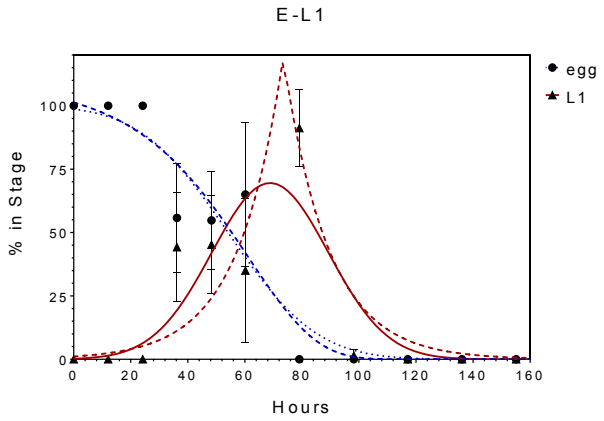
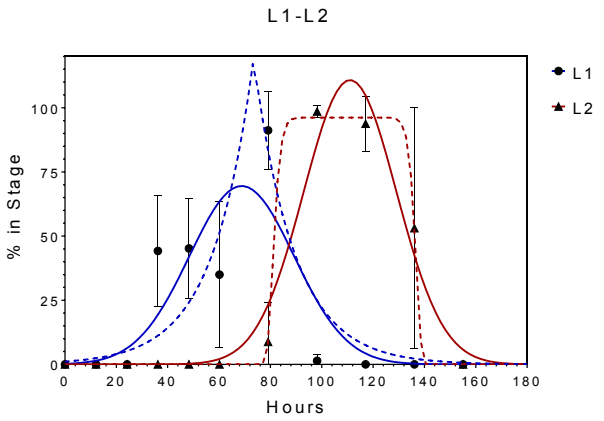
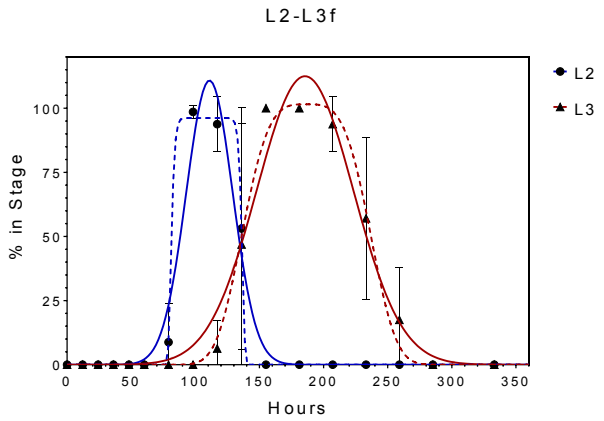
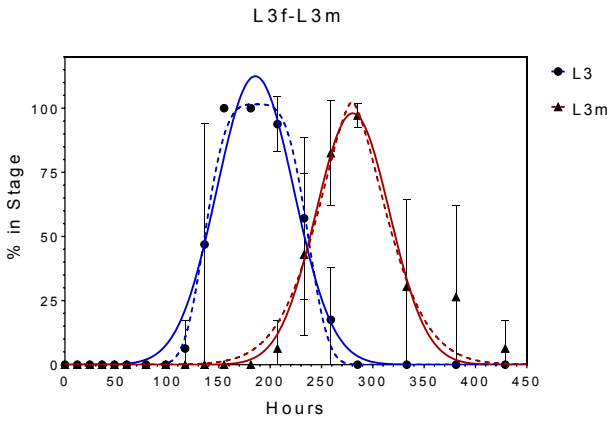


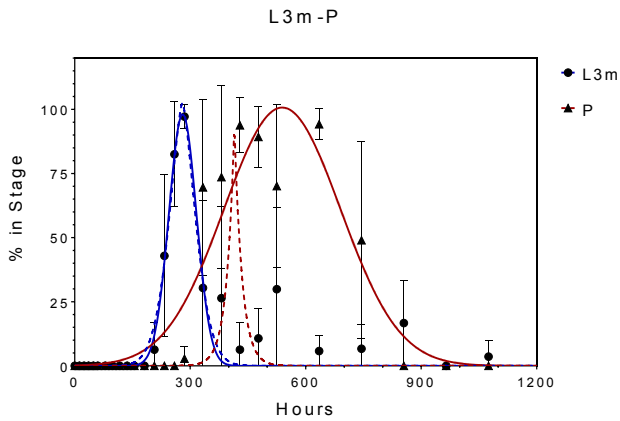
Fig. 4. Stage distributions *Lucilia sericata*, 17.5° C.











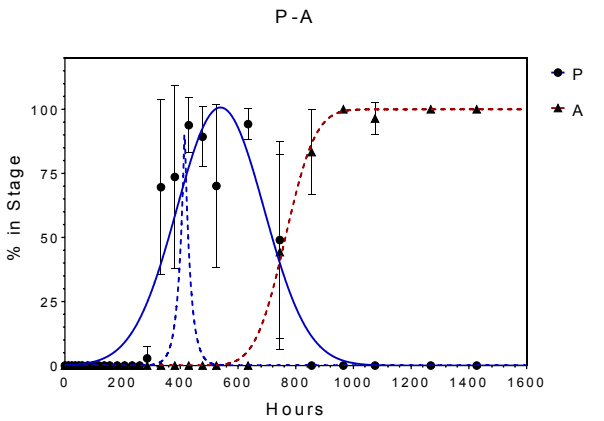


Fig. 5. Stage distributions *Lucilia sericata*, 20.0° C.

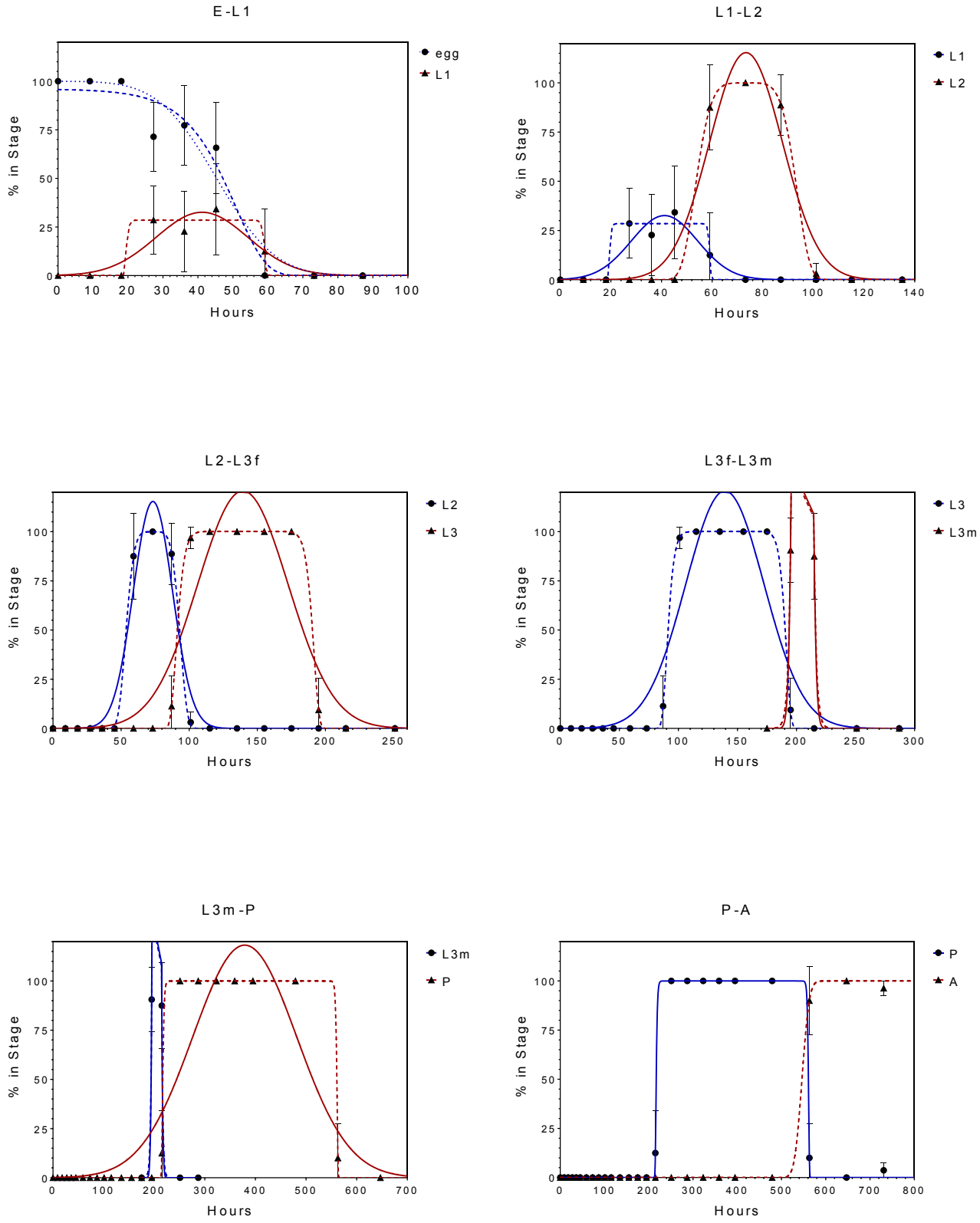


Fig. 6. Stage distributions *Lucilia sericata*, 22.5° C.

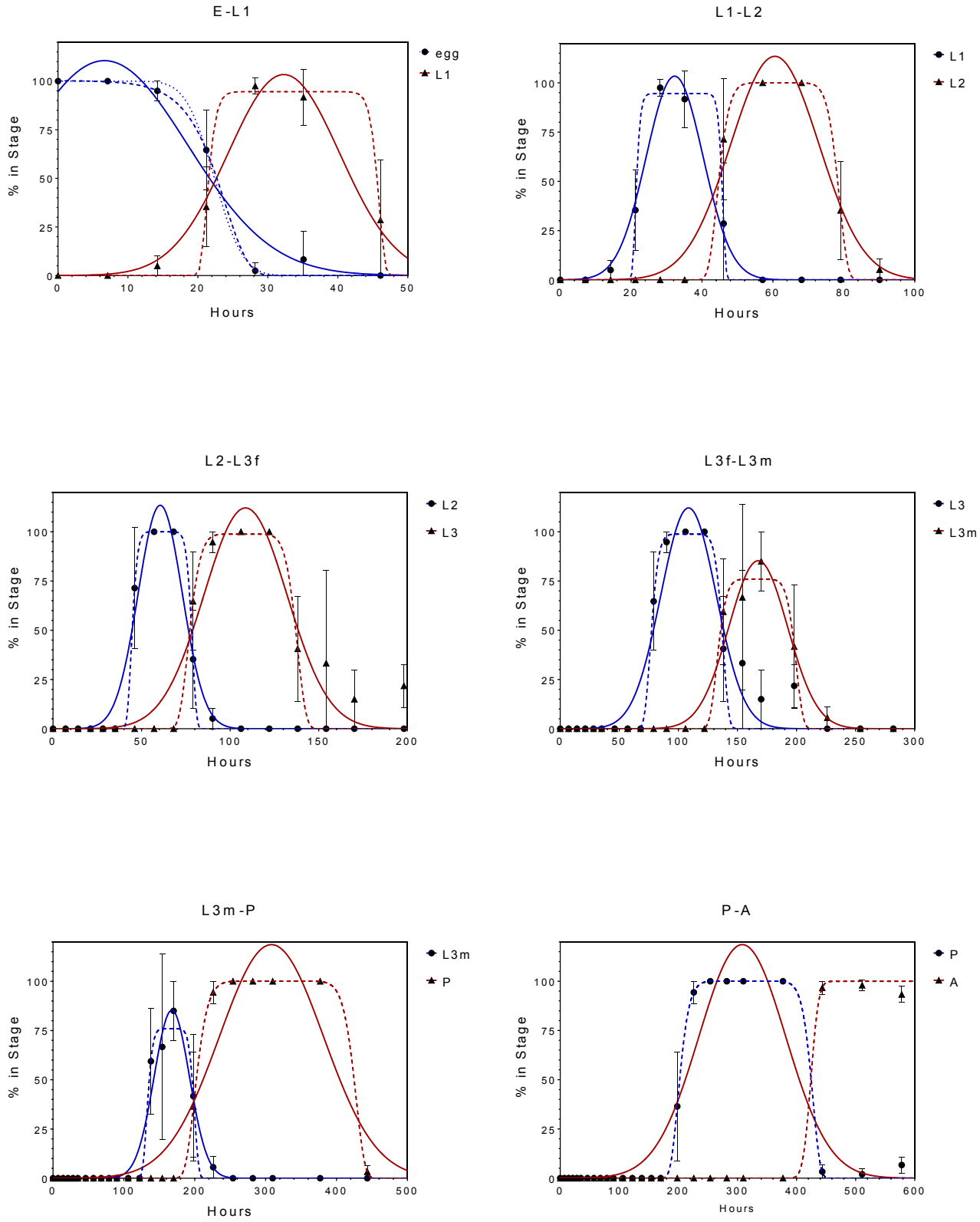


Fig. 7. Stage distributions *Lucilia sericata*, 25.0° C.

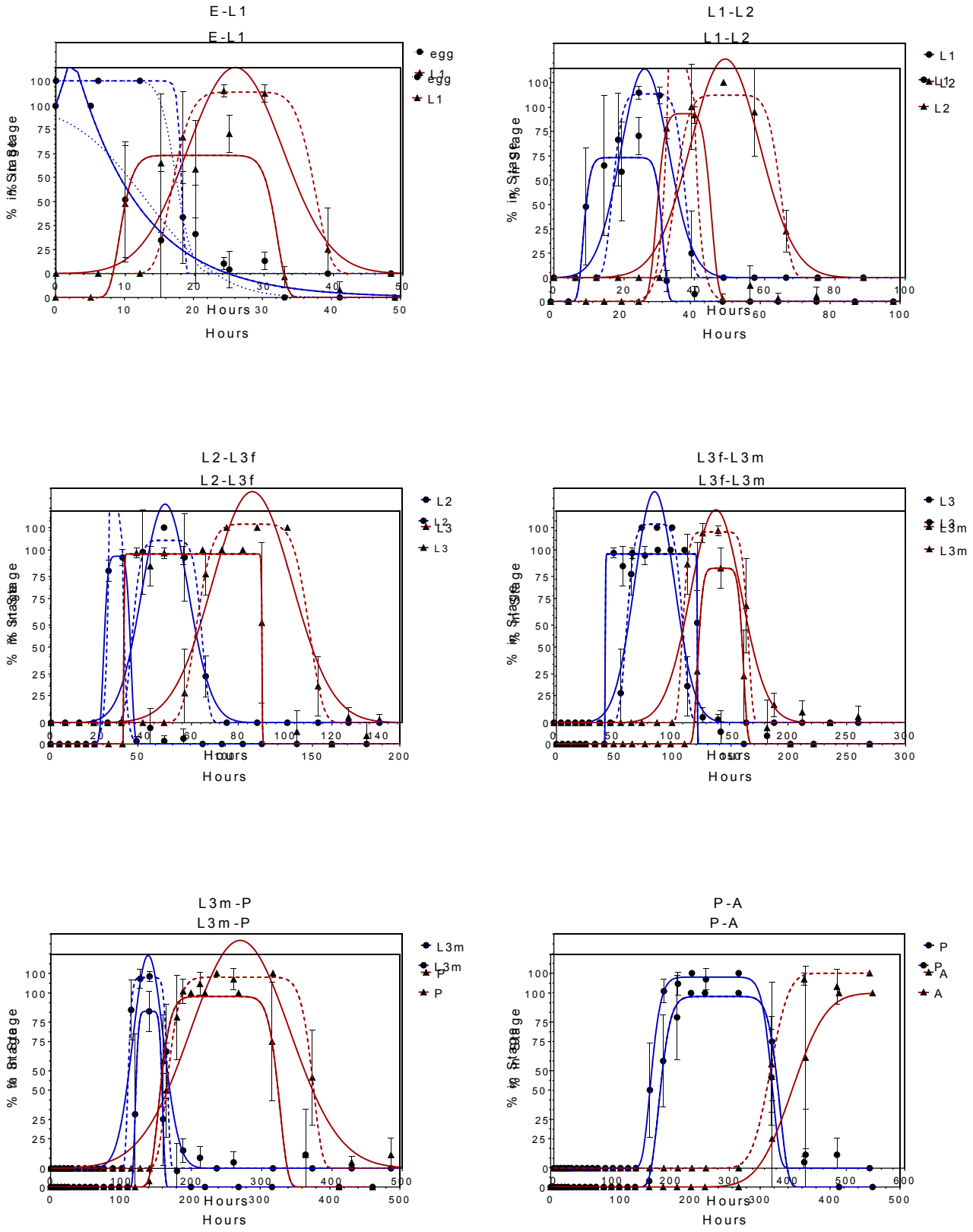


Fig. 8. Stage distributions *Lucilia sericata*, 27.5° C.

Fig. 9. Stage distributions *Lucilia sericata*, 30.0° C.

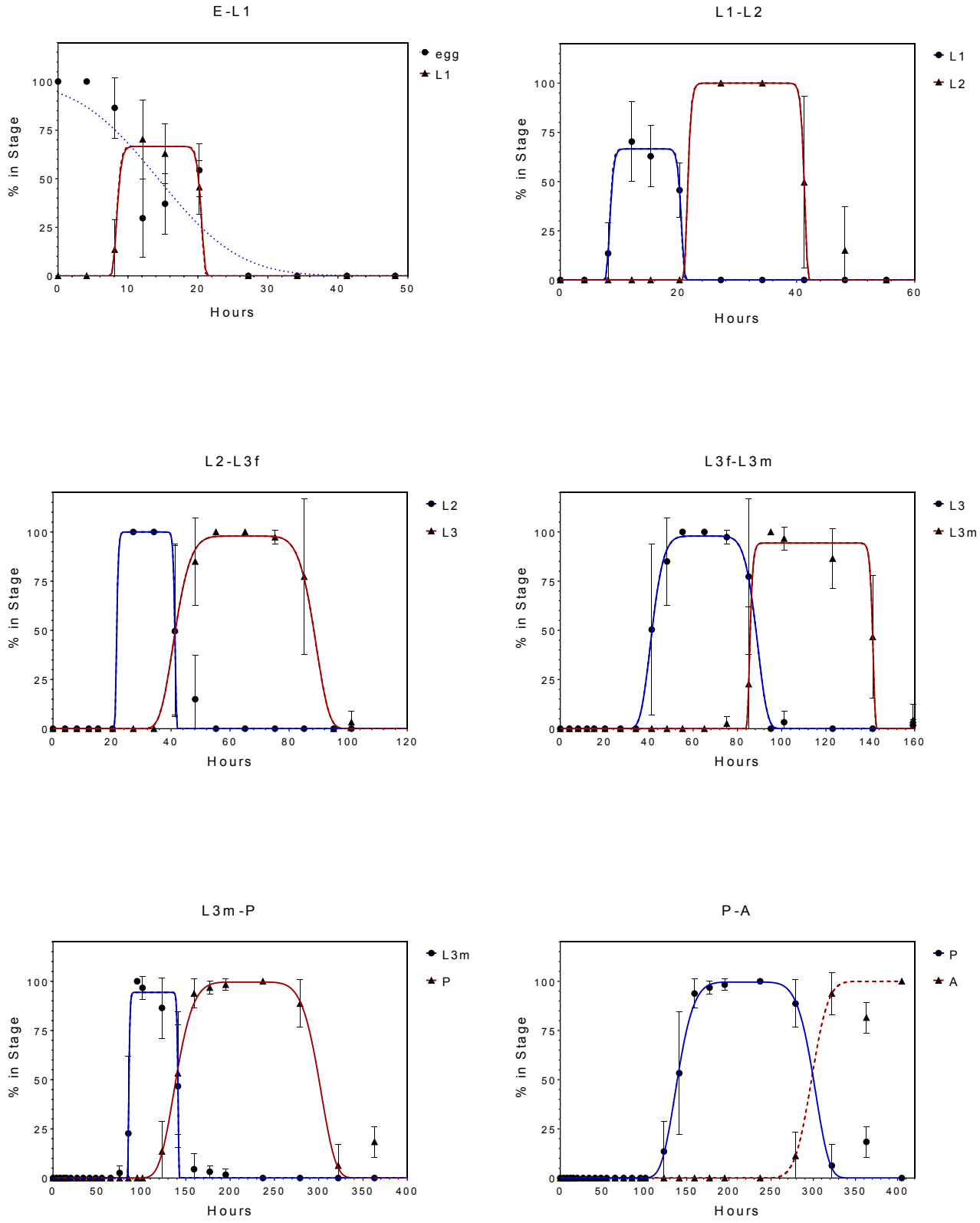


Fig. 10. Stage distributions *Lucilia sericata*, 32.5° C.

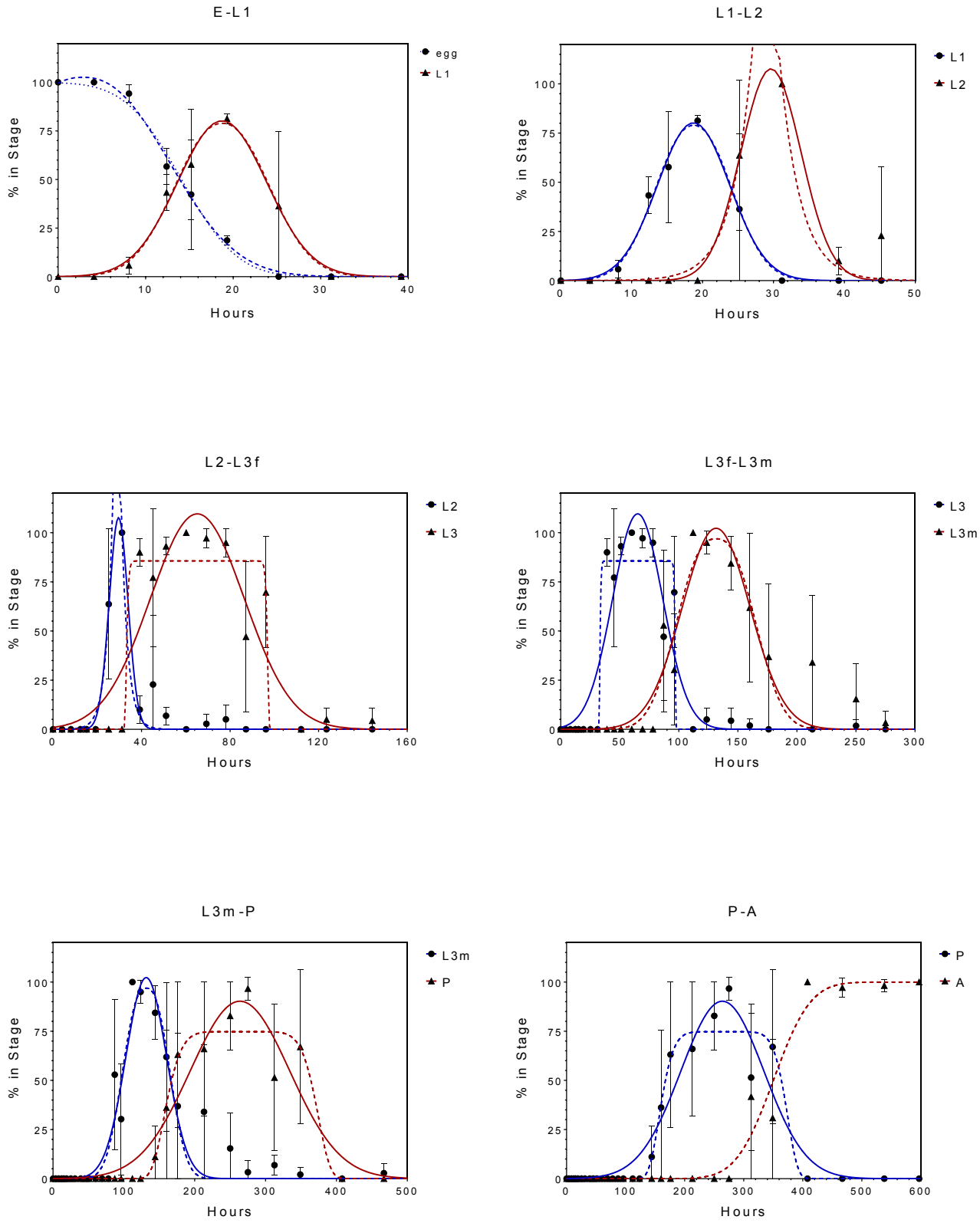


Fig. 11. *Lucilia sericata* stage distribution from egg to adult, 10.0° C.

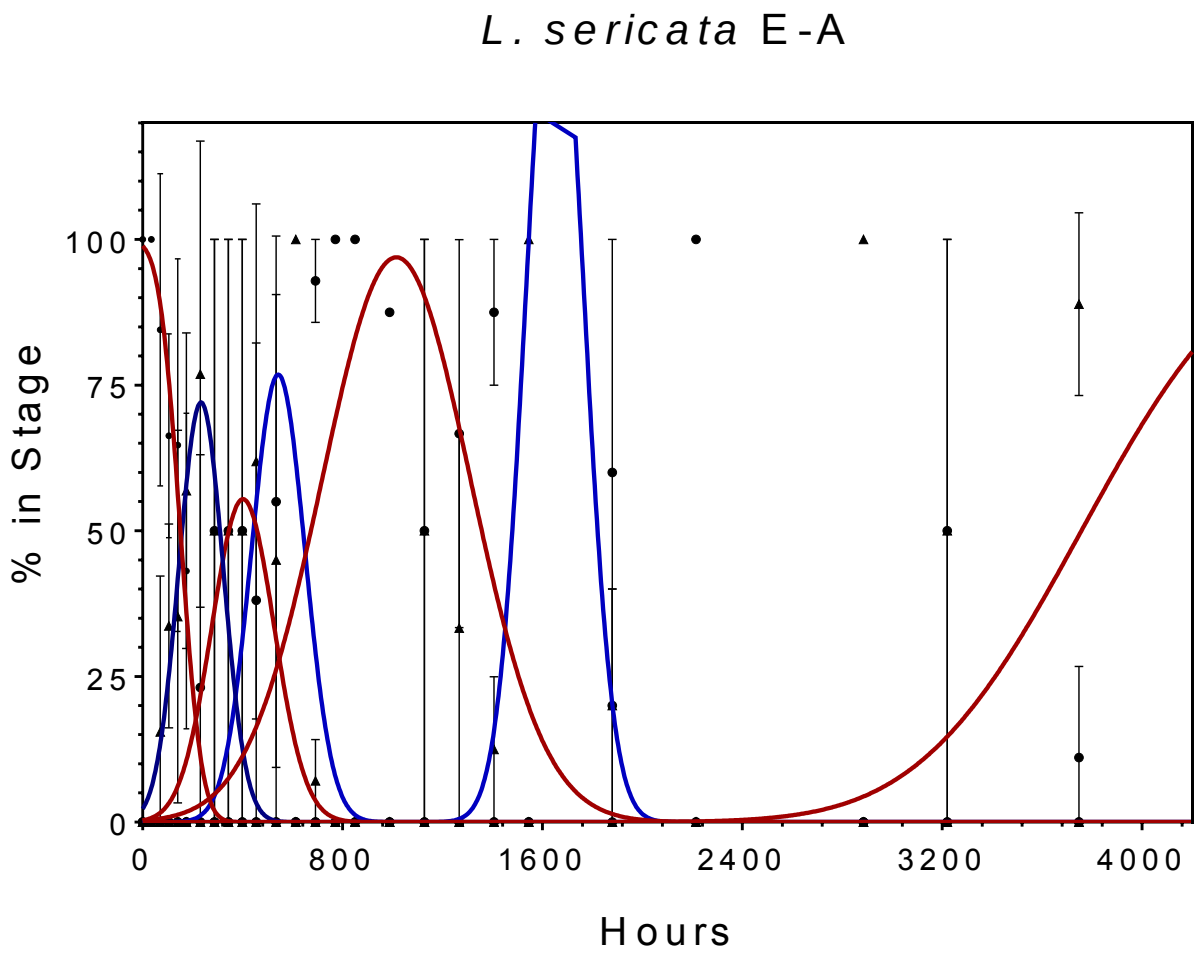


Fig. 12. *Lucilia sericata* stage distribution from egg to adult, 12.5° C.

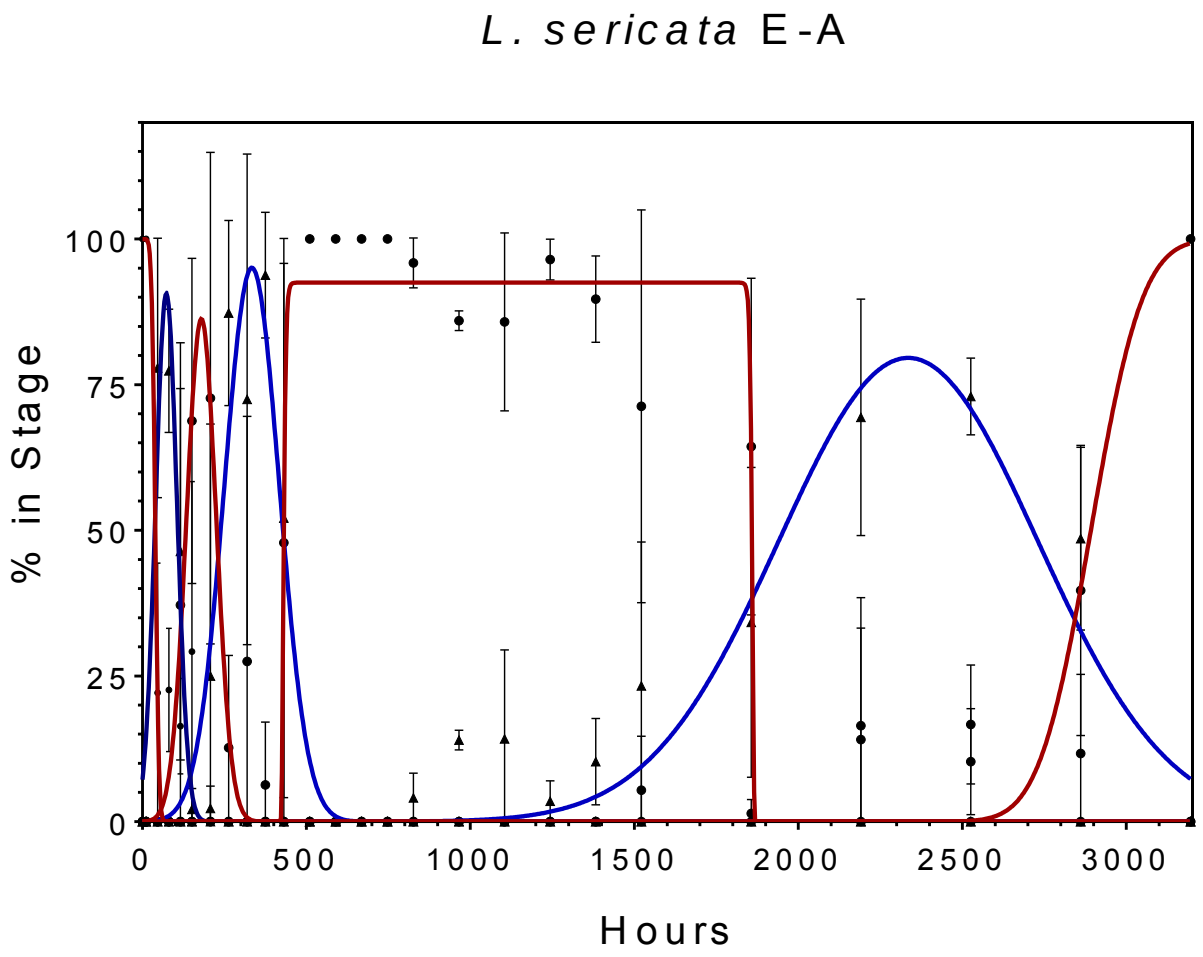


Fig. 13. *Lucilia sericata* stage distribution from egg to adult, 15.0° C.

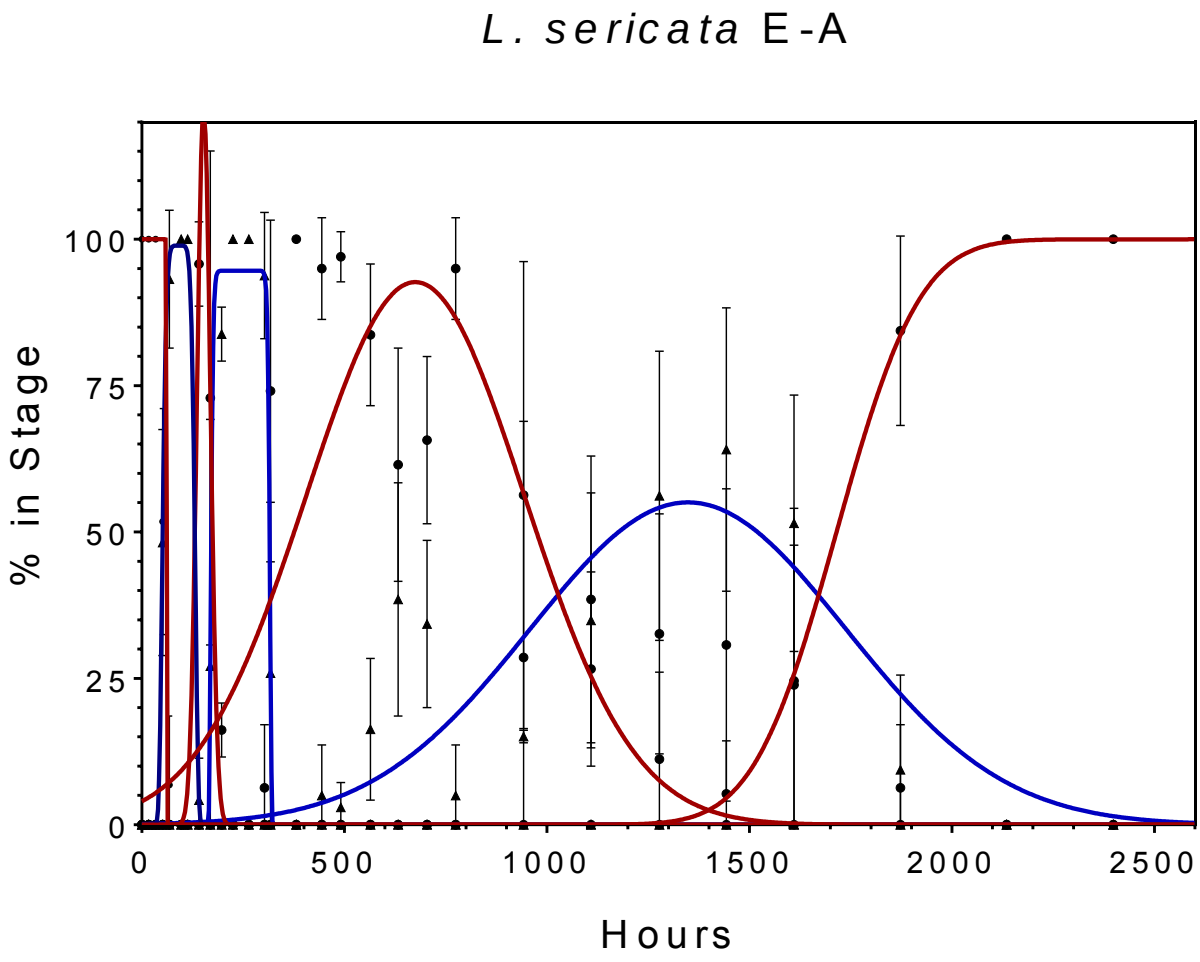


Fig. 14. *Lucilia sericata* stage distribution from egg to adult, 17.5° C.

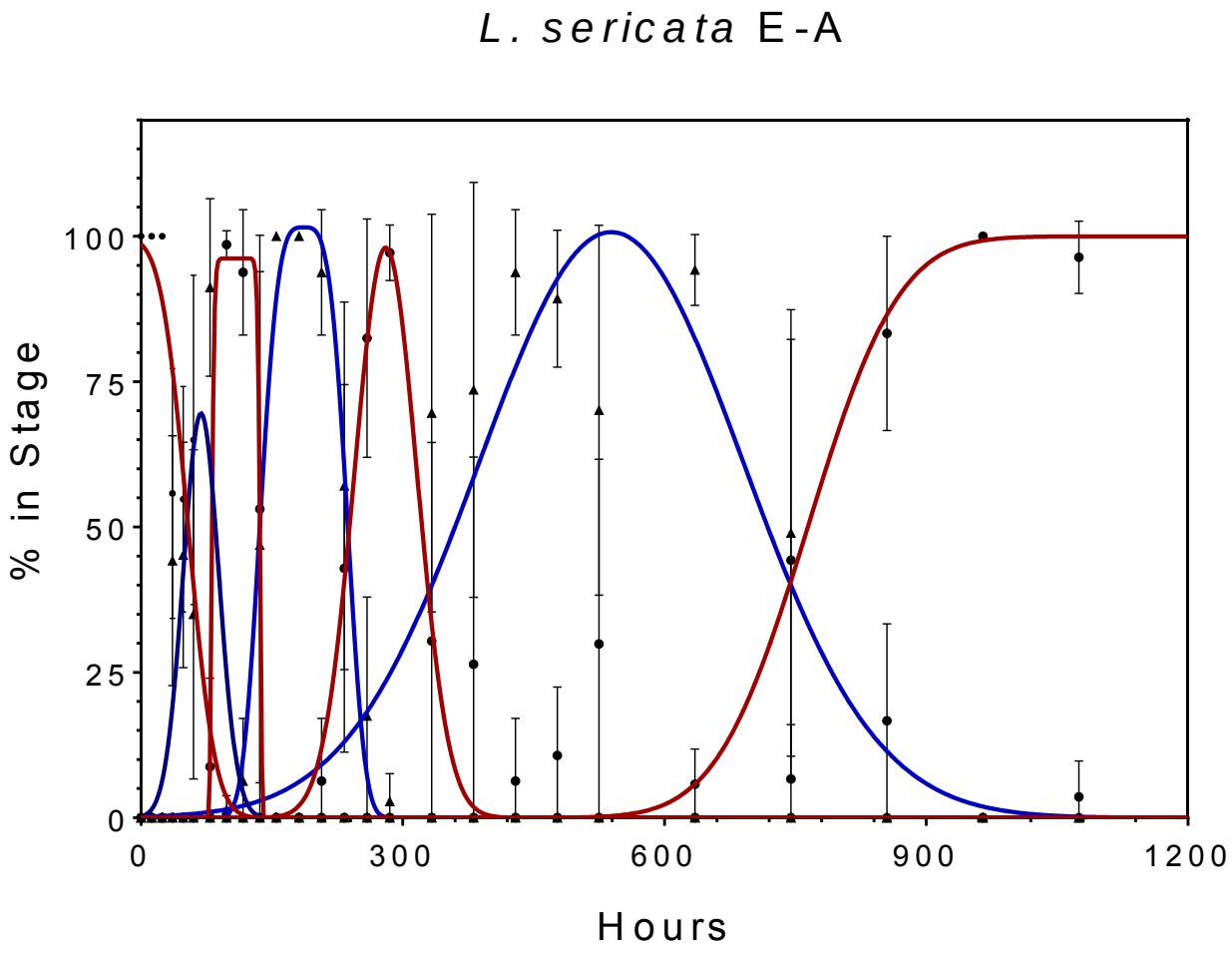


Fig. 15. *Lucilia sericata* stage distribution from egg to adult, 20.0° C.

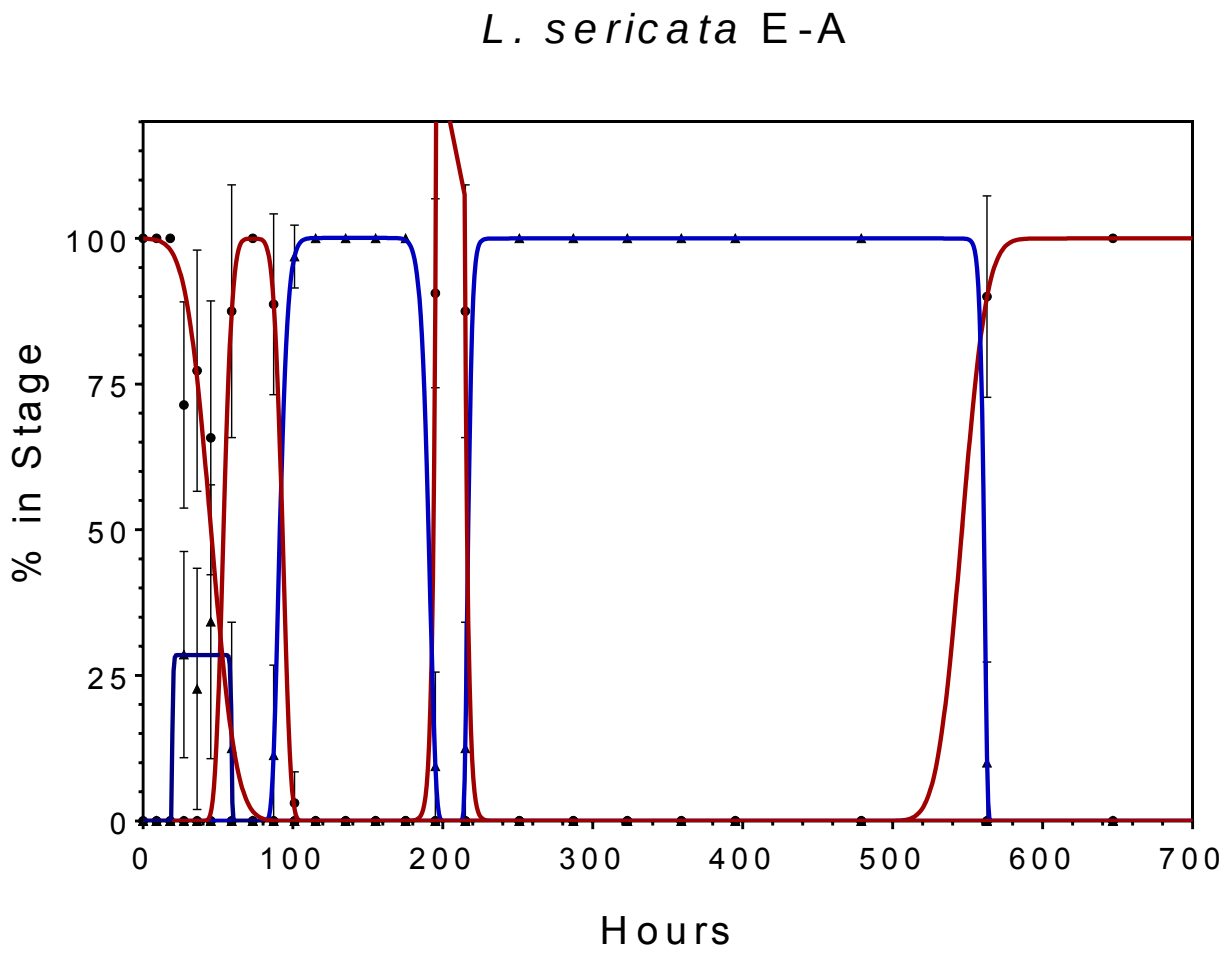


Fig. 16. *Lucilia sericata* stage distribution from egg to adult, 22.5° C.

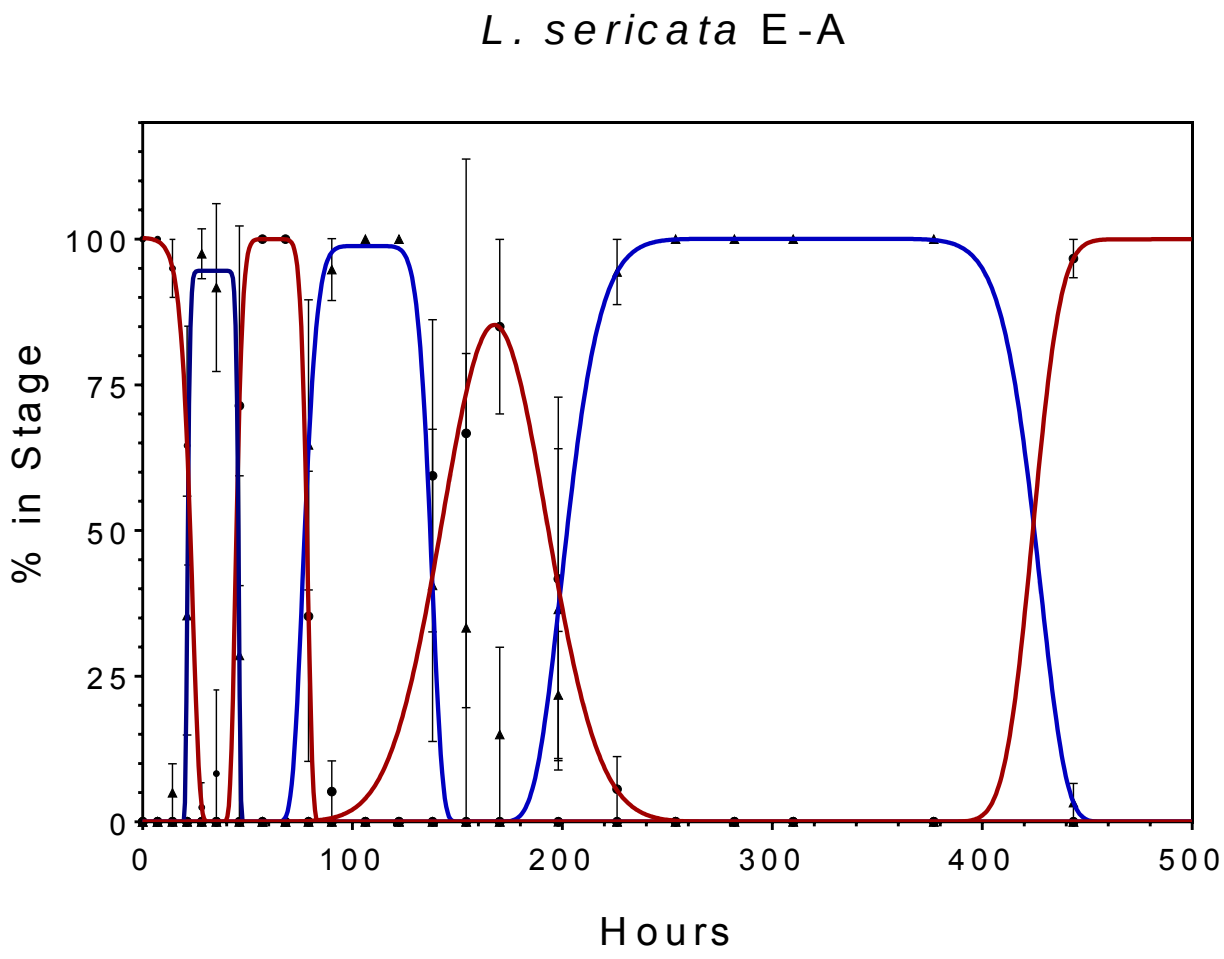


Fig. 17. *Lucilia sericata* stage distribution from egg to adult, 25.0° C.

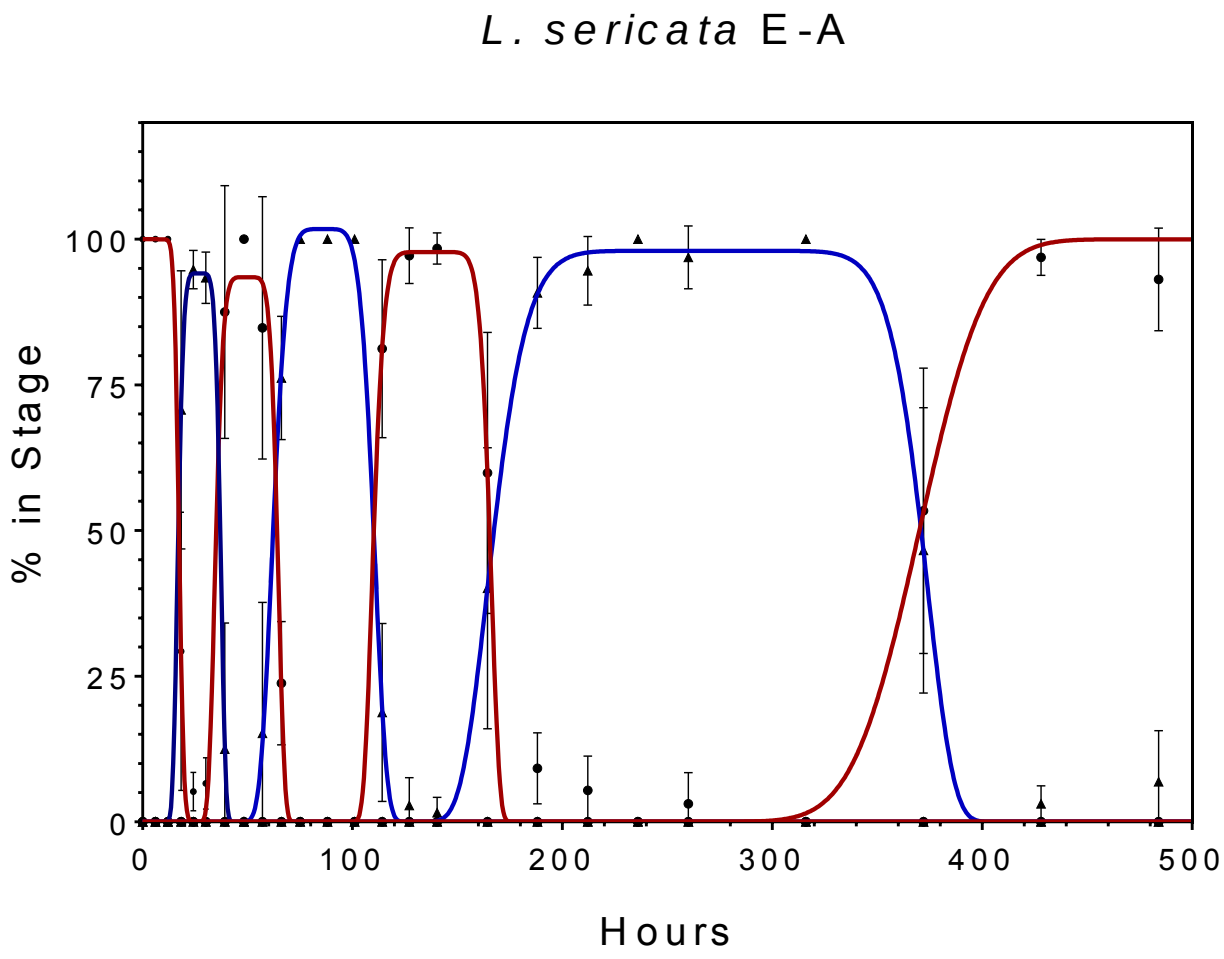


Fig. 18. *Lucilia sericata* stage distribution from egg to adult, 27. 5° C.

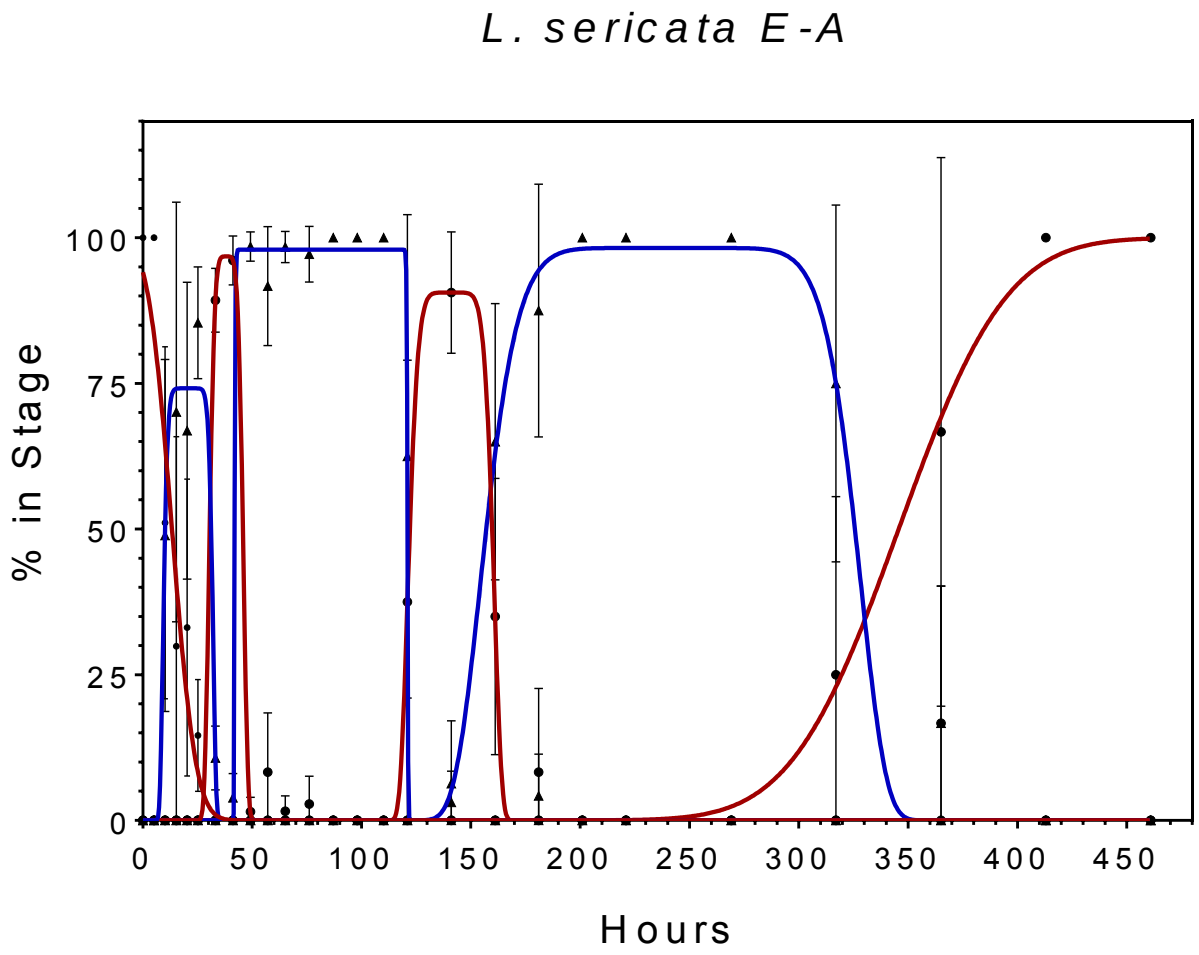


Fig. 19. *Lucilia sericata* stage distribution from egg to adult, 30.0° C.

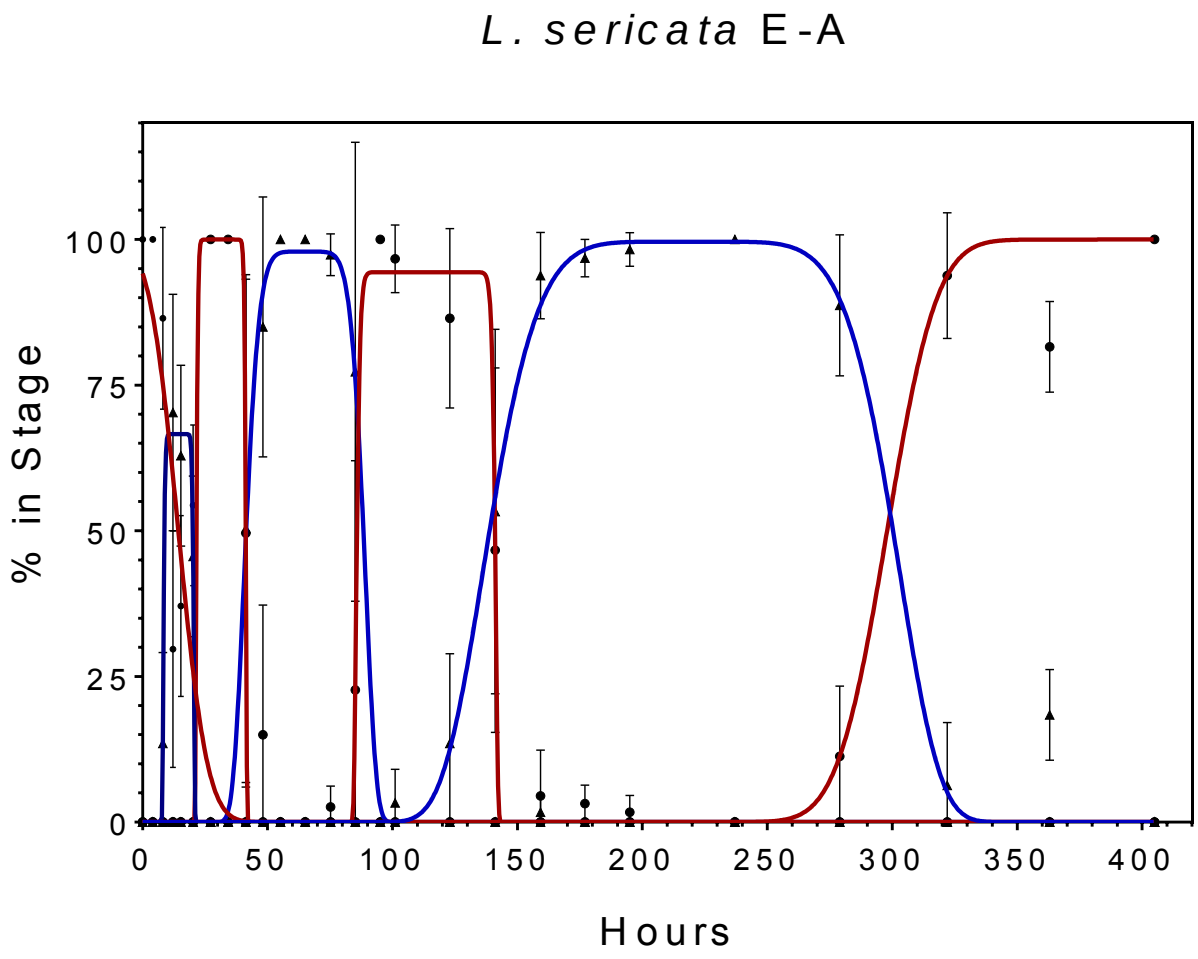


Fig. 20. *Lucilia sericata* stage distribution from egg to adult, 32.5° C.

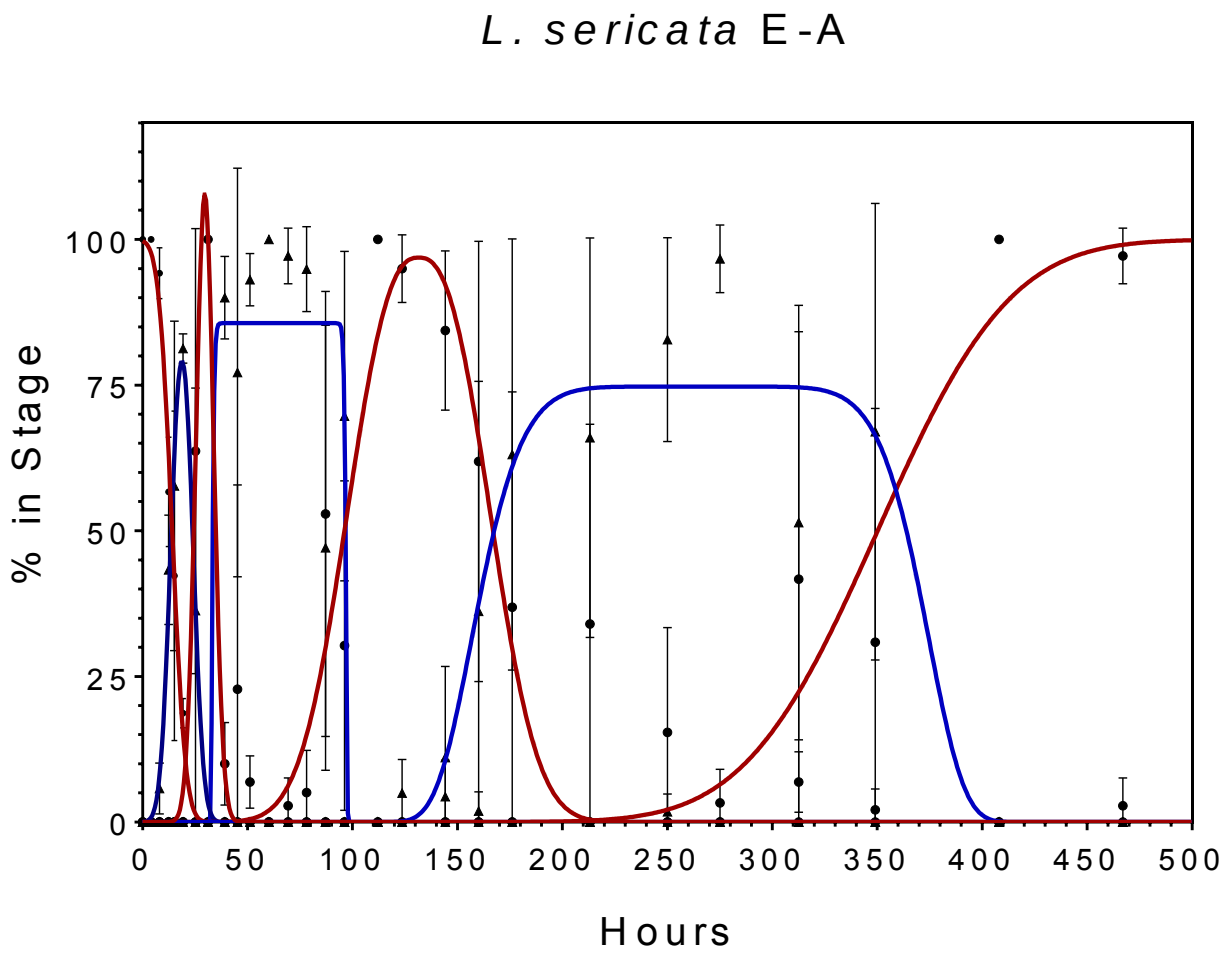


Fig. 21. *Lucilia sericata* stage transition fit to dose response curve, 10.0° C.

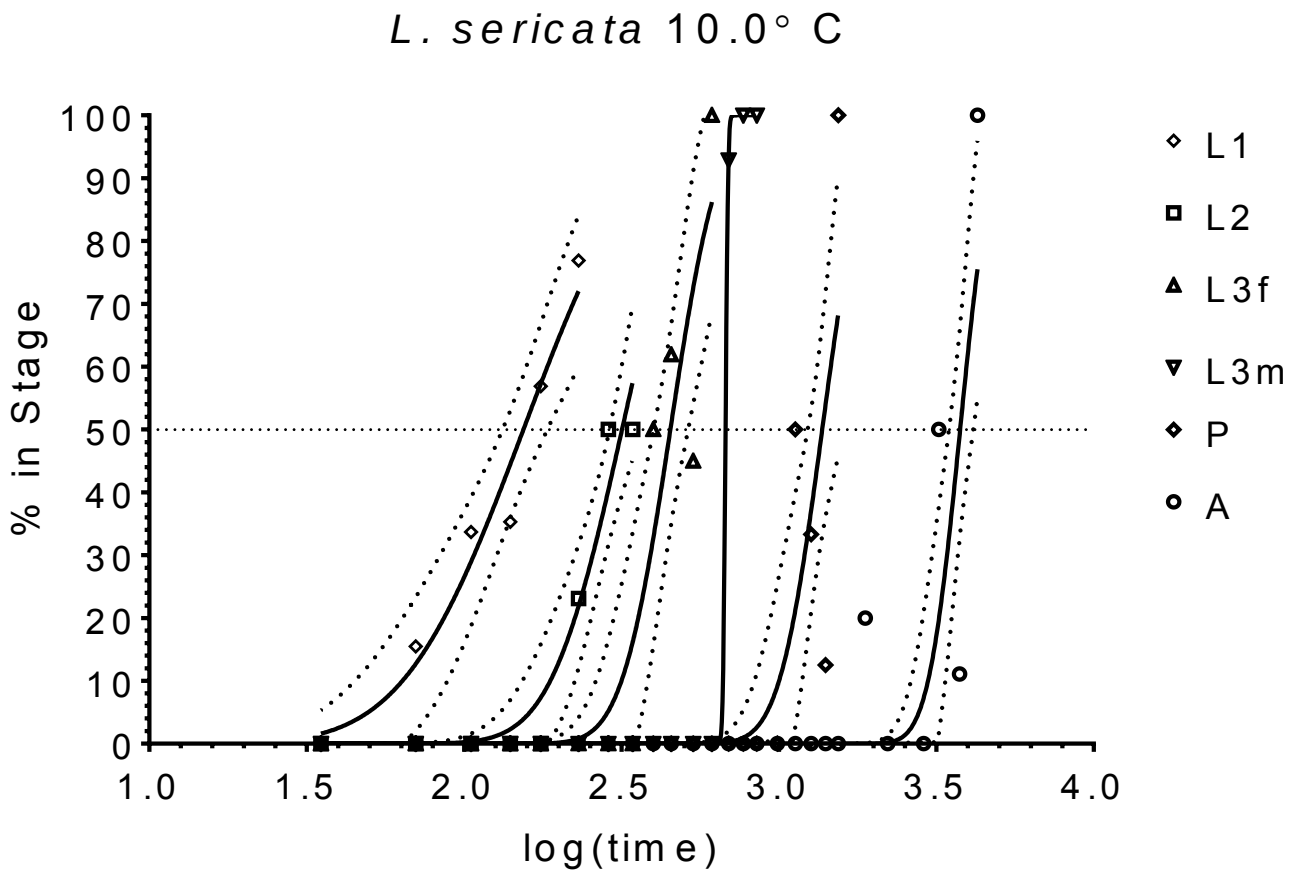


Fig. 22. *Lucilia sericata* stage transition fit to dose response curve, 12.5° C.

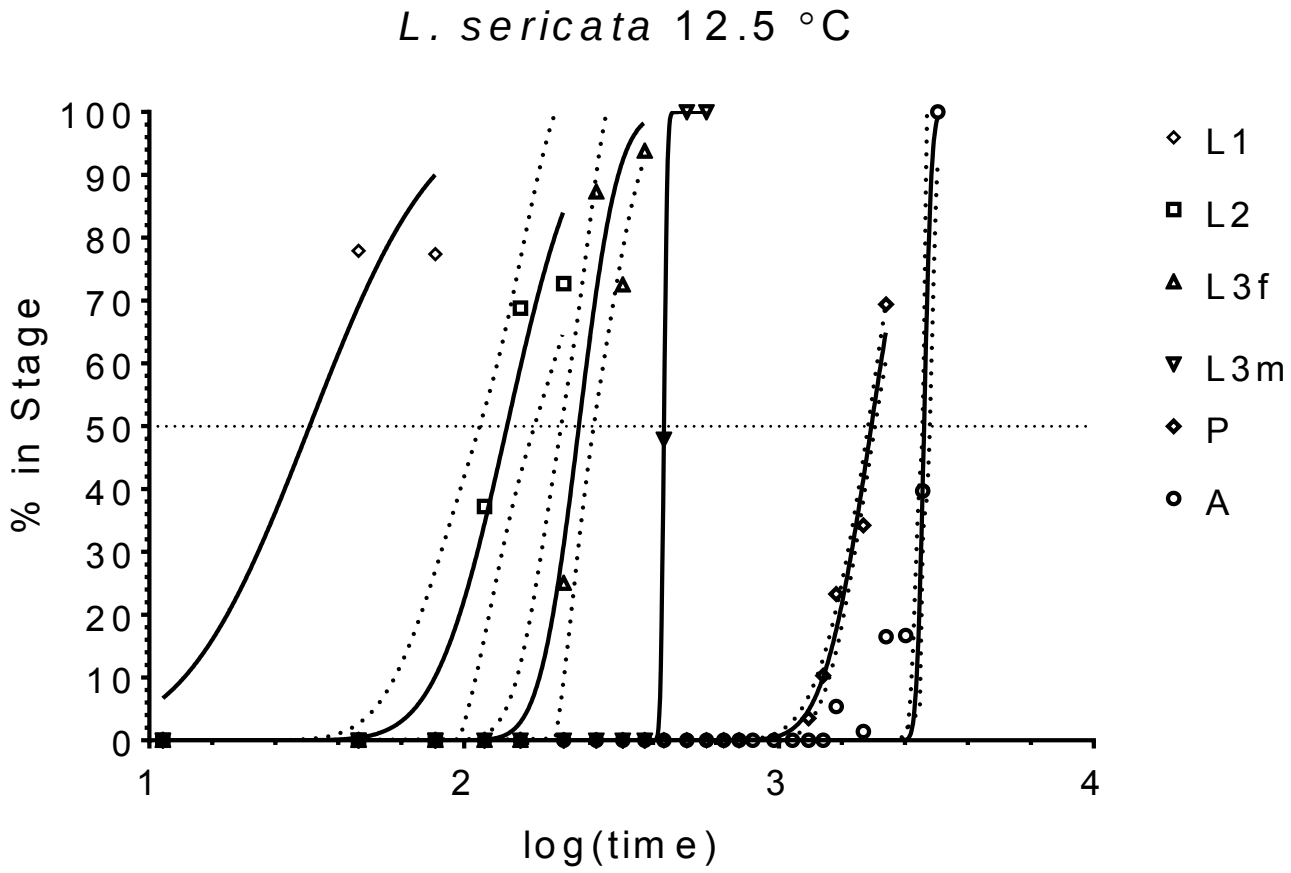


Fig. 23. *Lucilia sericata* stage transition fit to dose response curve, 15.0° C.

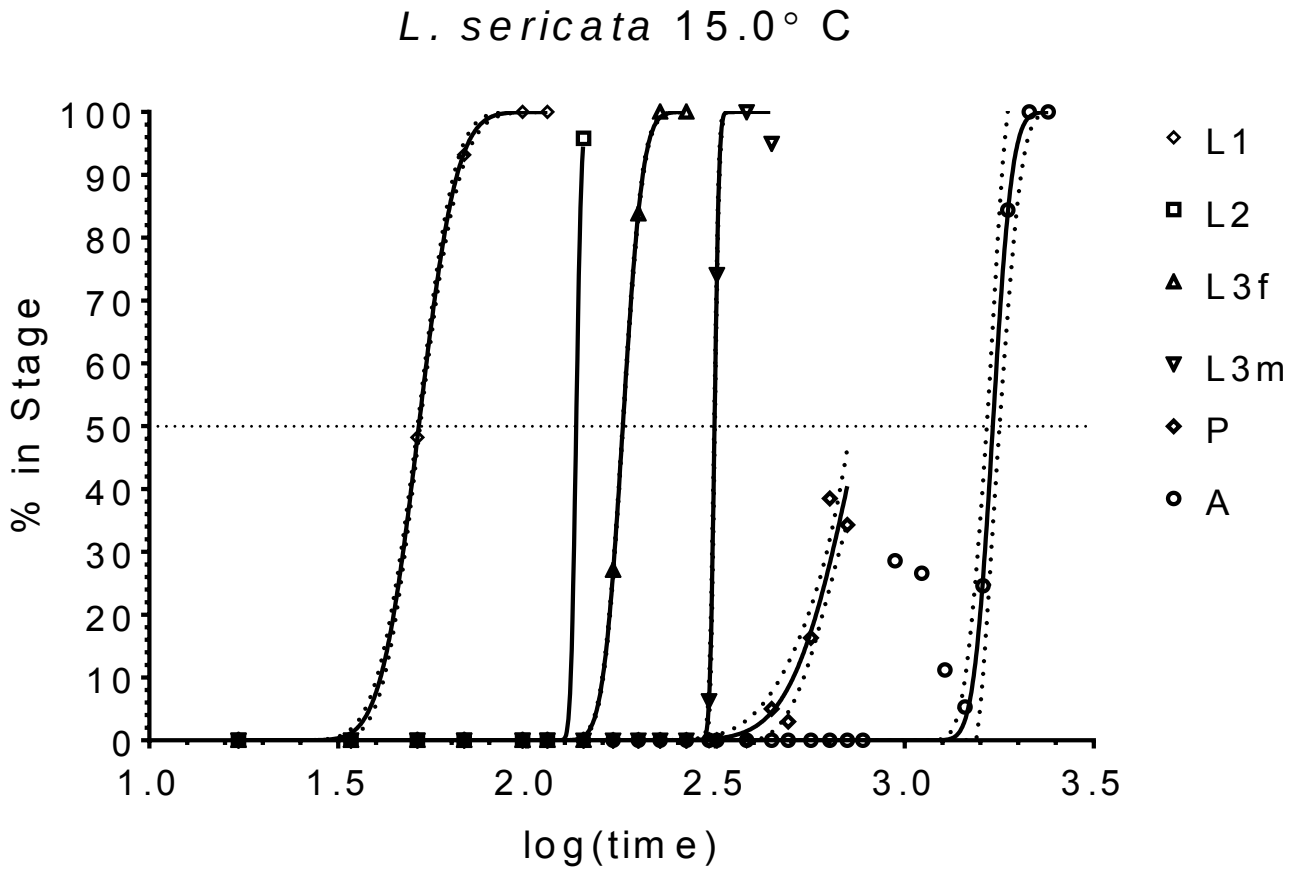


Fig. 24. *Lucilia sericata* stage transition fit to dose response curve, 17.5° C.

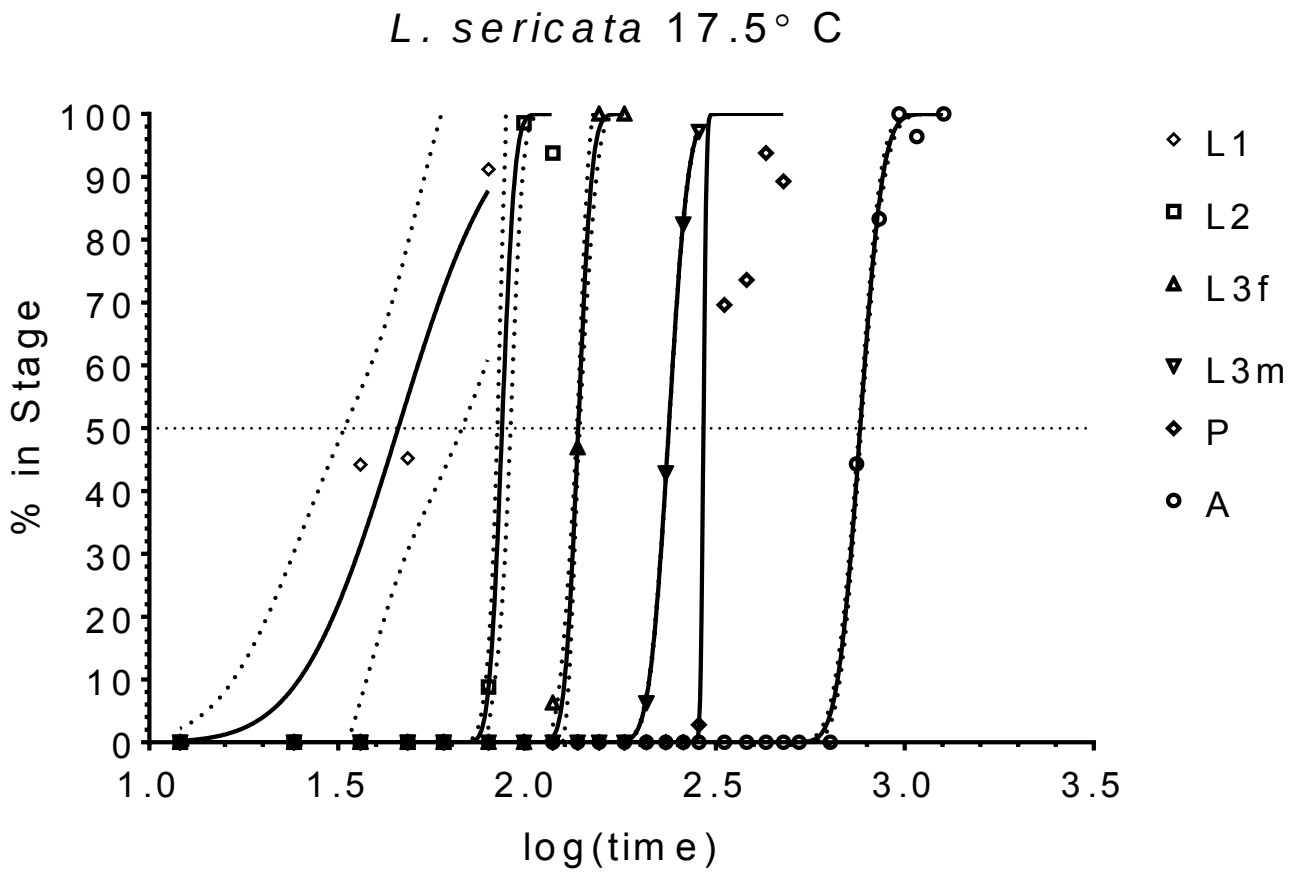


Fig. 25. *Lucilia sericata* stage transition fit to dose response curve, 20.0° C.

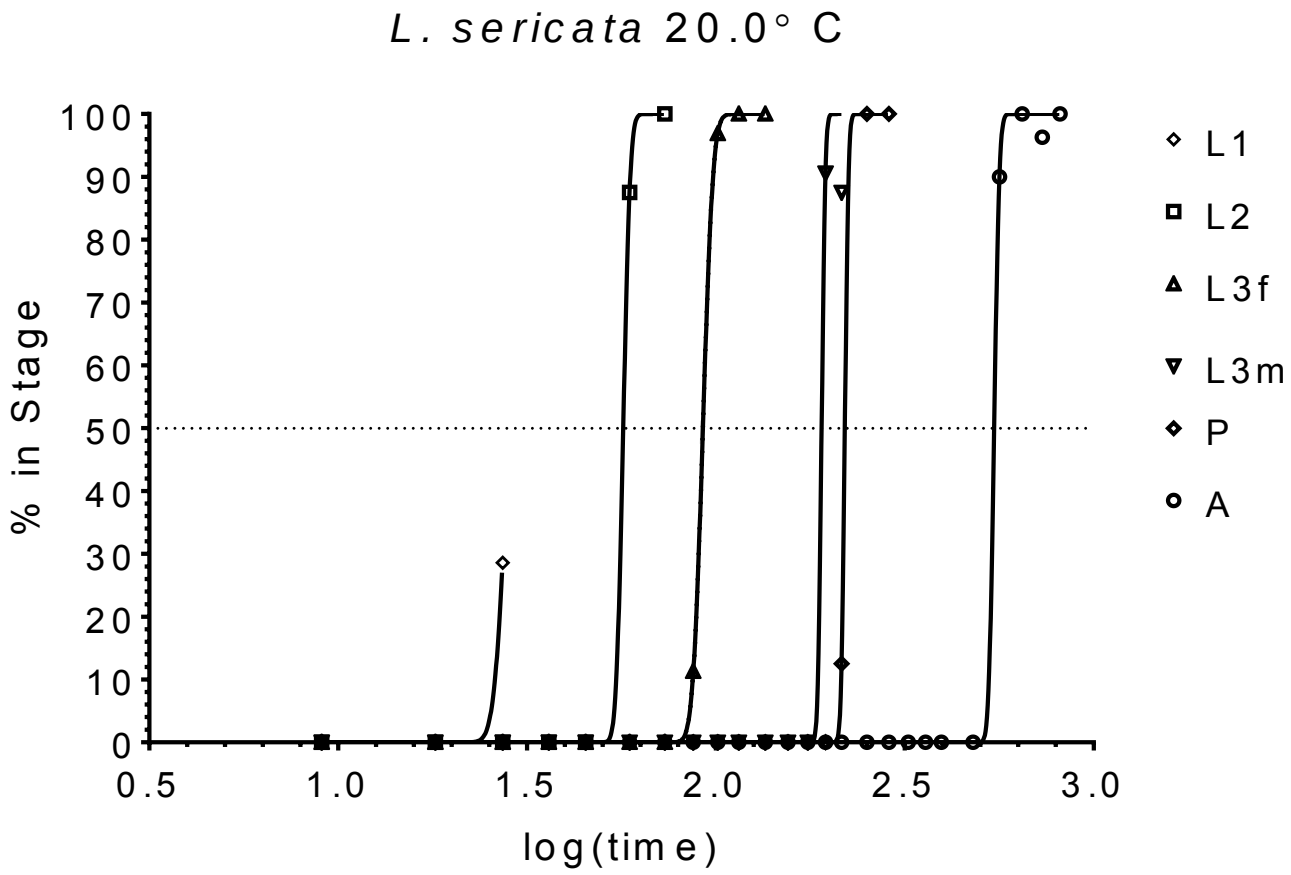


Fig. 26. *Lucilia sericata* stage transition fit to dose response curve, 22.5° C.

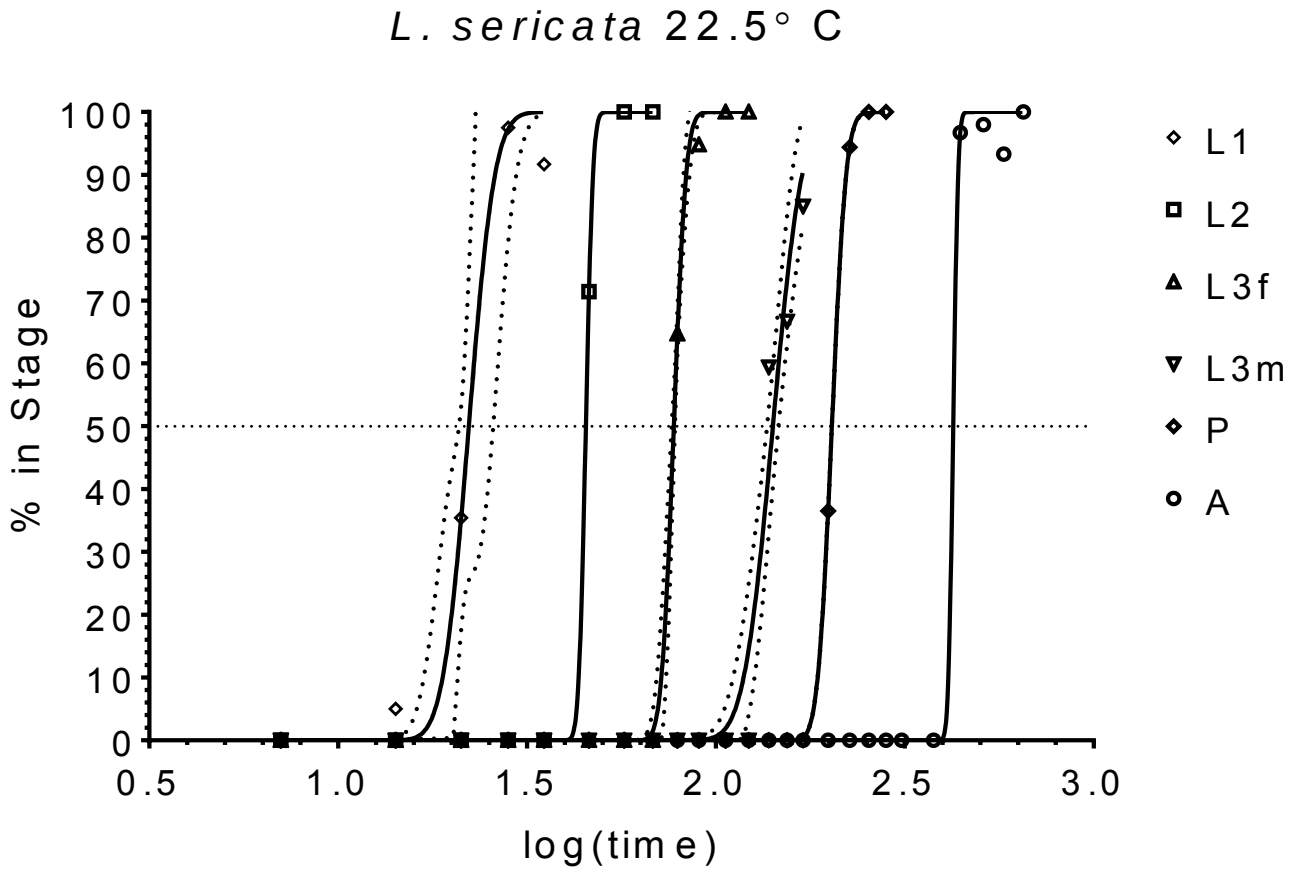


Fig. 27. *Lucilia sericata* stage transition fit to dose response curve, 25.0° C.

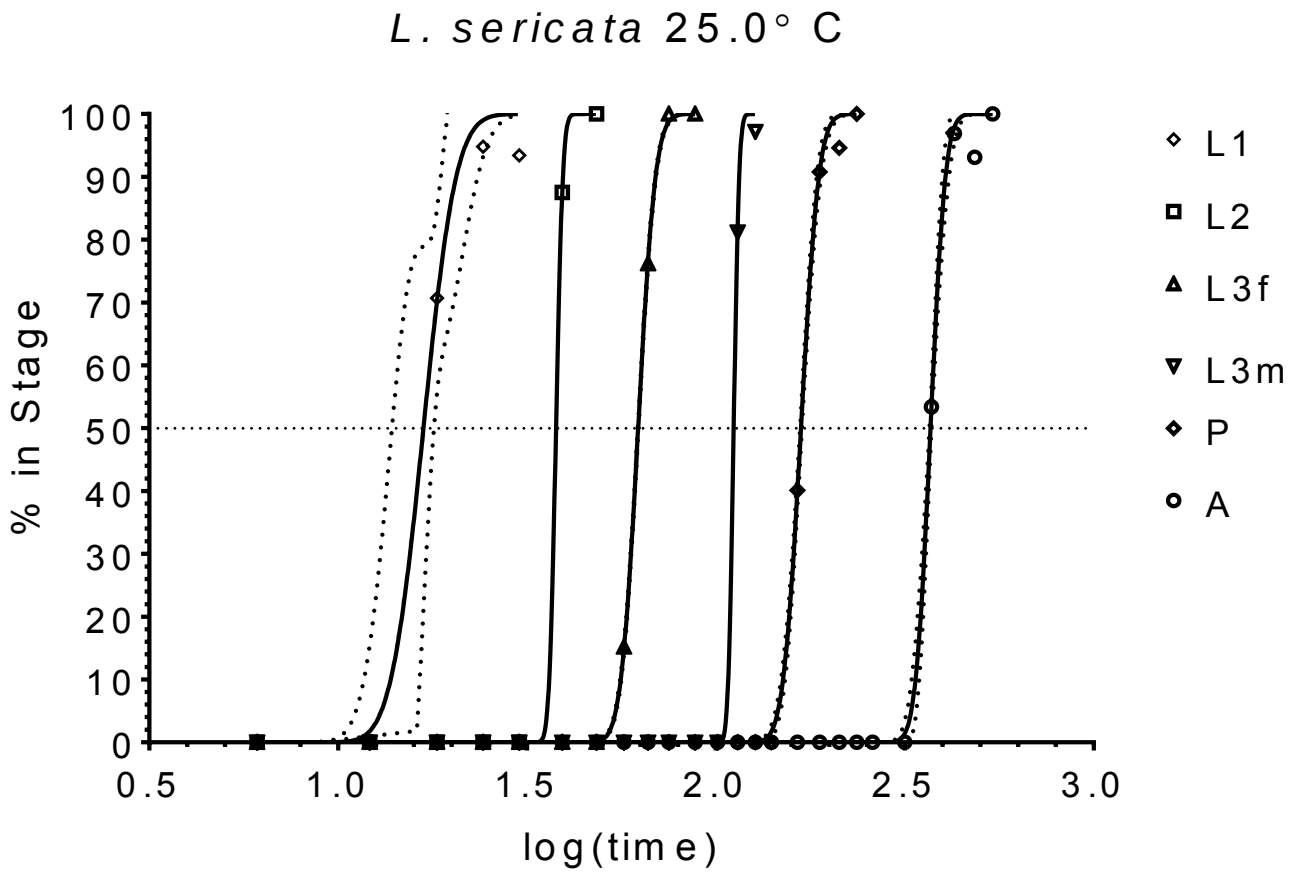


Fig. 28. *Lucilia sericata* stage transition fit to dose response curve, 27.5° C.

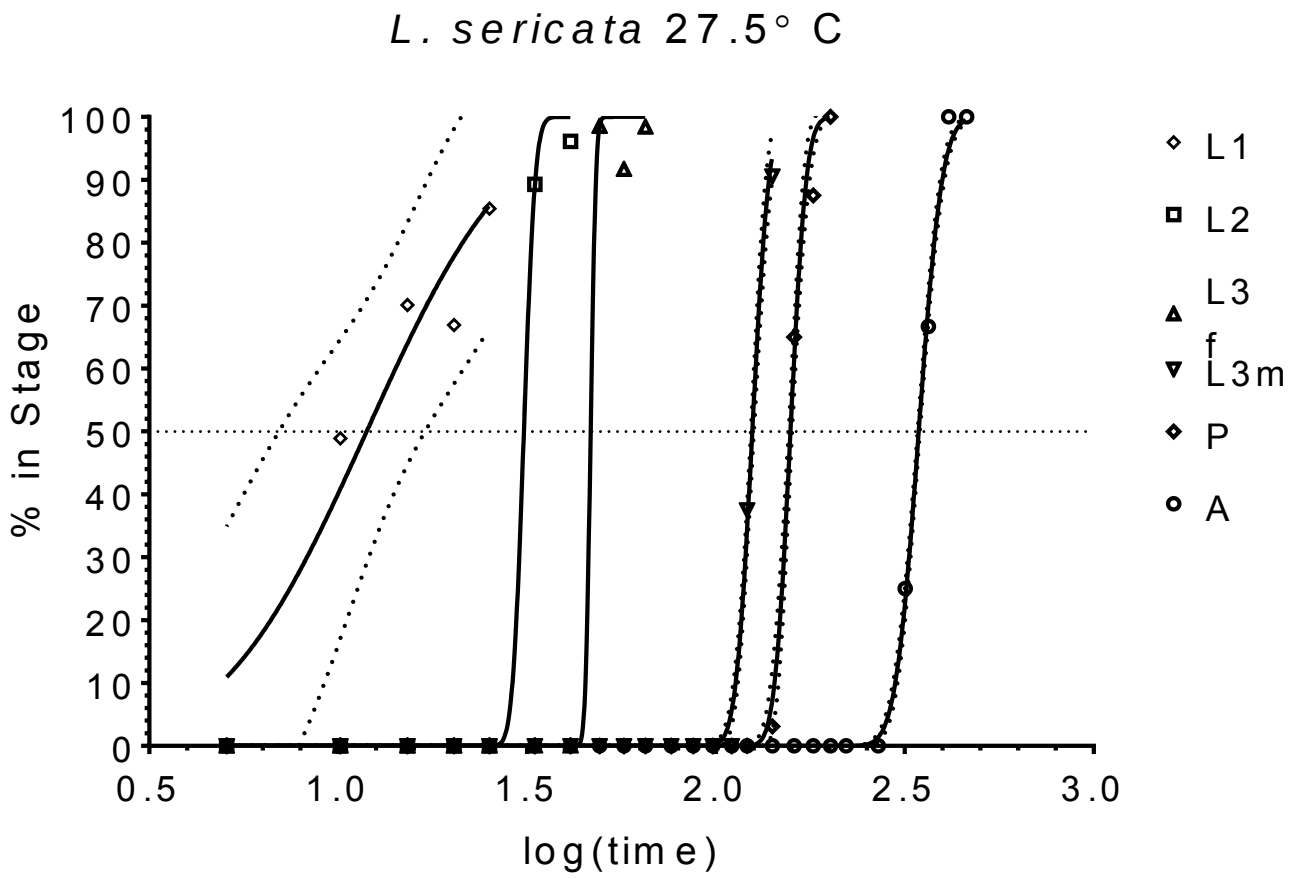


Fig. 29. *Lucilia sericata* stage transition fit to dose response curve, 30.0° C.

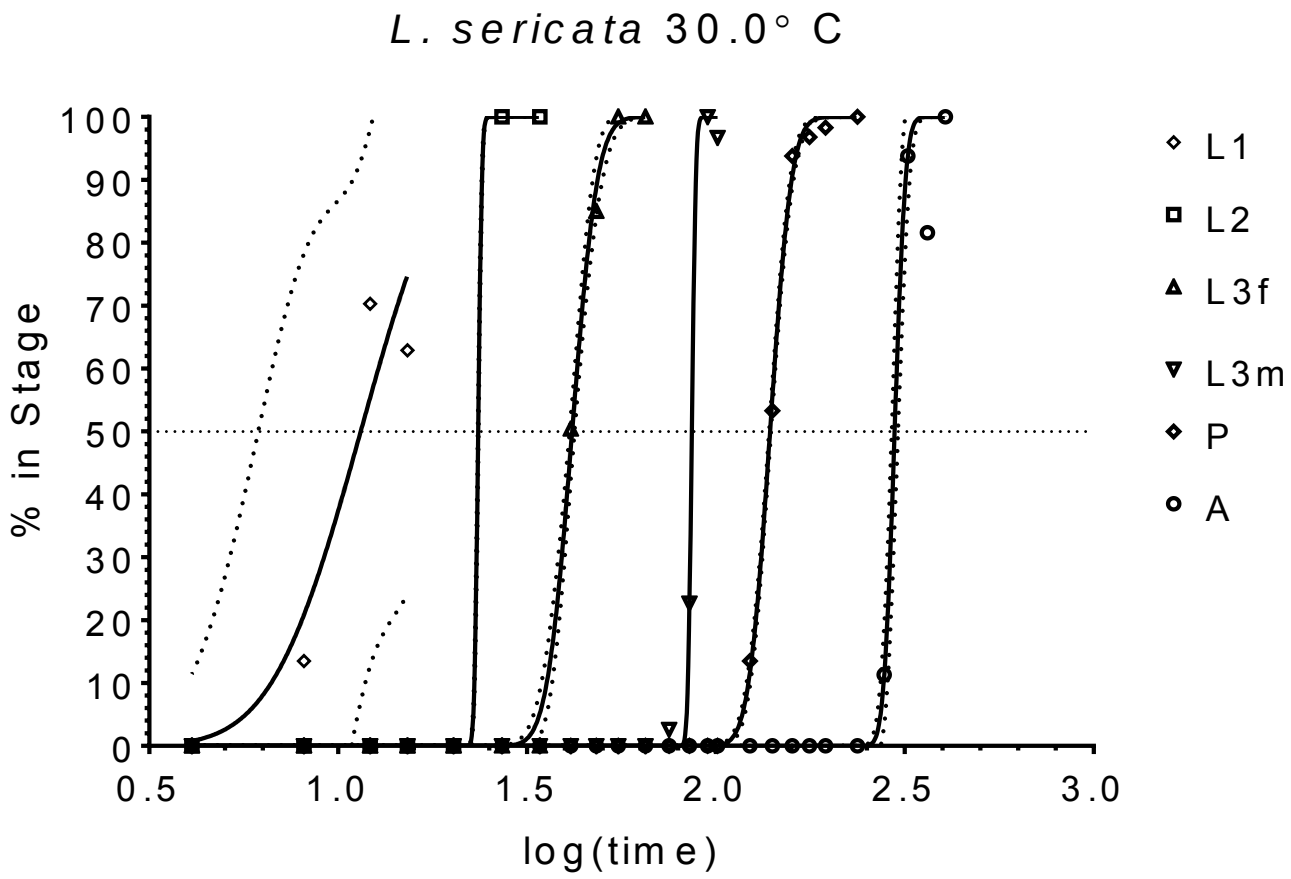


Fig. 30. *Lucilia sericata* stage transition fit to dose response curve, 32.5° C.

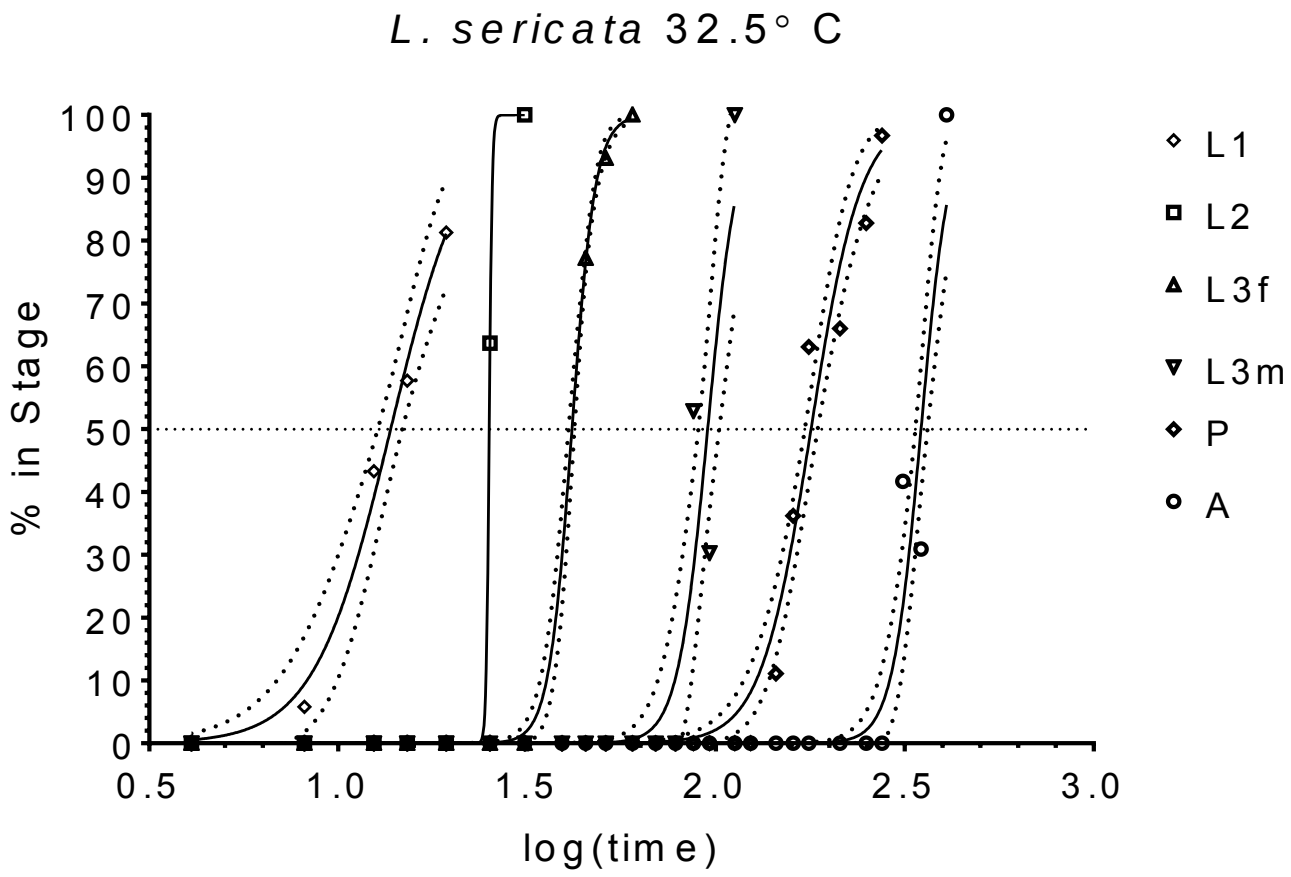


Fig. 31. *Lucilia sericata* development rates (as 1/days) by stage (as estimated by linear regressions).

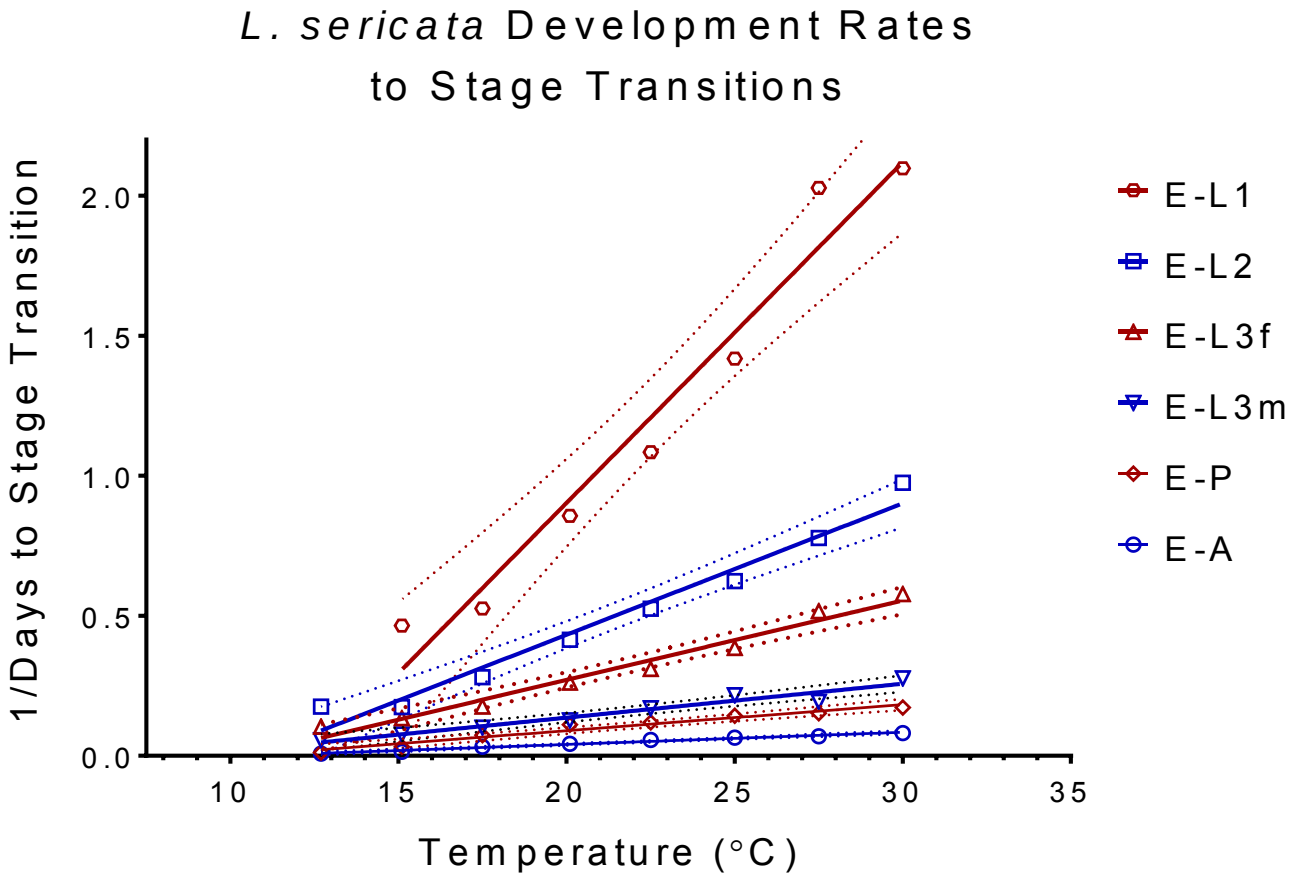


Fig. 32. *Lucilia sericata* development rate to stage (as estimated by linear regressions).

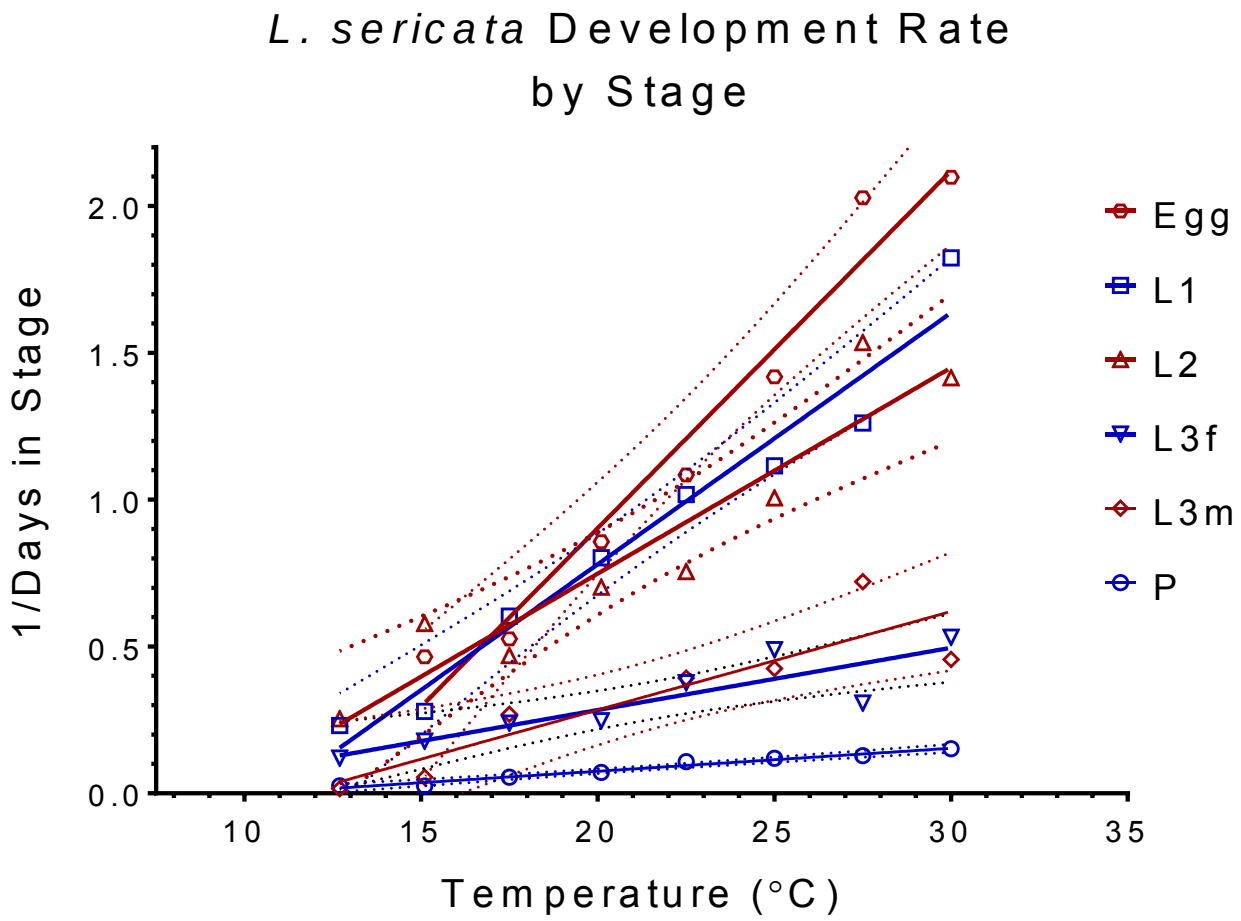


Fig. 33. *Lucilia sericata* calculated ADD for stage duration across temperatures (this should produce a line with zero slope).

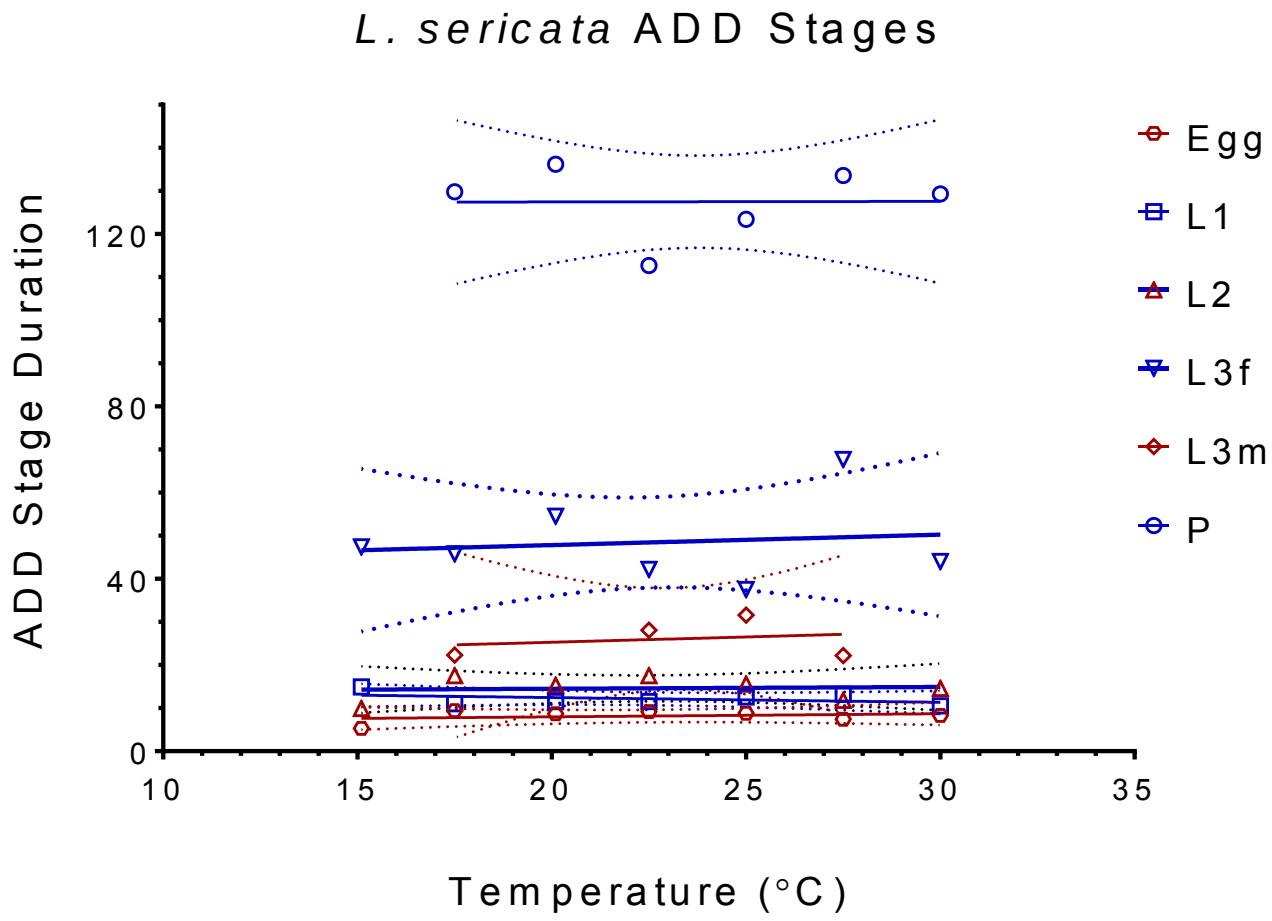


Fig. 34. *Lucilia sericata* development modeled based on exponential decay function, and evidence temperature threshold and time dependence migrating third stage maggots (L3m).

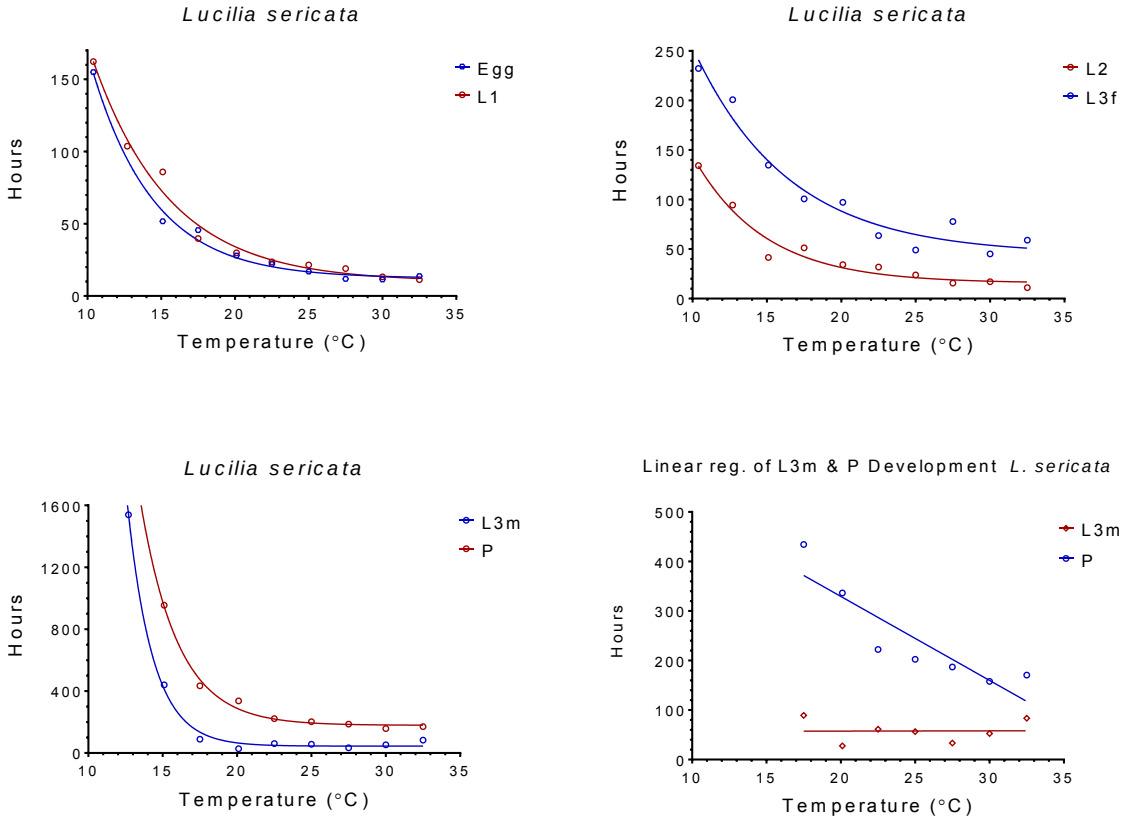


Fig. 35. Stage distributions *Phormia regina*, 10.0° C.

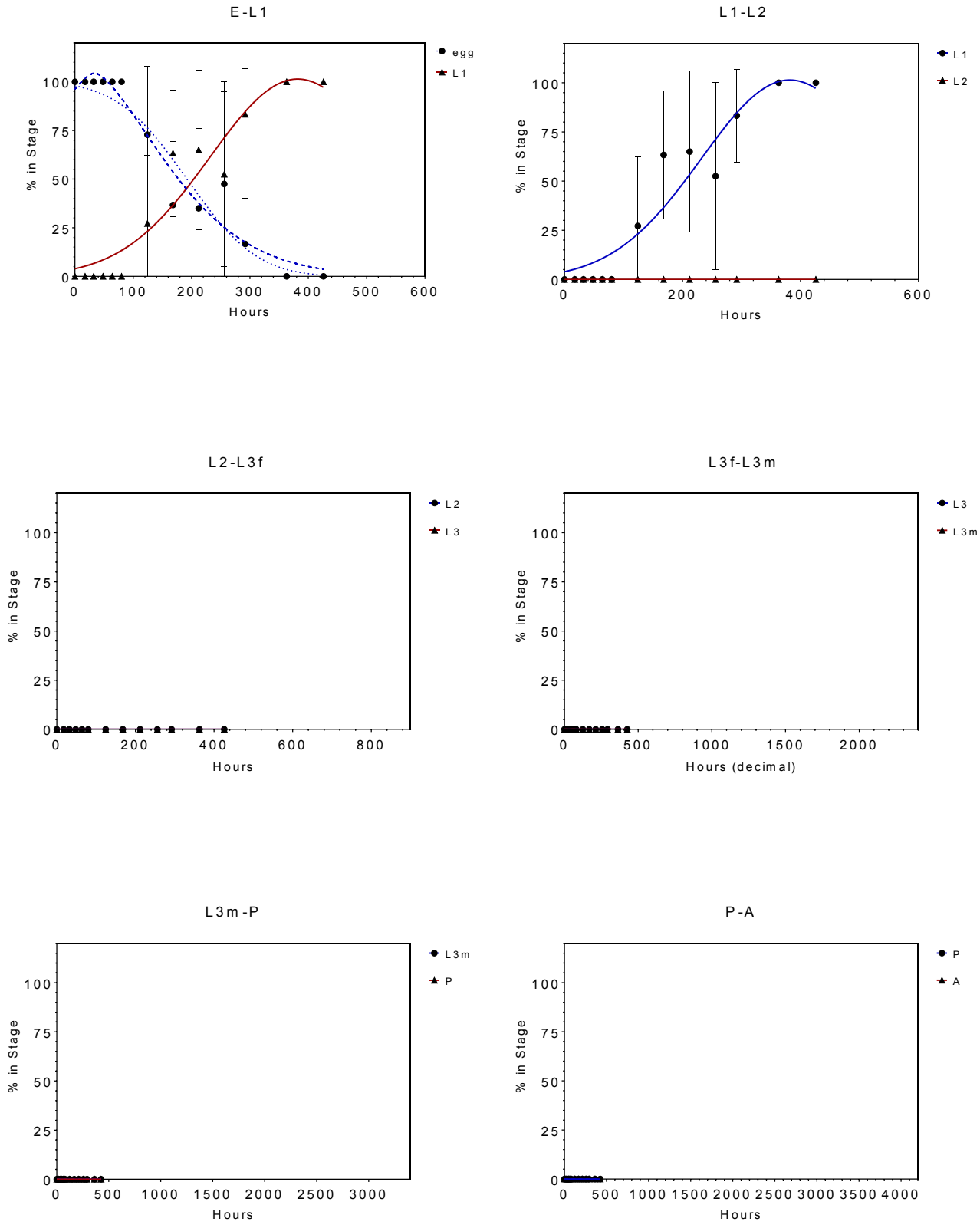


Fig. 36. Stage distributions *Phormia regina*, 12.5° C.

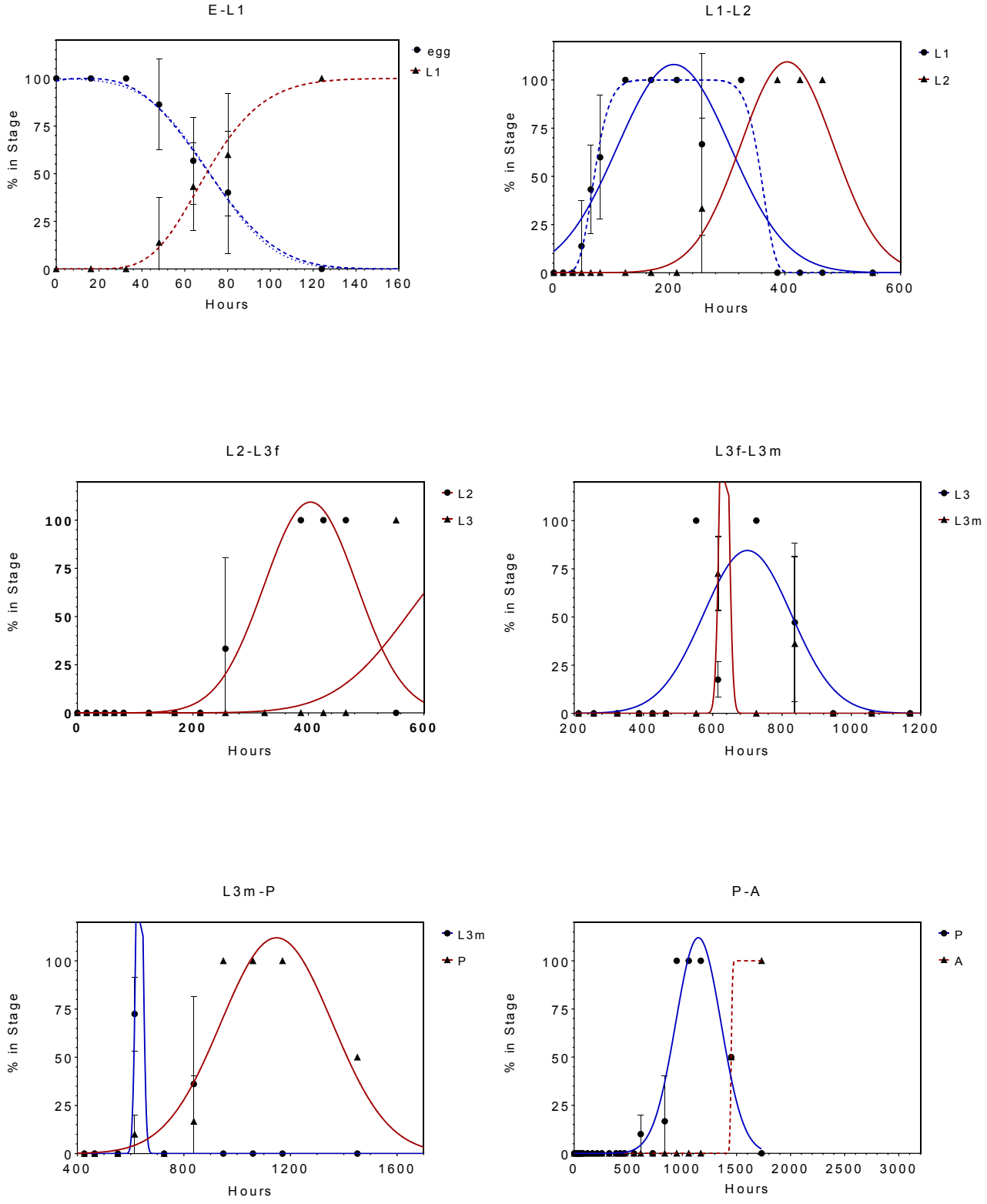


Fig. 37. Stage distributions *Phormia regina*, 15.0° C

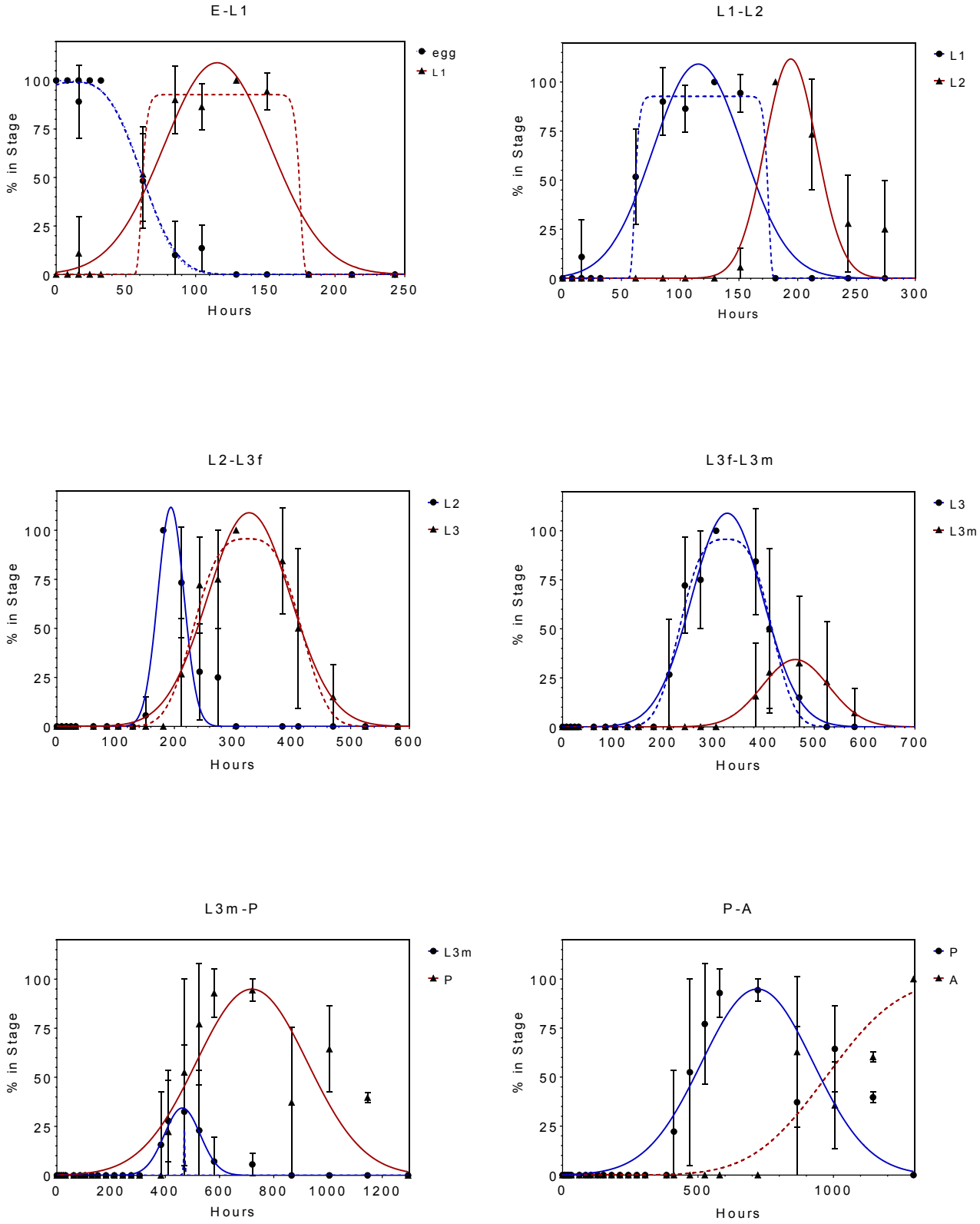


Fig. 38. Stage distributions *Phormia regina*, 17.5° C.

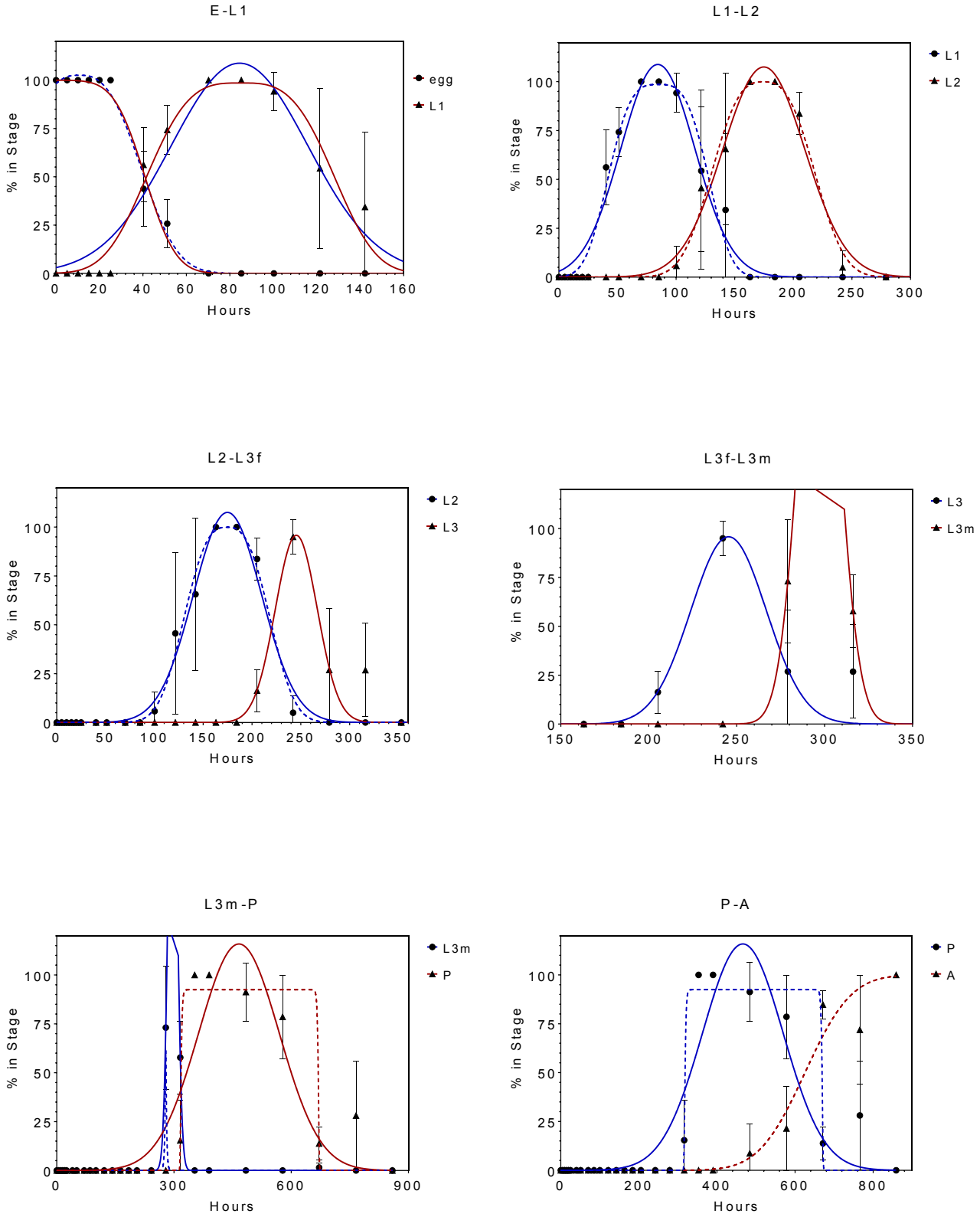


Fig. 39. Stage distributions *Phormia regina*, 20.0° C.

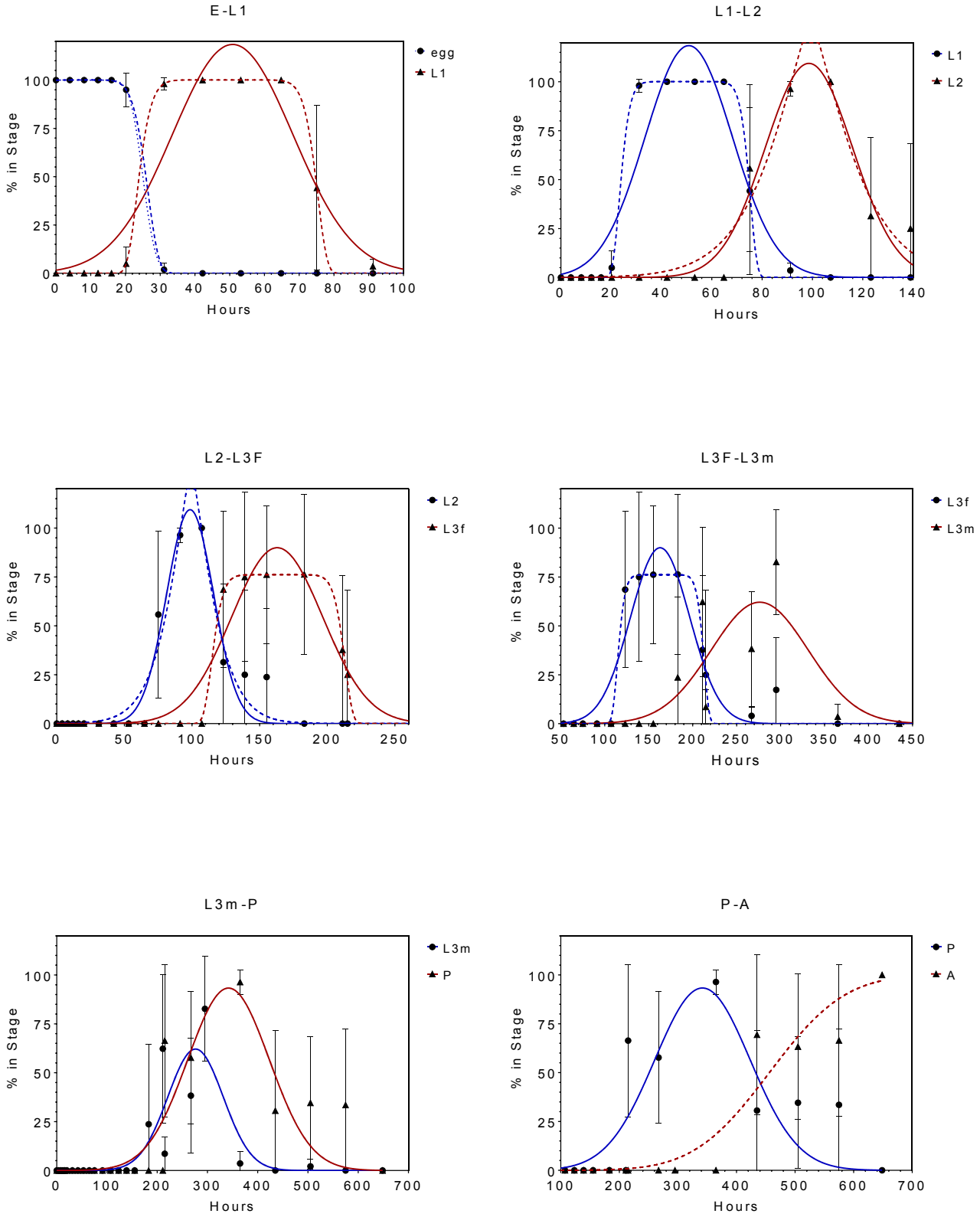


Fig. 40. Stage distributions *Phormia regina,* 22.5° C.

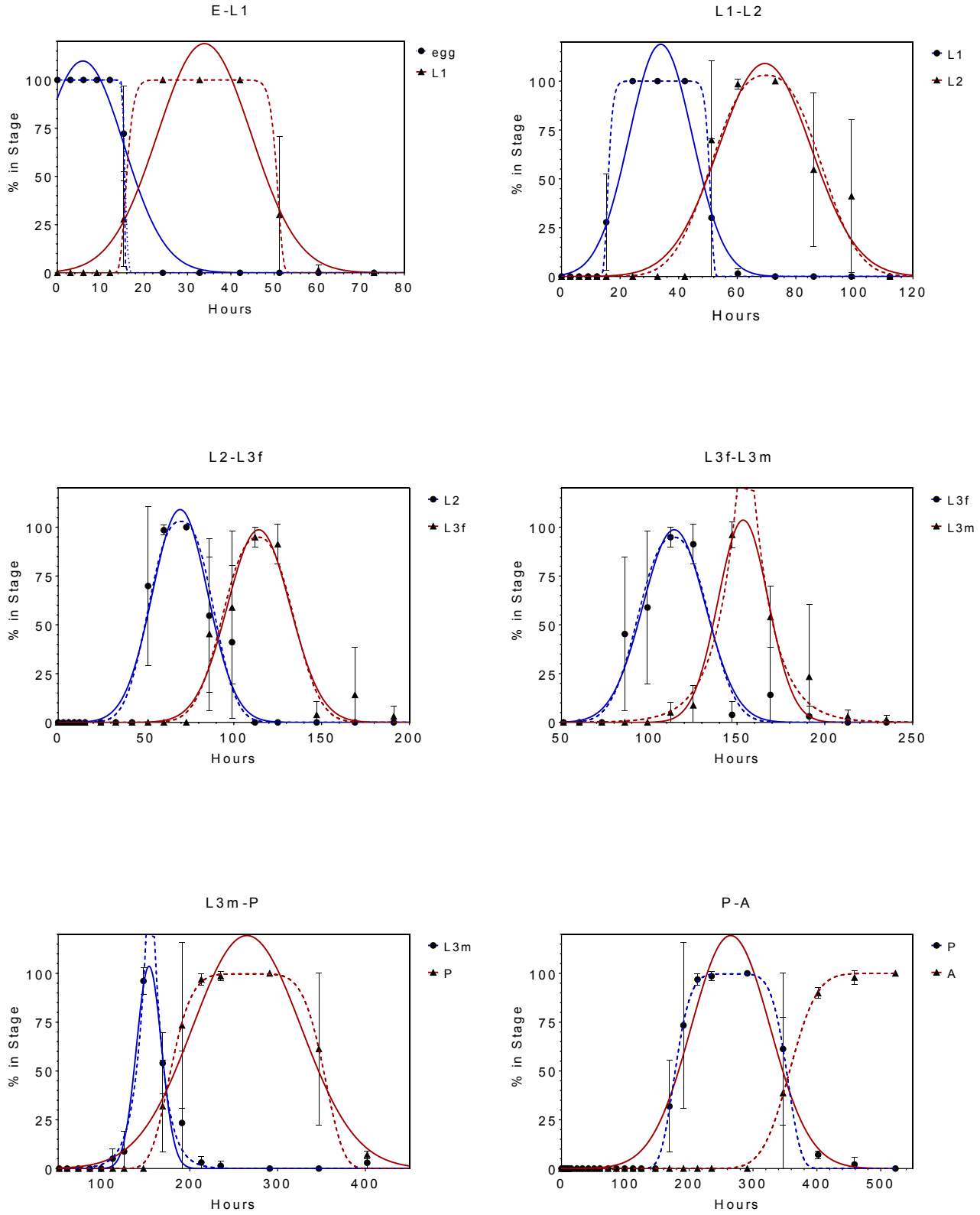


Fig. 41. Stage distributions *Phormia regina,* 25.0° C.

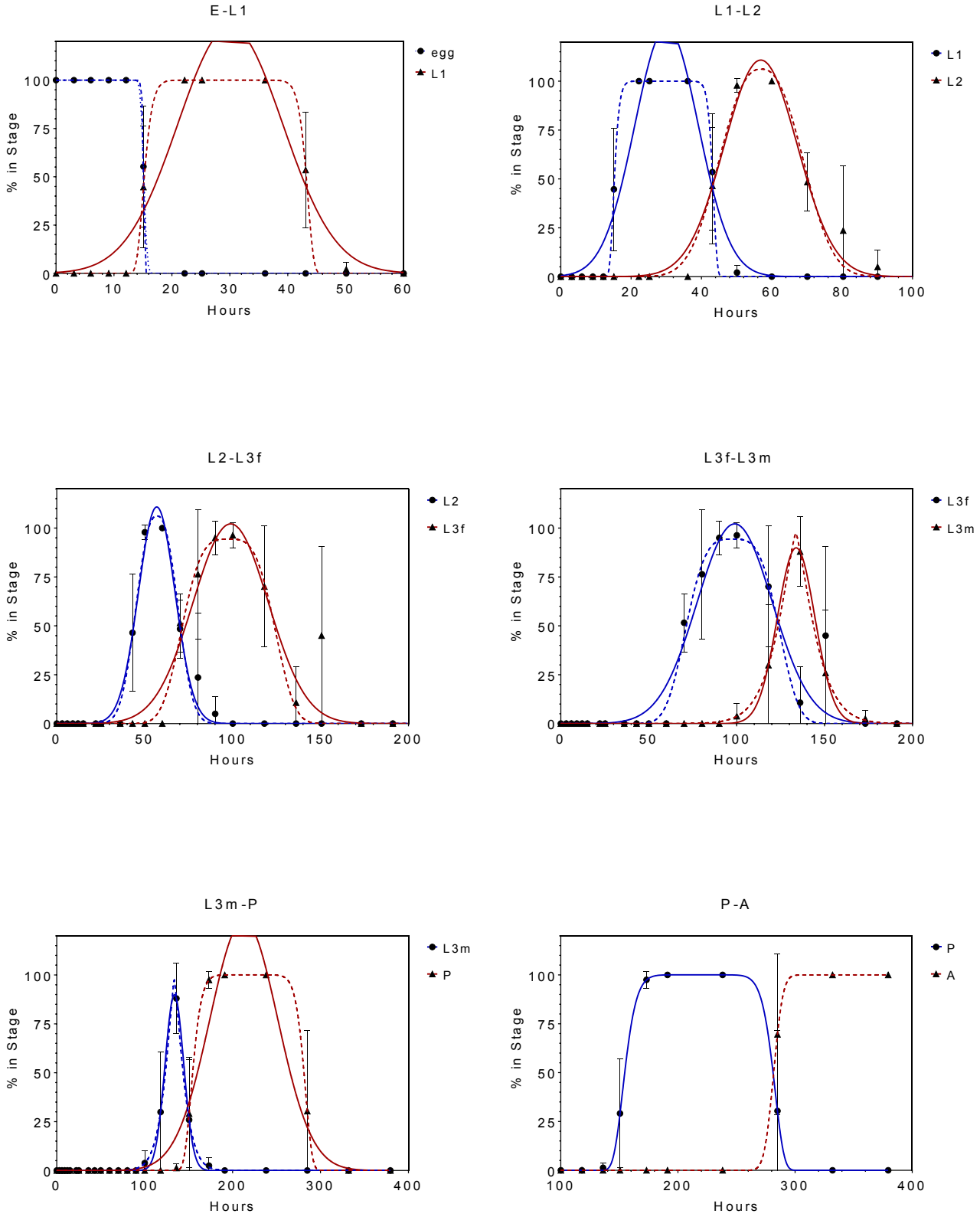


Fig. 42. Stage distributions *Phormia regina*, 27.5° C.

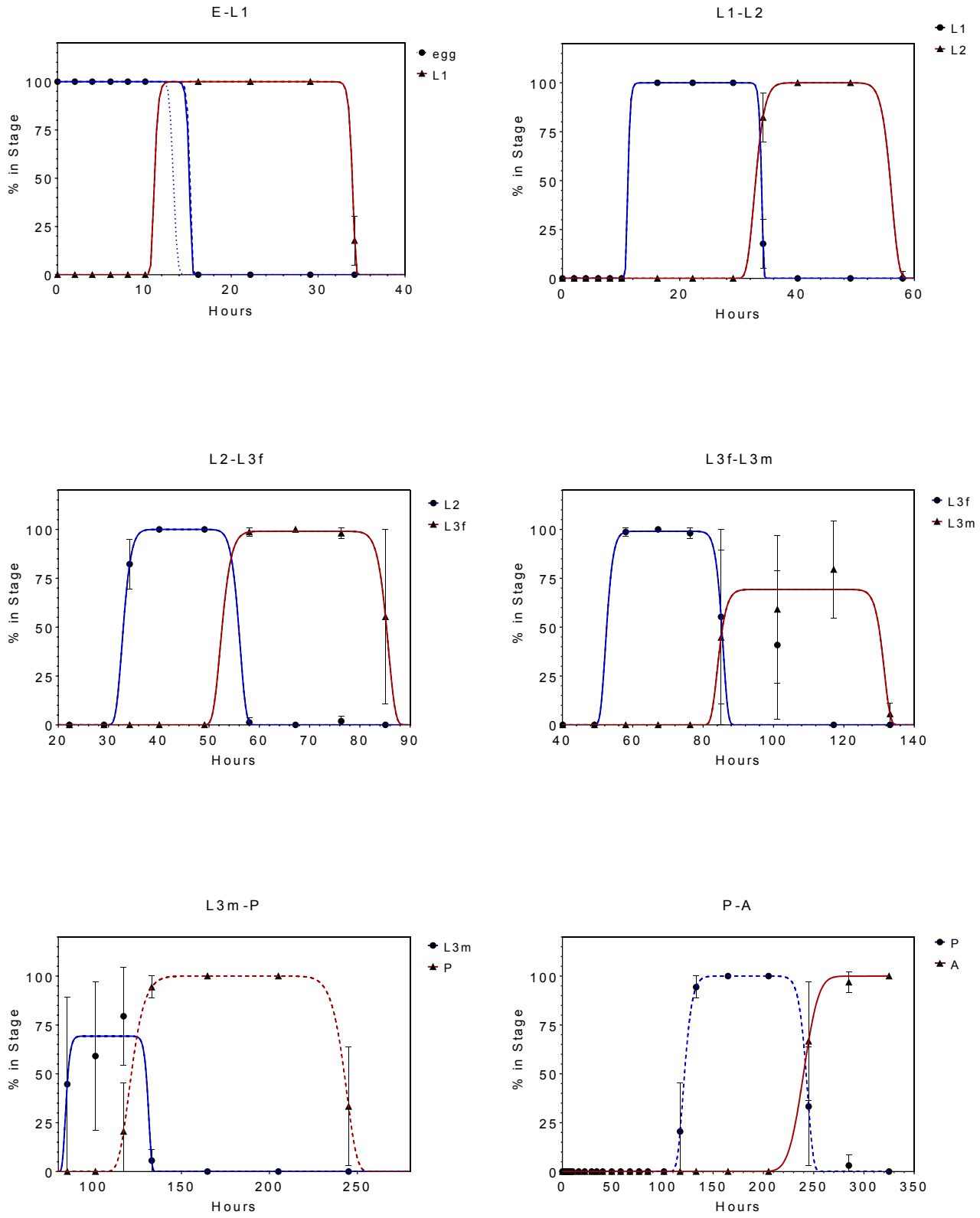


Fig. 43. Stage distributions *Phormia regina*, 30.0° C.

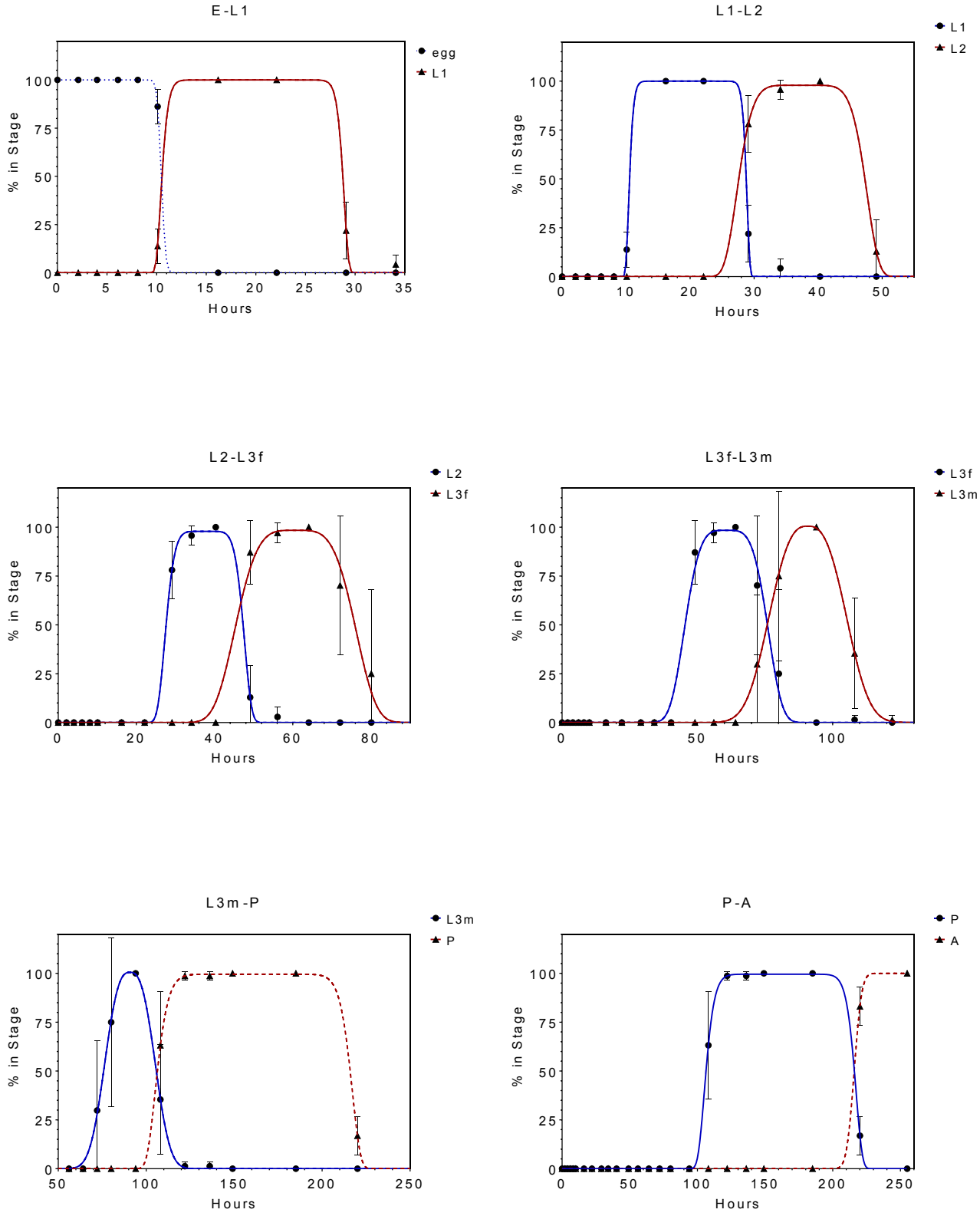


Fig. 44. Stage distributions *Phormia regina,* 32.5° C.

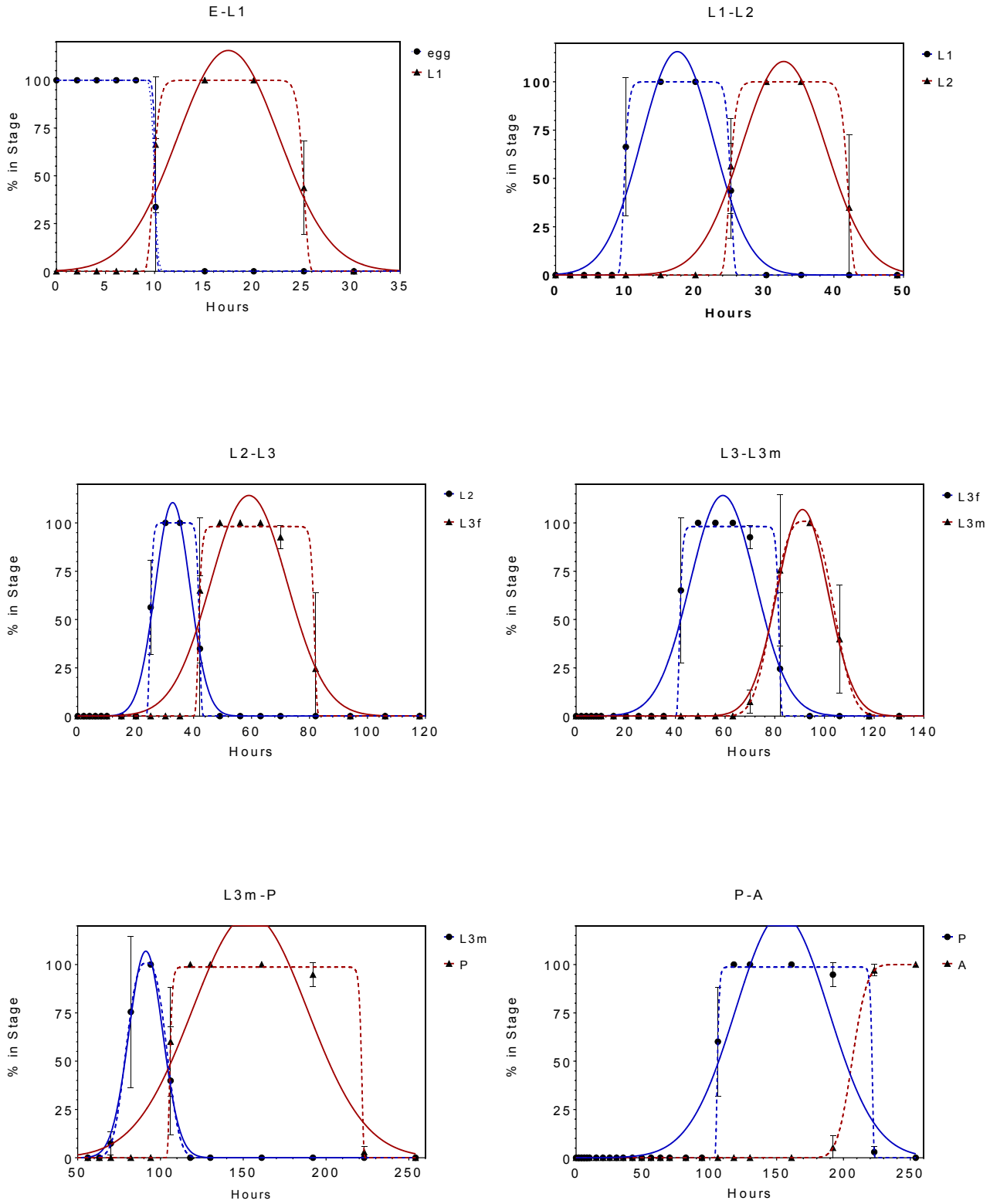


Fig. 45. *Phormia regina* stage distribution from egg to adult, 12.5° C.

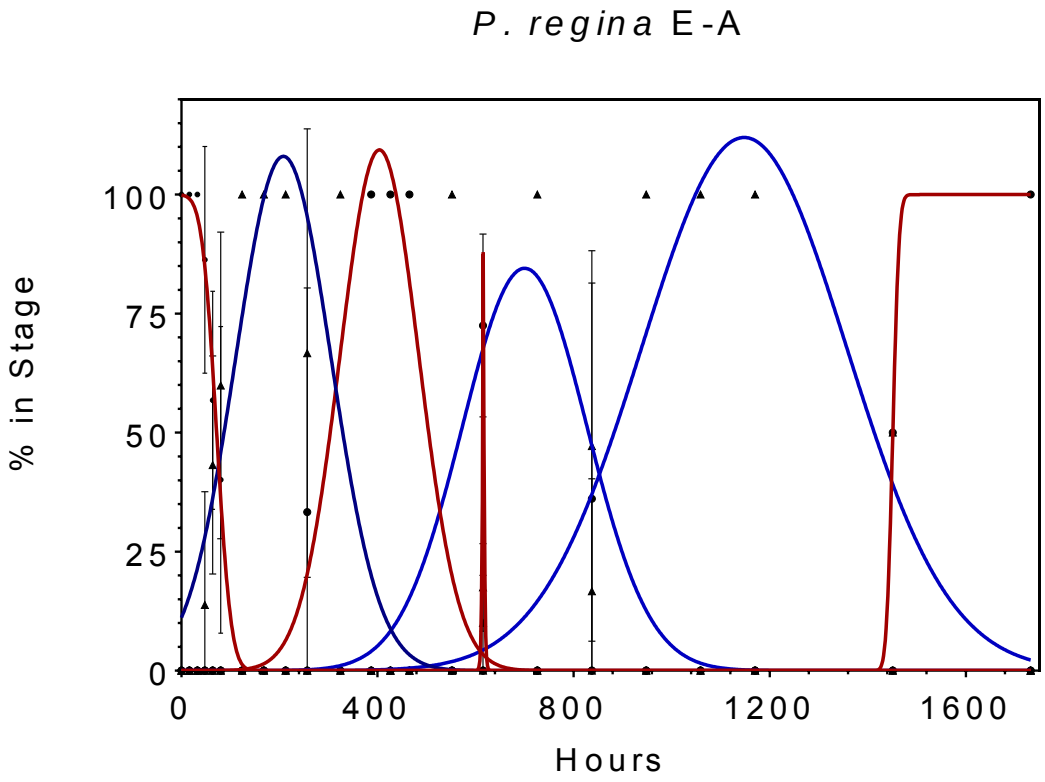


Fig. 46. *Phormia regina* stage distribution from egg to adult, 15.0° C.

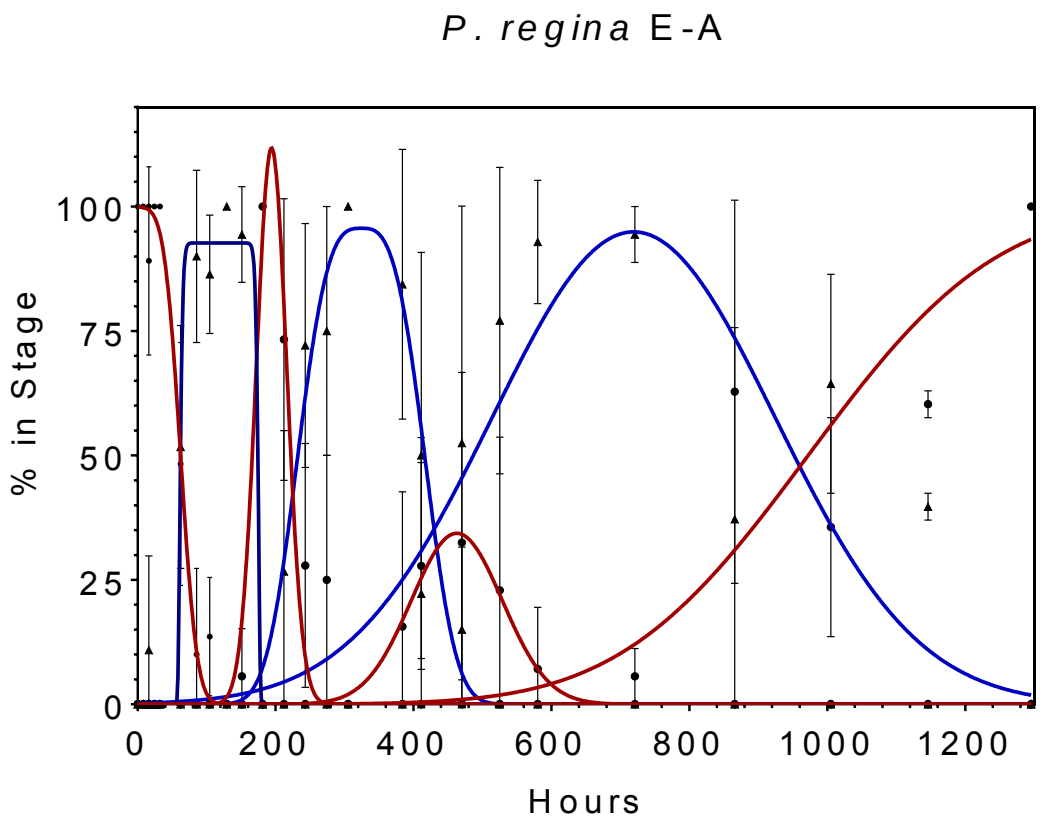


Fig. 47. *Phormia regina* stage distribution from egg to adult, 17.5° C.

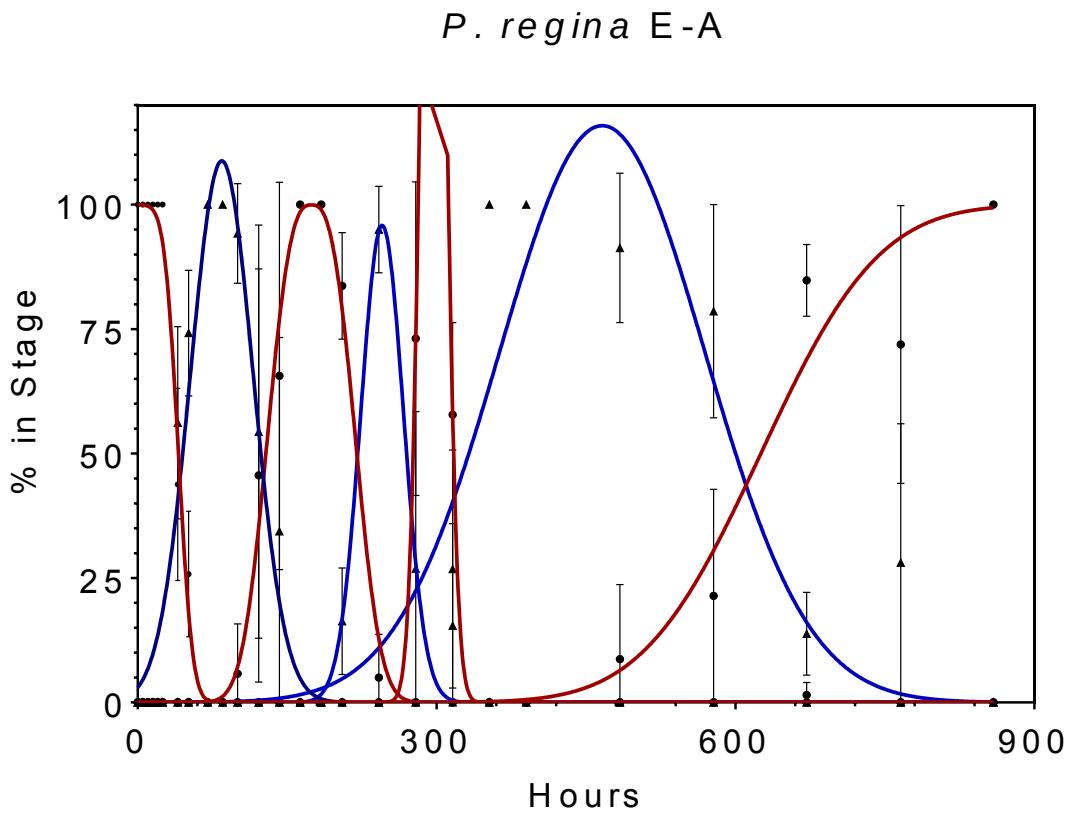


Fig. 48. *Phormia regina* stage distribution from egg to adult, 20.0° C.

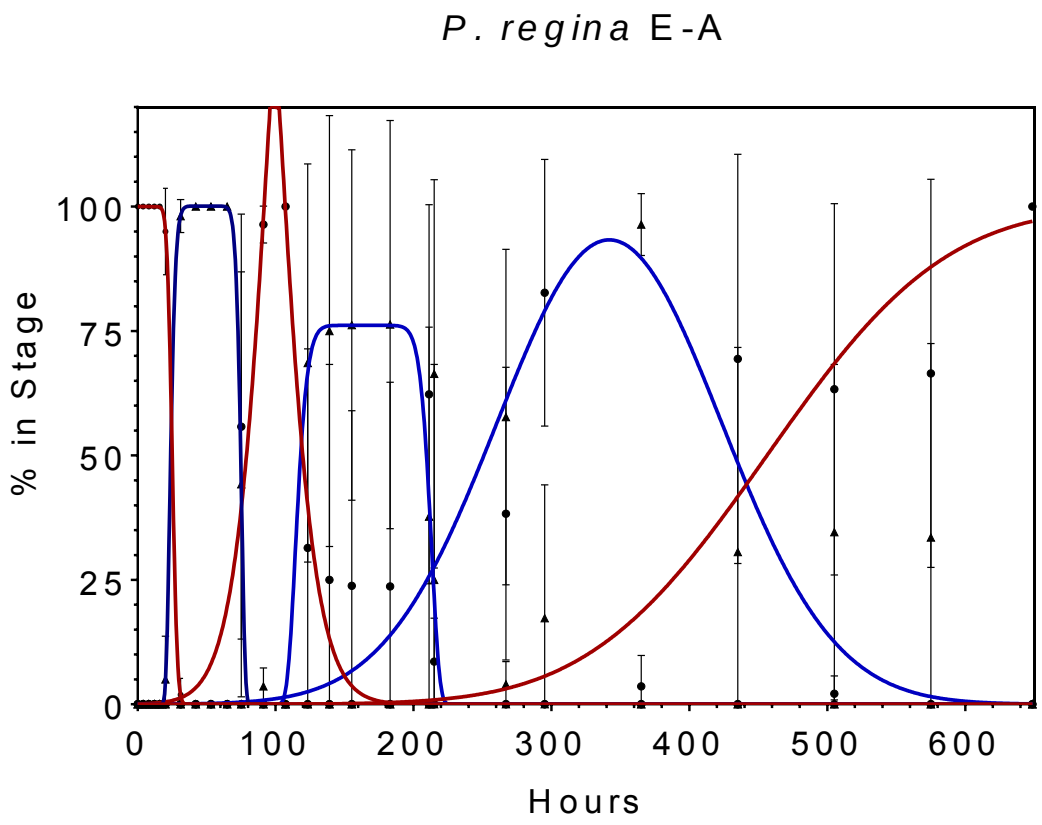


Fig. 49. *Phormia regina* stage distribution from egg to adult, 22.5° C.

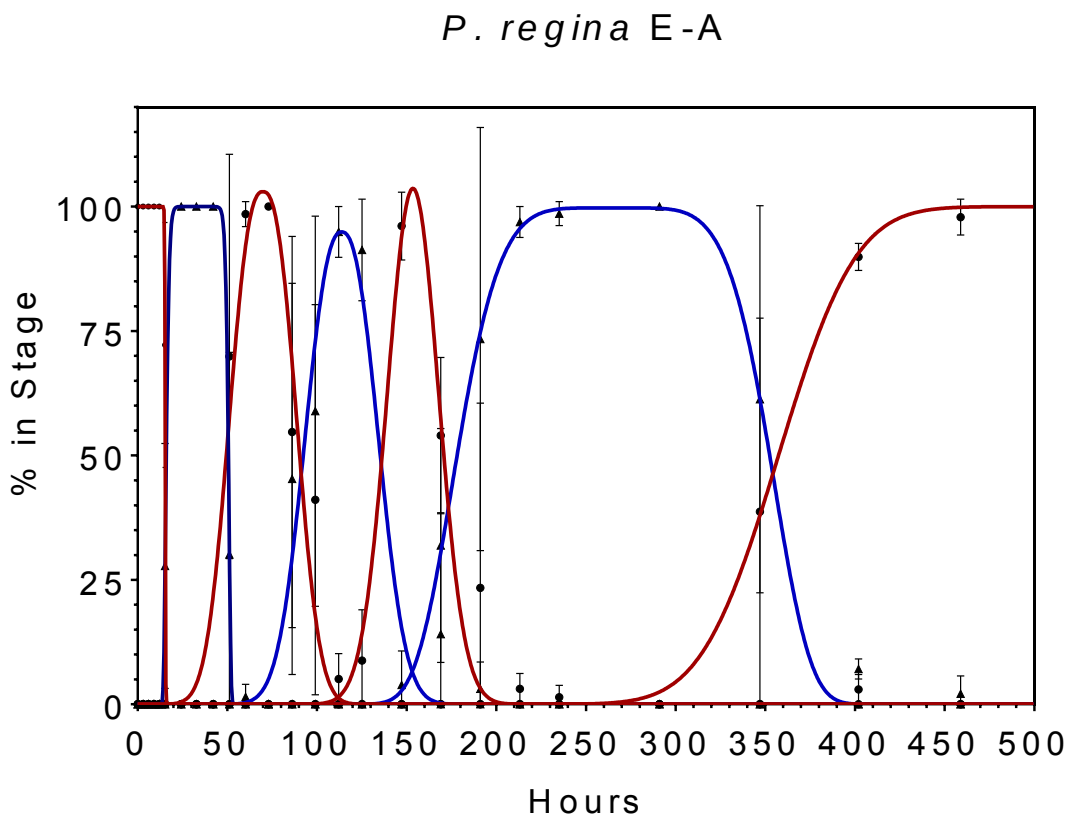


Fig. 50. *Phormia regina* stage distribution from egg to adult, 25.0° C.

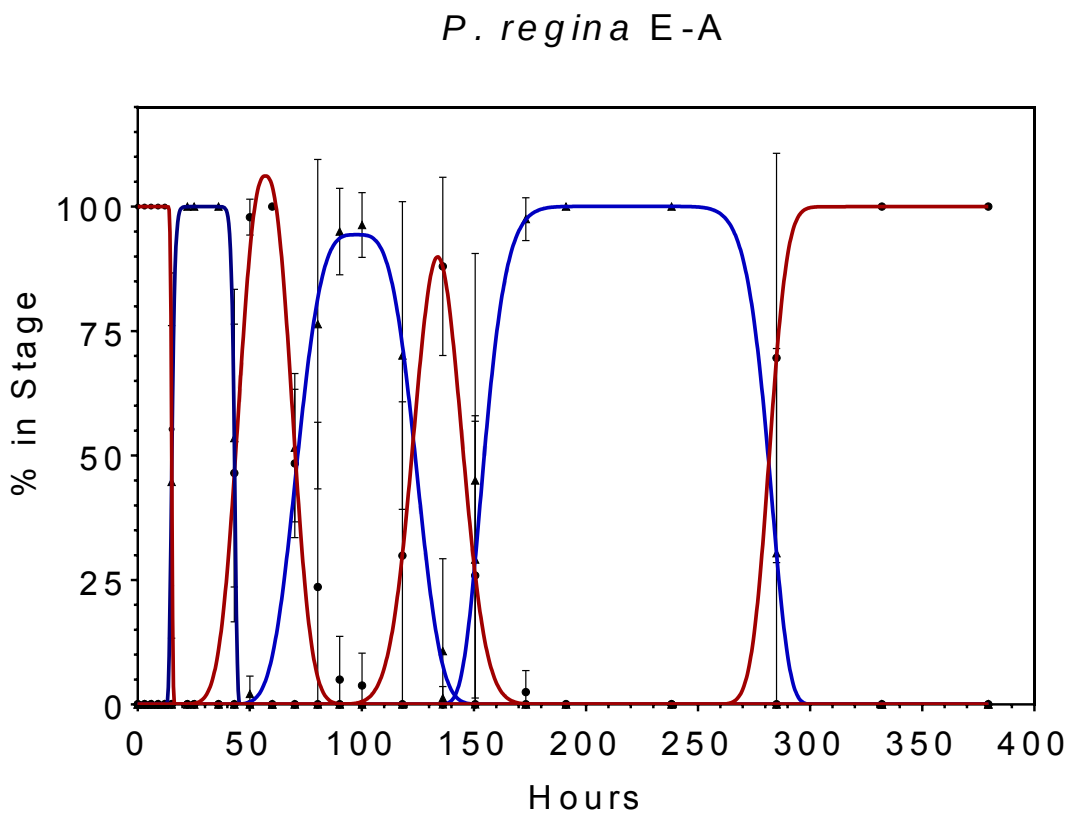


Fig. 51. *Phormia regina* stage distribution from egg to adult, 27.5° C.

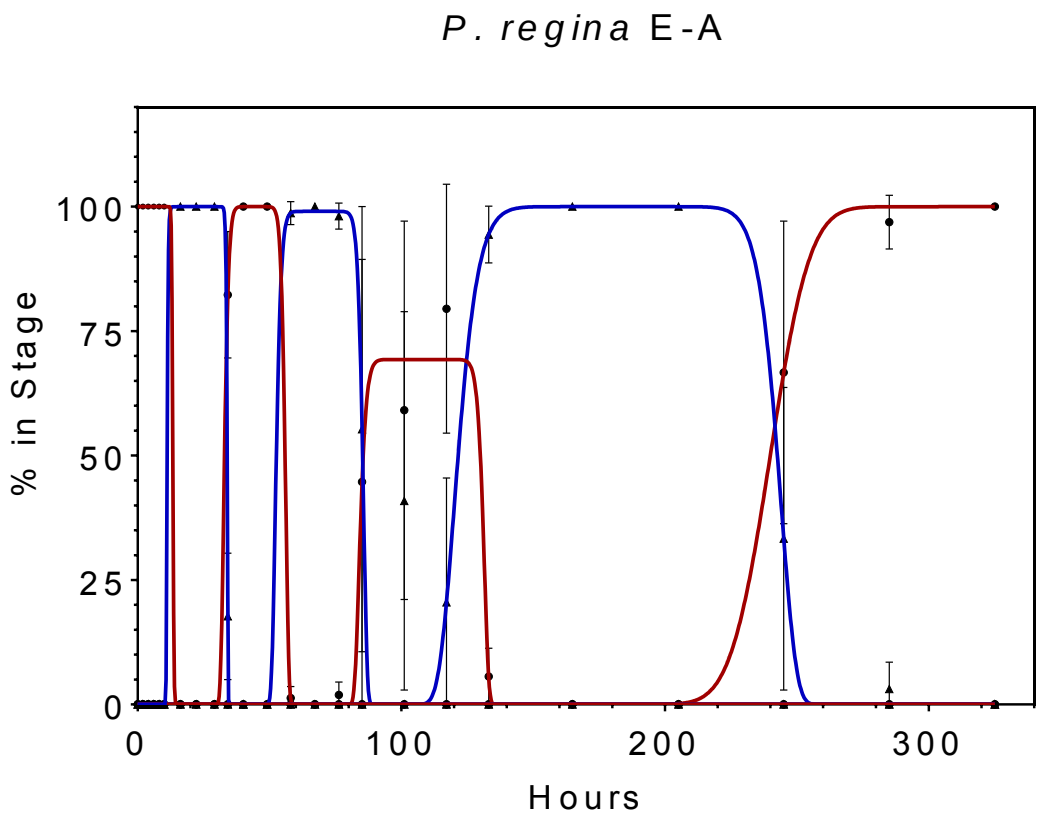


Fig. 52. *Phormia regina* stage distribution from egg to adult, 30.0° C.

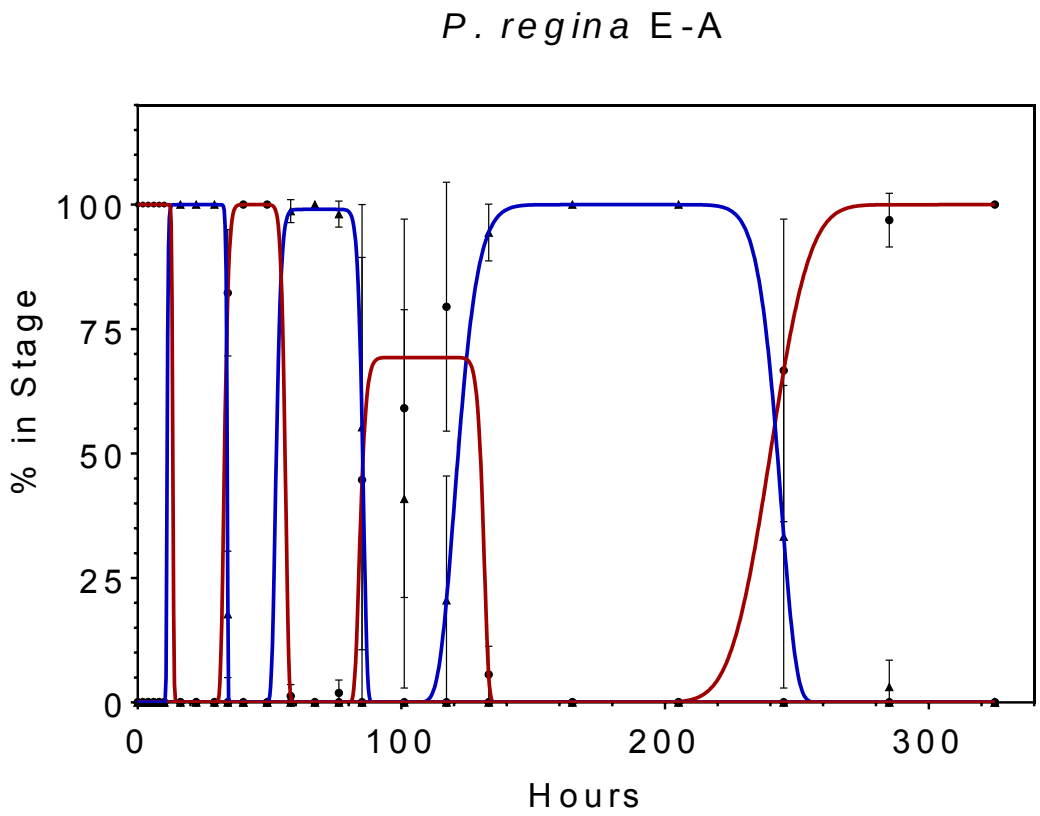


Fig. 53. *Phormia regina* stage distribution from egg to adult, 32.5° C.

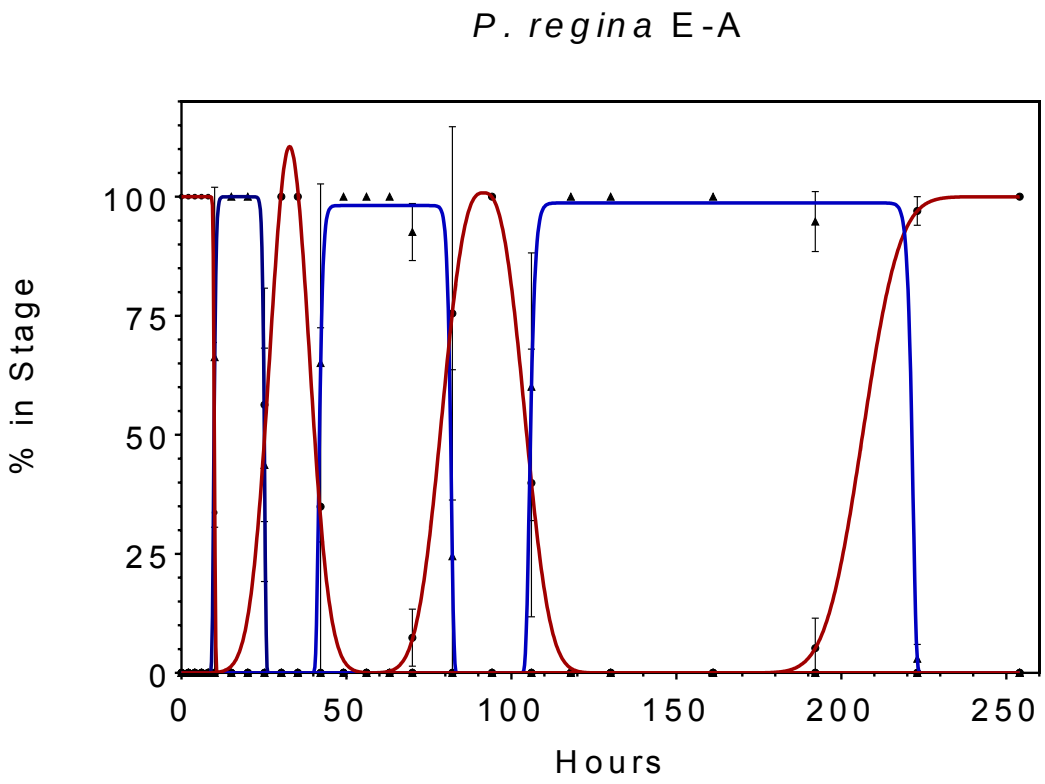


Fig. 54. *Phormia regina* stage transition fit to dose response curve, 10.0° C.

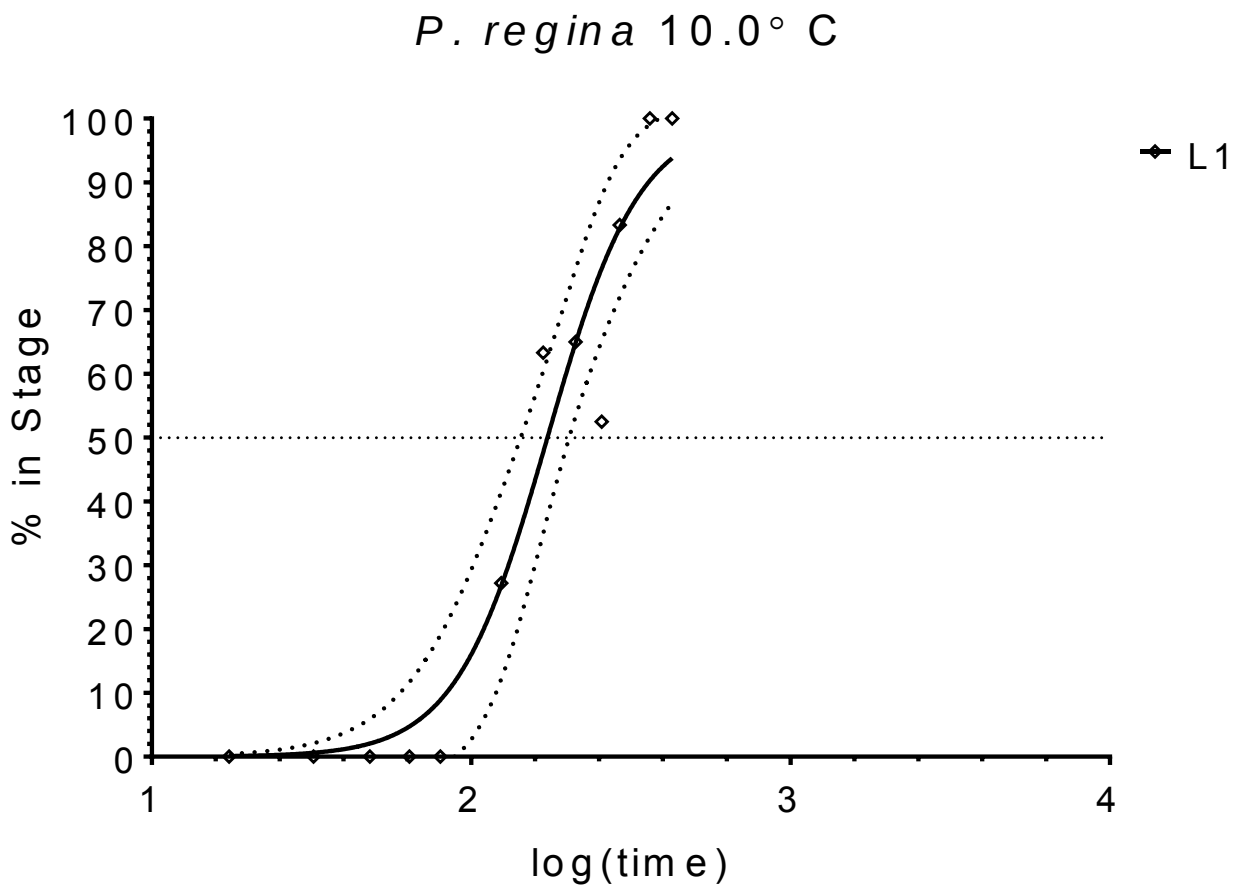


Fig. 55. *Phormia regina* stage transition fit to dose response curve, 12.5° C.

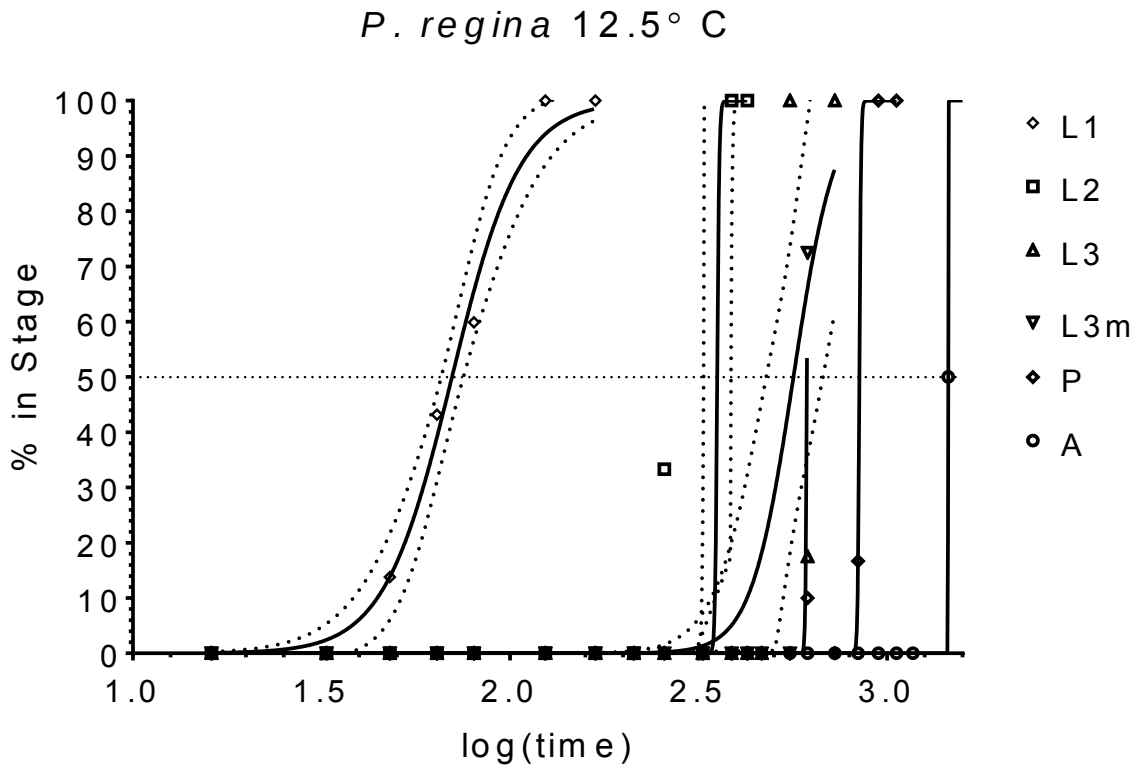


Fig. 57. *Phormia regina* stage transition fit to dose response curve, 15.0° C.

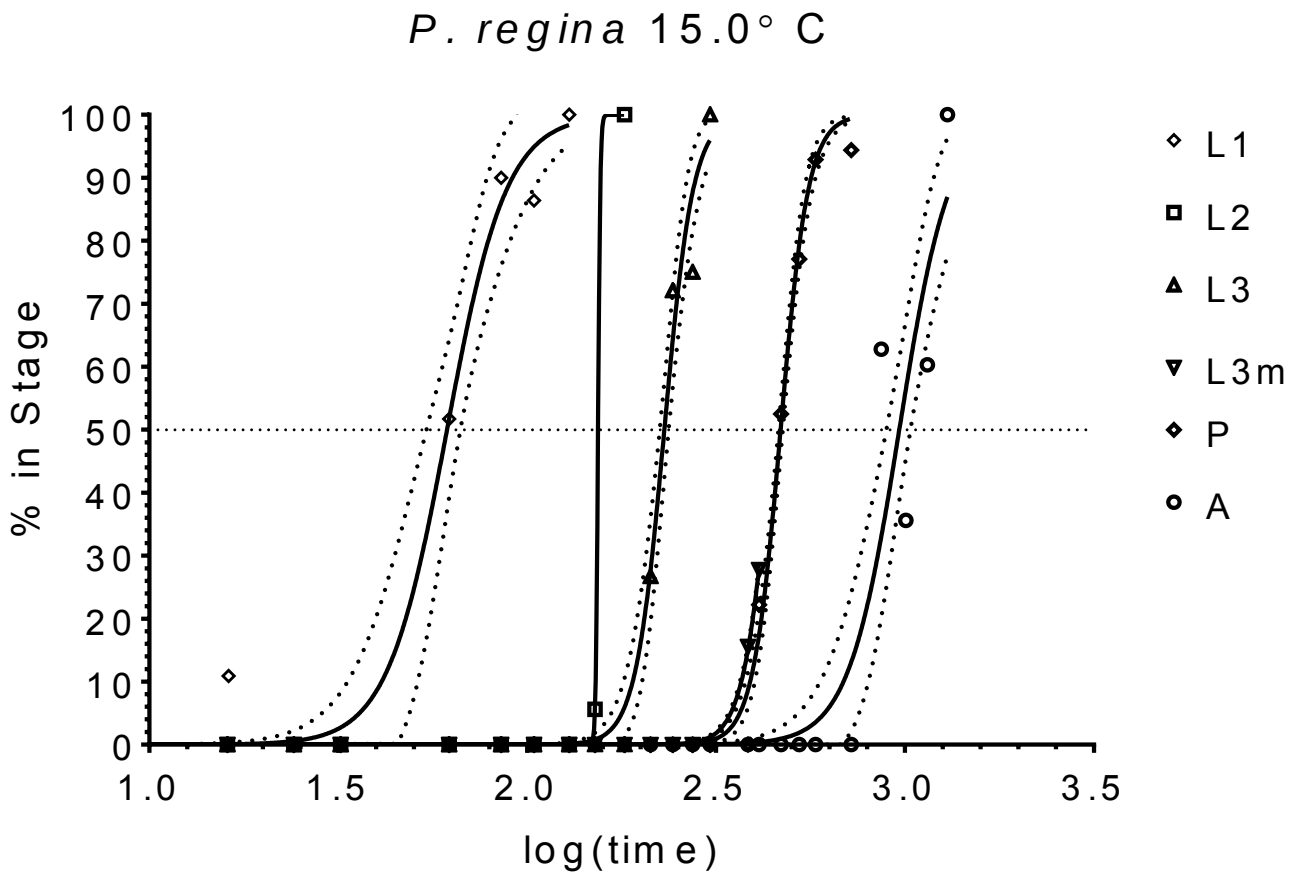


Fig. 58. *Phormia regina* stage transition fit to dose response curve, 17.5° C.

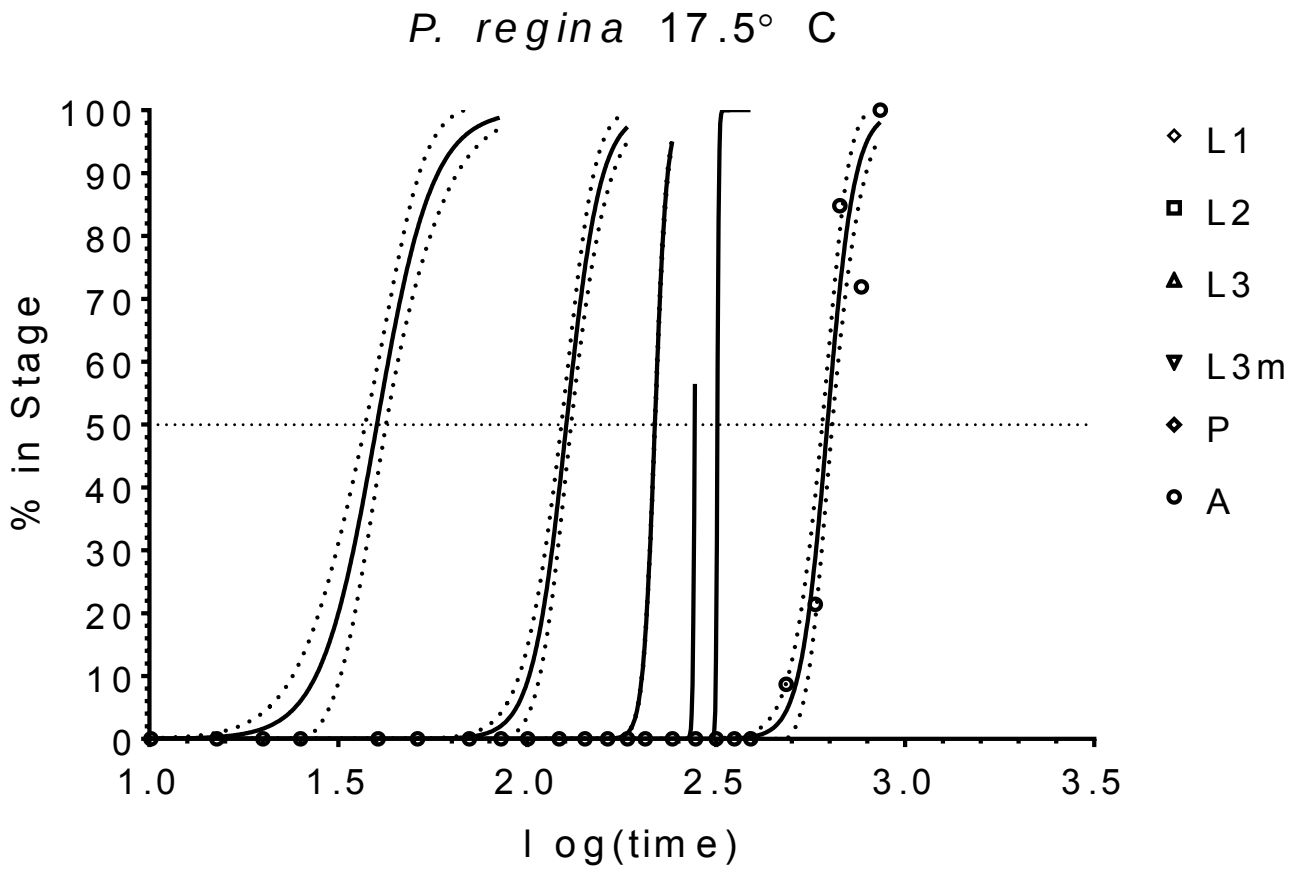


Fig. 60. *Phormia regina* stage transition fit to dose response curve, 20.0° C.

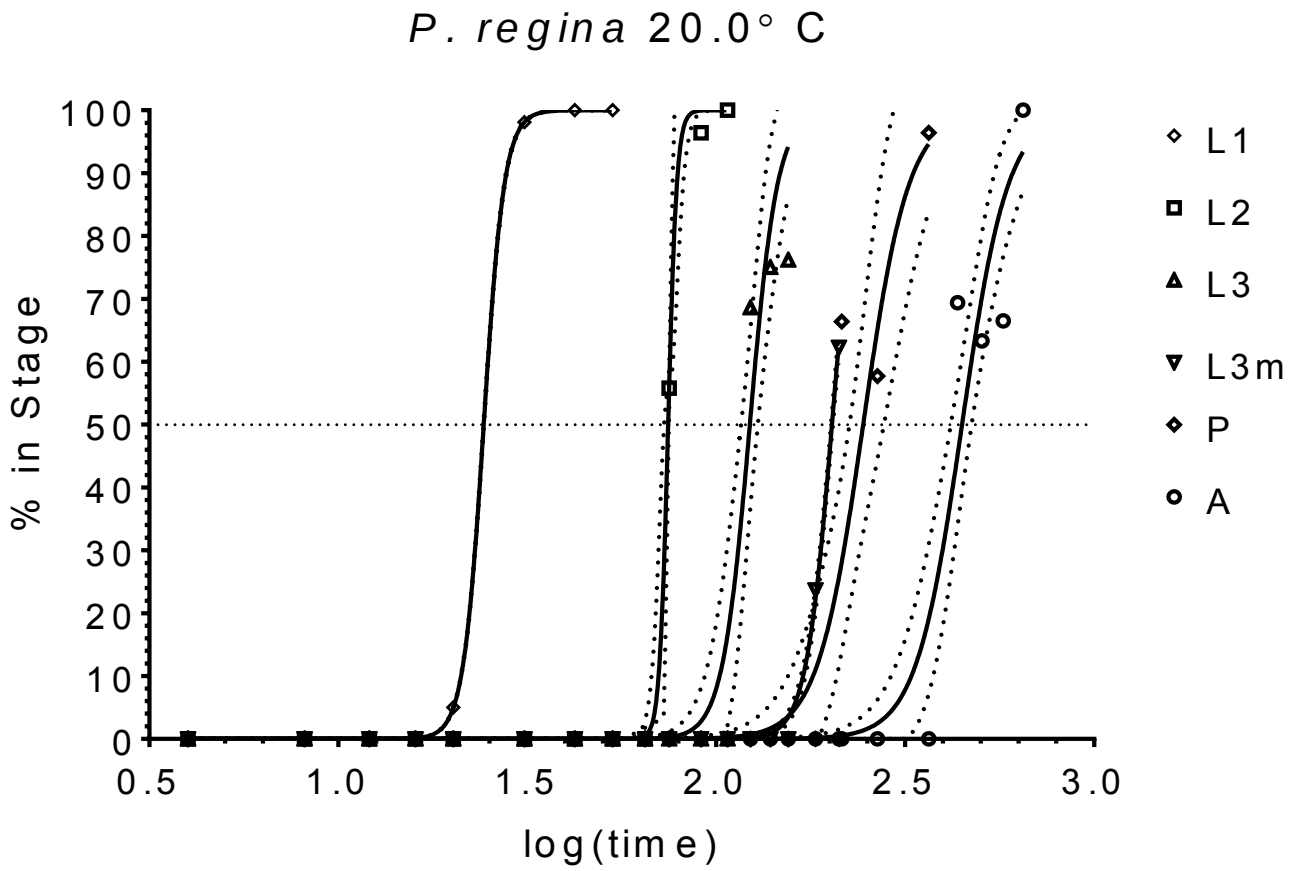


Fig. 61. *Phormia regina* stage transition fit to dose response curve, 22.5° C.

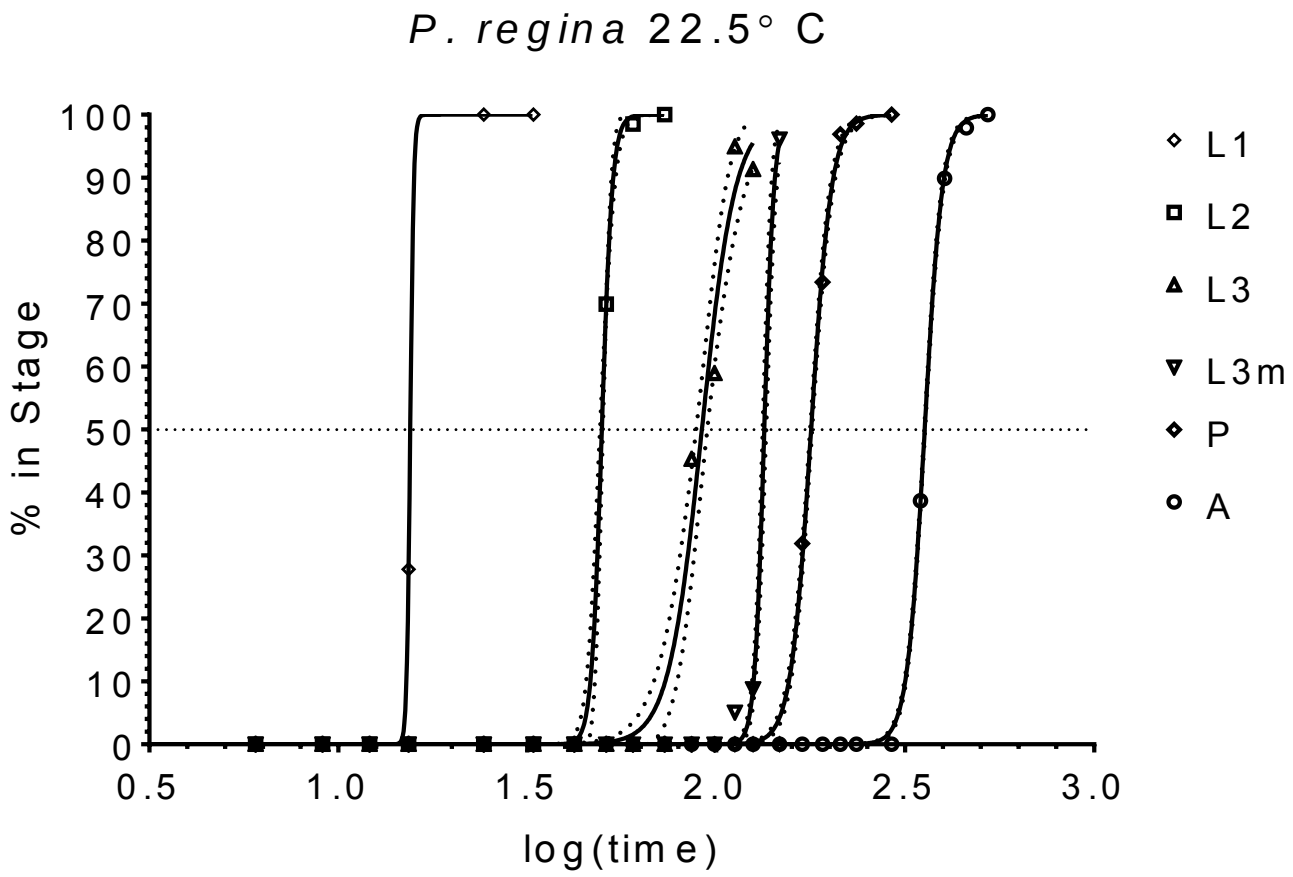


Fig. 62. *Phormia regina* stage transition fit to dose response curve, 25.0° C.

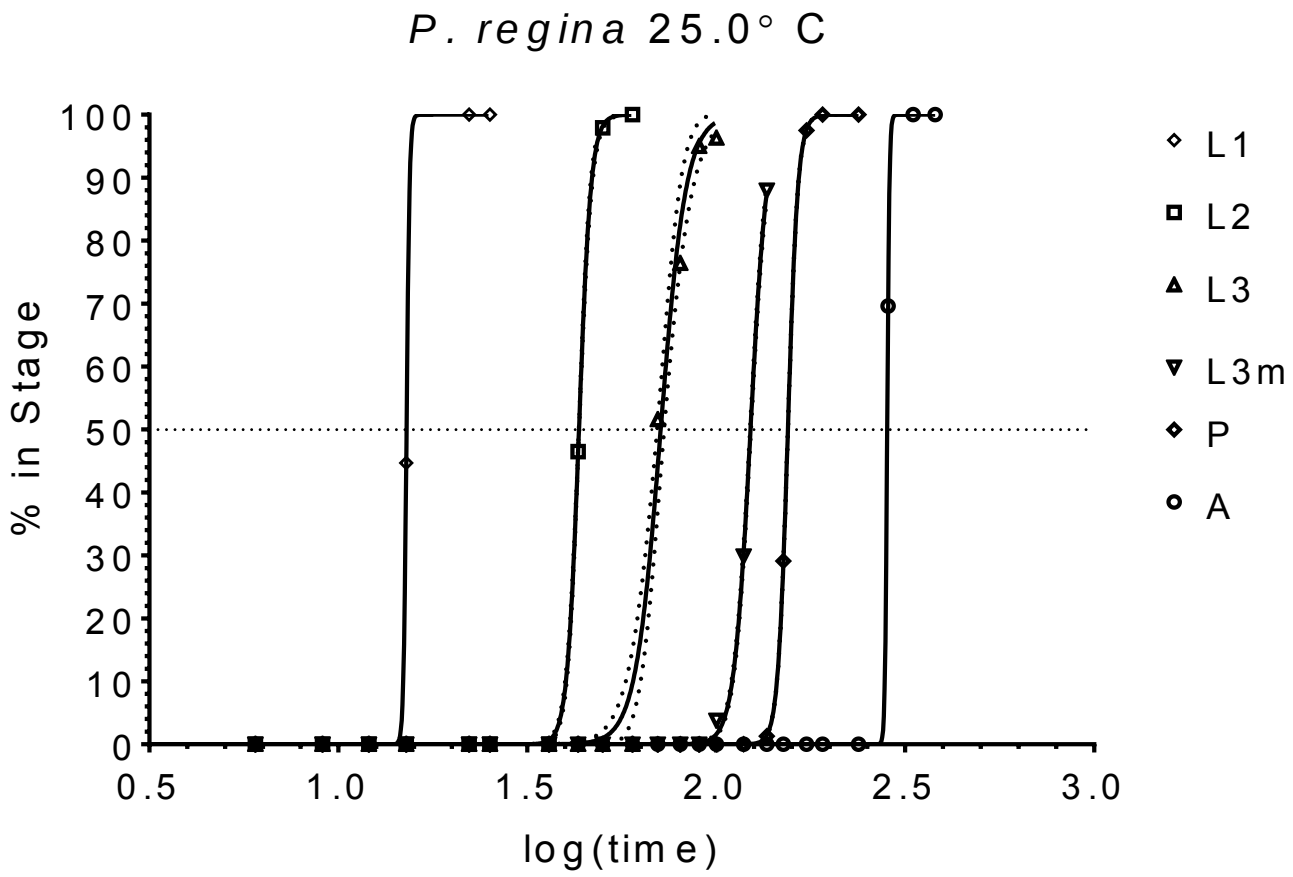


Fig. 63. *Phormia regina* stage transition fit to dose response curve, 27.5° C.

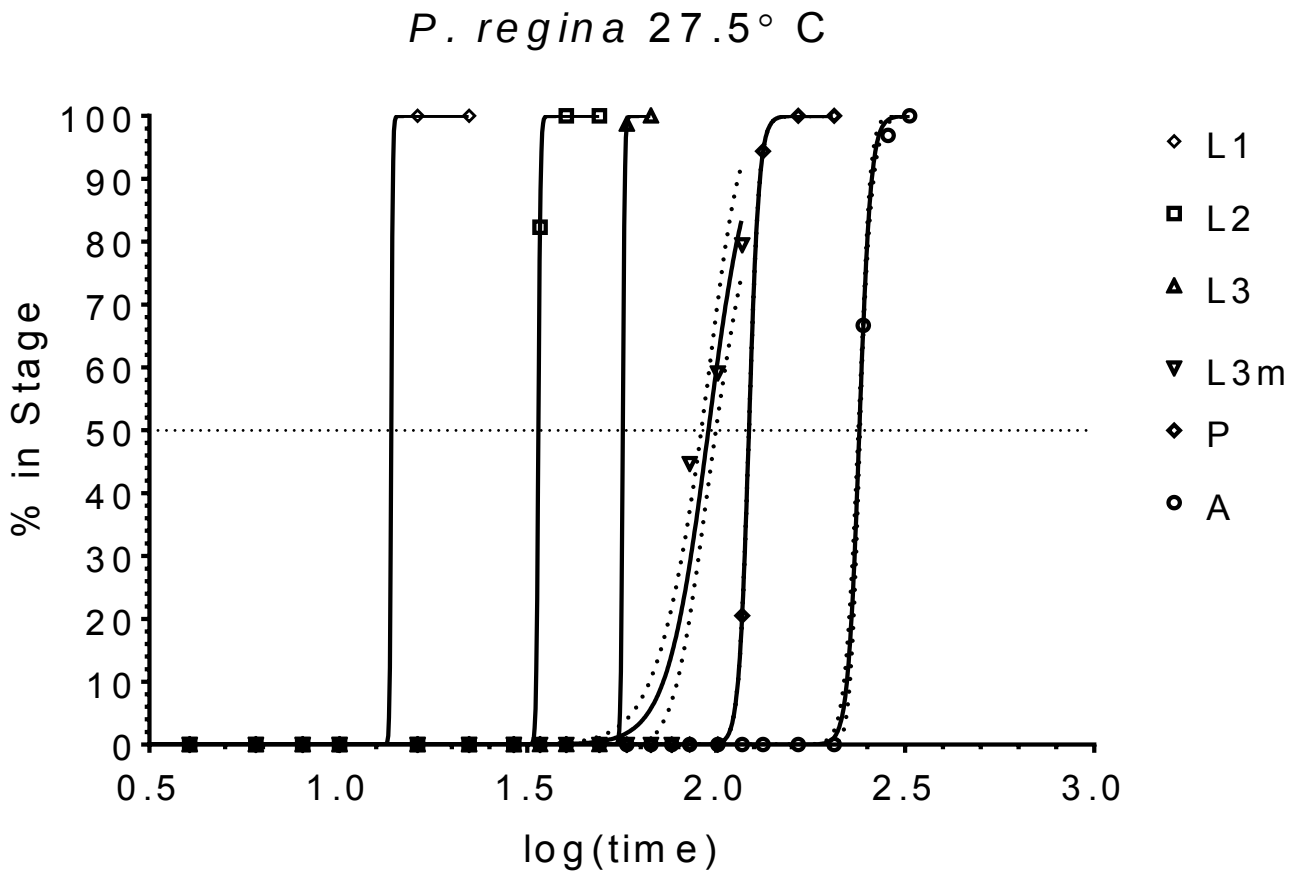


Fig. 64. *Phormia regina* stage transition fit to dose response curve, 30.0° C.

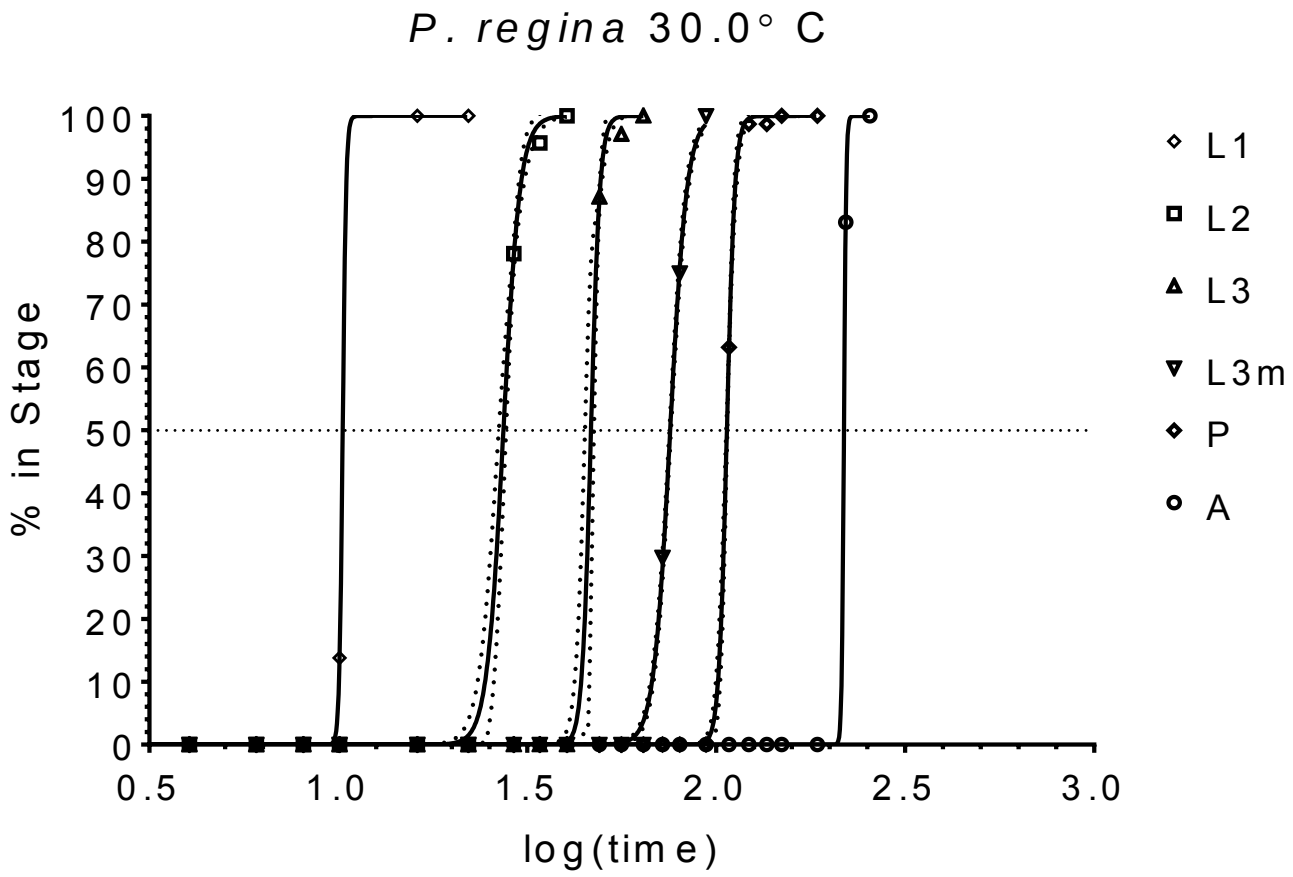


Fig. 65. *Phormia regina* stage transition fit to dose response curve, 32.5° C.

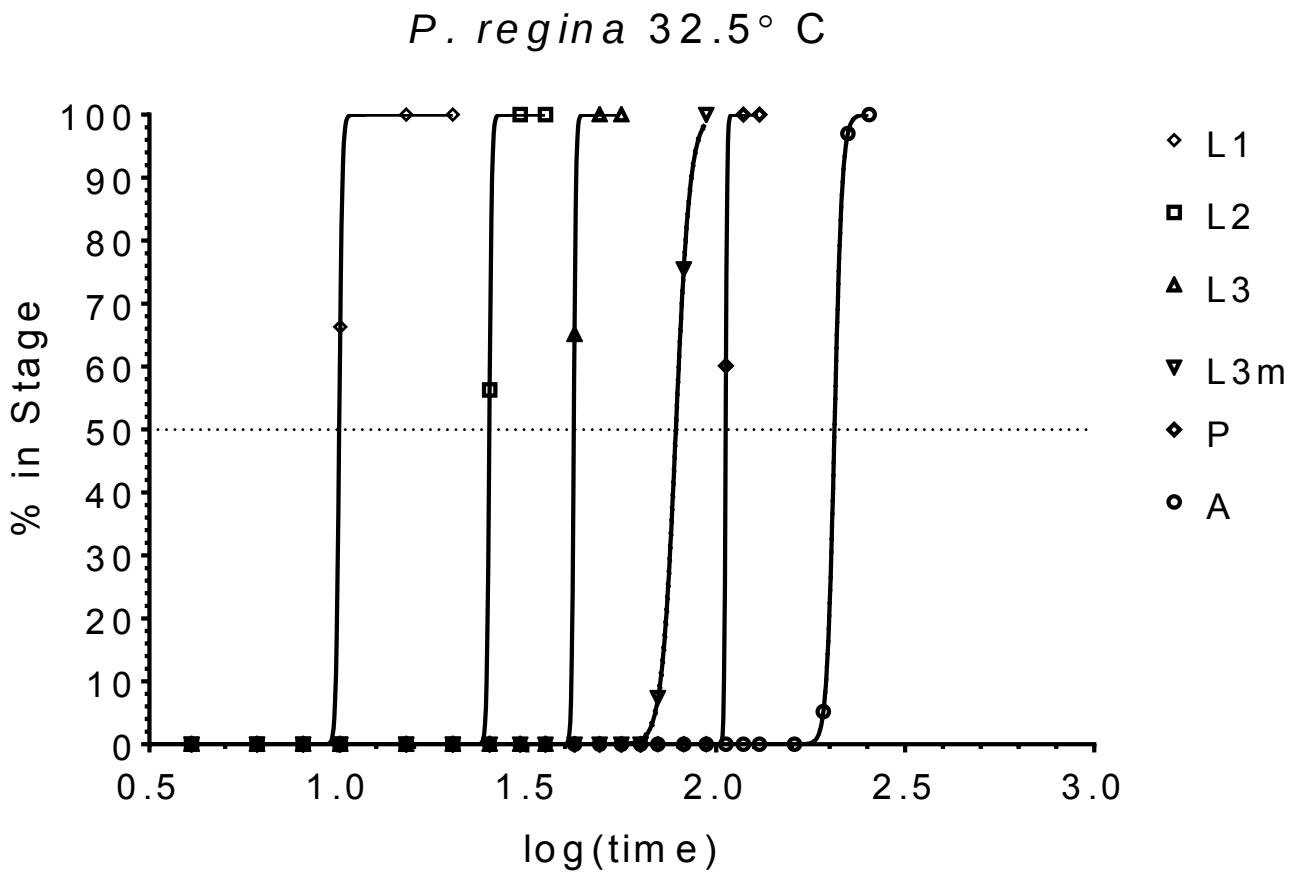


Fig. 66. *Phormia regina* development rates (as 1/days) to stage (as estimated by linear regressions).

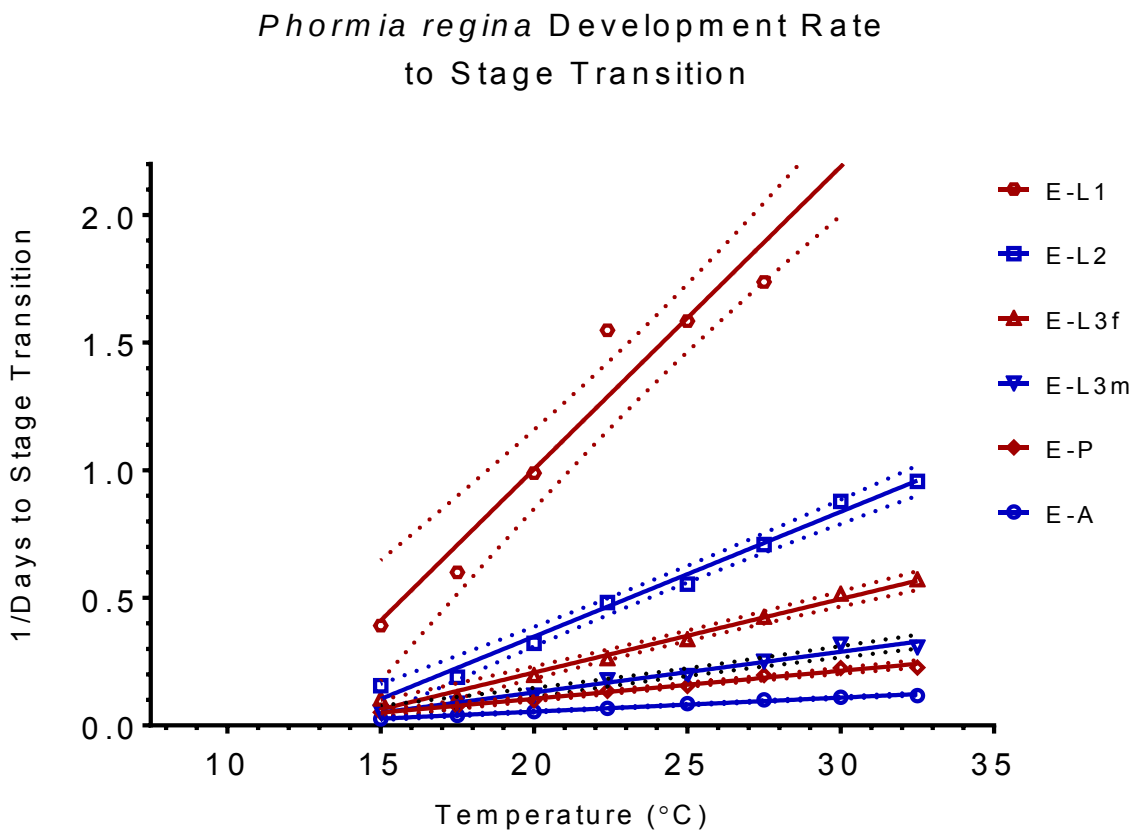


Fig. 67. *Phormia regina* development rates by stage (as estimated by linear regressions).

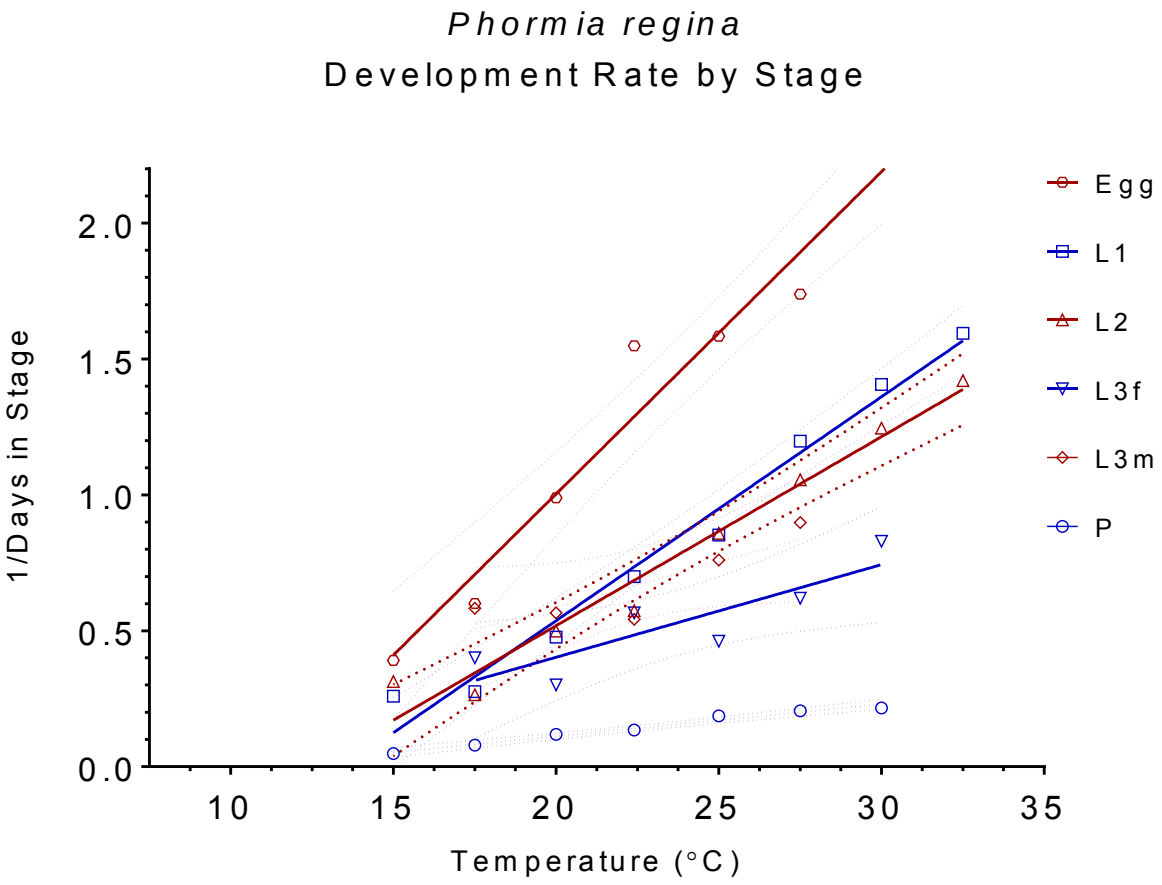


Fig. 68. *Phormia regina* calculated ADD for stage duration across temperatures (this should produce a line with zero slope).

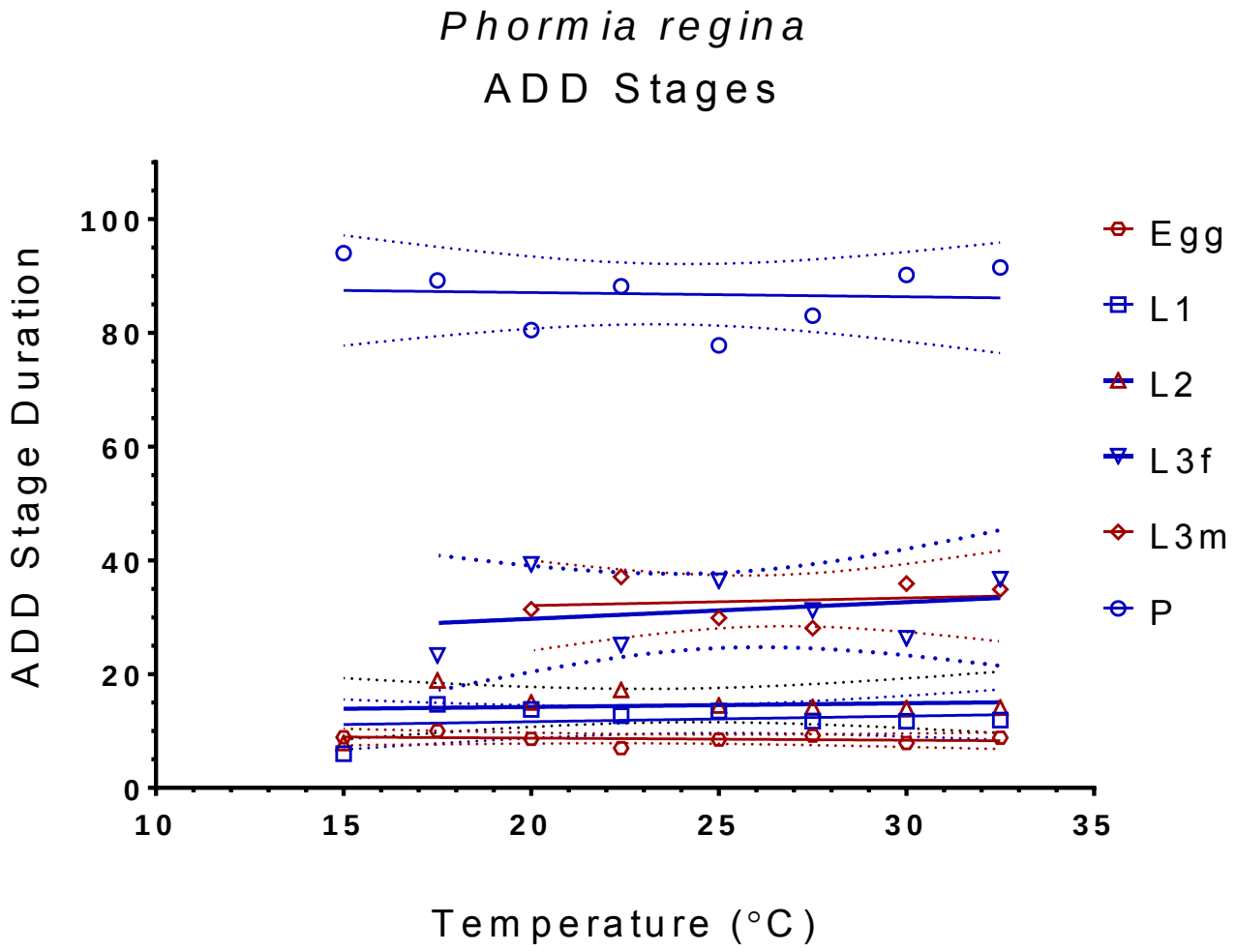


Fig. 69. *Phormia regina* development modeled based on exponential decay function, and evidence temperature threshold and time dependence migrating third stage maggots (L3m).

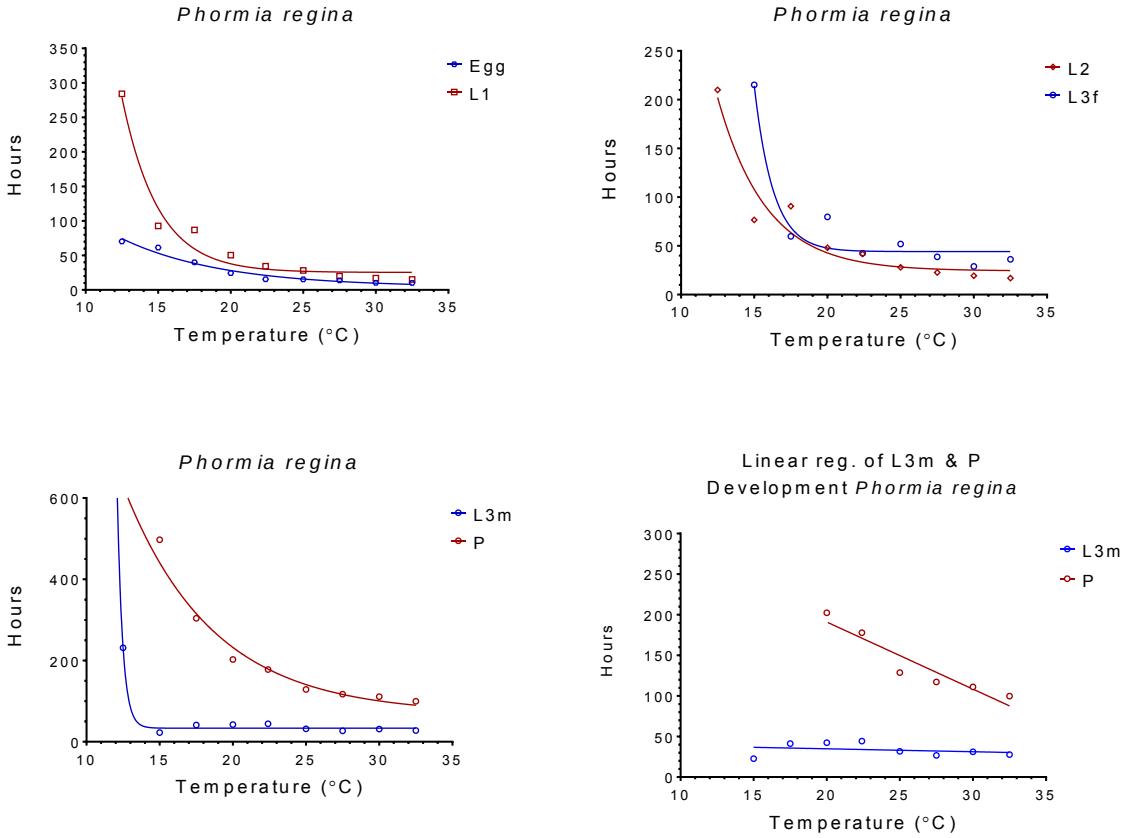


Fig. 70. Lethal time for mortality in 50% (LT 50) of test populations of four forensic calliphorid species (*Calliphora vicina*,, *Cochliomyia macellaria*, *Lucilia sericata*, and *Phormia regina*) under hypoxia (nitrogen atmosphere).

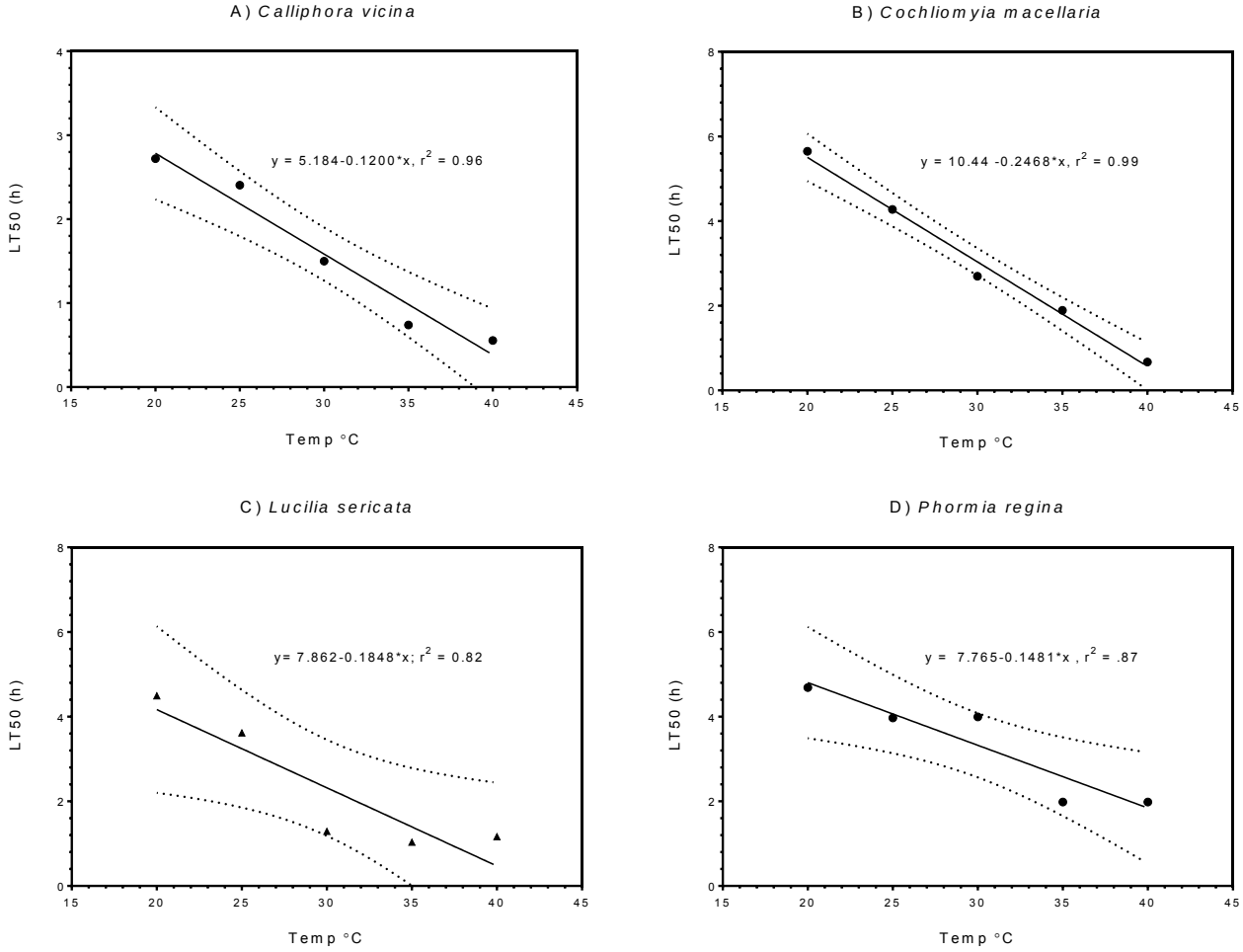
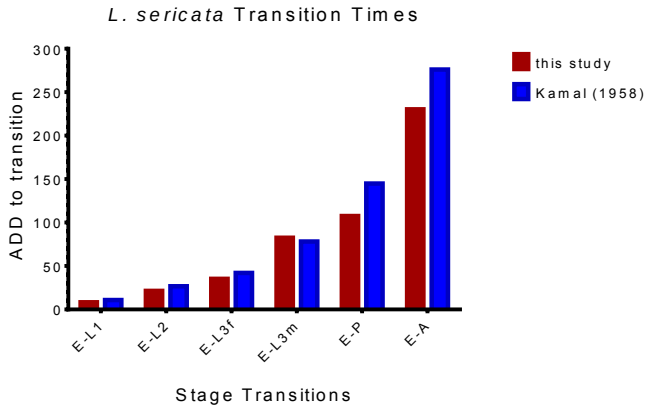
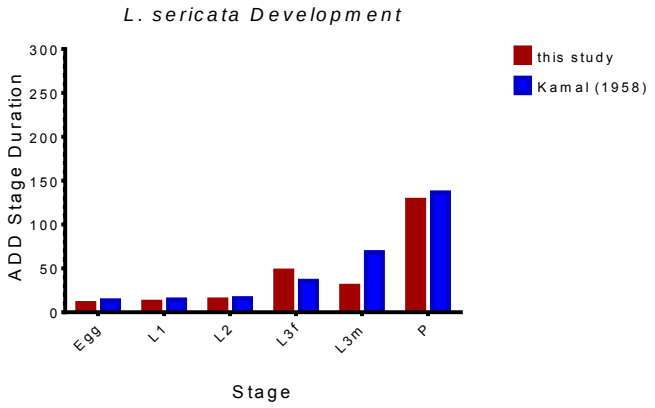
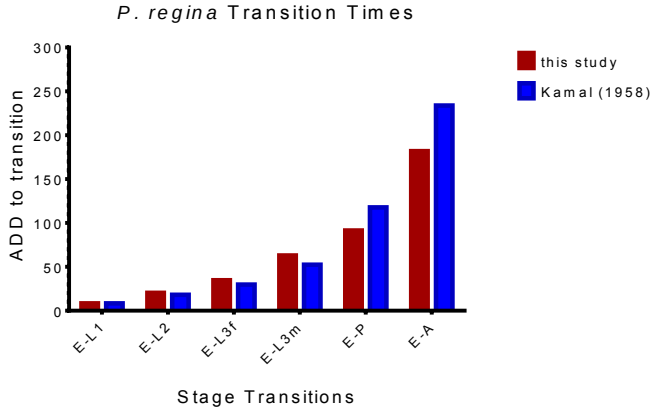
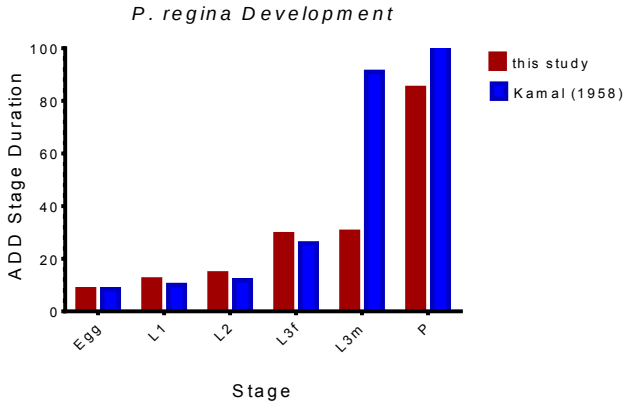


Fig. 71. Comparison of results from this study at 27.5° C versus data from Kamal (1958) at 26.7° C, with degree days in Kamal calculated from 50% transition to 50% transition (with points calculated from minima and maxima data) and using developmental minima calculated in this study.









Conclusions

The development data for *Lucilia sericata* and *Phormia regina* represent the most comprehensive datasets ever obtained for forensic insects; indeed the data obtained for just one of these species exceeds that of all published development data for all other forensically important blow flies combined. With these data it has been possible to develop a new method for calculating conventional degree days that ensures internal validity within known confidence levels. Additionally, data allowed the development of curvilinear models that avoid the intrinsic limitations of linear methods like degree days. Developing these models was the core objective of this study, and similar datasets for additional species are needed. Our stated intention was to develop such datasets for at least five blow fly species, and this remains our goal. Unfortunately, the complexity and difficulty of getting these data means that work on additional species (specifically, *Calliphora vicina*, *Chrysomya megacephala*, and *Cochliomyia macellaria*) will continue beyond the end of this project. Because *Lucilia sericata* and *Phormia regina* are thought to be the fastest developing blow fly species in North America (Kamal 1958), the developmental data and model of *L. sericata* is especially valuable in putting a lower limit on potential development times of any North American species (*L. sericata* is of more practical value than *P. regina* because *P. regina* oviposition occurs later after death than with *L. sericata*). This point is especially pertinent in cases where blow fly species identification is not possible.

Characterizing stage transition periods provides a new tool for assessing the age of sampled insects in various forensic situations. The collection of samples with only one stage or mixed stages can be used with the known transition data to reduce the uncertainty of insect ages within a stage. Practically, this technique can improve estimates by hours to days depending upon the insect stages and ambient temperatures. Additionally, the importance of stage transitions in assessing development is not currently indicated in the scientific literature, and in many instances data sets used in estimating the post mortem interval (PMI) offer no indication of transition times. Without an explicit consideration of stage transition times, it is possible to systematically over or under estimate a PMI. Obtaining detailed data on stage transitions was the most time consuming, expensive, and frustrating aspect of our research, so identifying more efficient methods would greatly accelerate research on this aspect of development.

Finding that the frequency of stage transitions follows a normal distribution (for all stages and species examined) has immediate and long term implications. Many forensic entomologists have argued that samples should be made of the "largest" larvae and that developmental models should be based on modes rather than means. Neither of these points is valid, and based on the evidence here we can show that such methods will bias estimates of PMI. In developing statistically valid sampling procedures, the normal distribution of stage transitions and time in stage will make sampling procedures relatively easy, provided sites of initial oviposition are sampled.

Besides the long duration of stage transitions, a number of other findings were surprising and potentially important. Although all experiments were conducted with fly populations with little or no genetic variability, ample evidence of individual variation was seen in as aspects of development with all species examined. If sex differences in larval development were behind this variation, then our frequency distributions for stage transitions or time in stage should have should evidence of bimodal distributions. In contrast, all transitions significantly fit normal distributions, which is what one would expect if individual variation in development is greater than variation by sex. Consequently, differences between males and females does not account for this variation. We have not yet looked at development of eggs from single females and confirmed lack of genetic variation through DNA testing, however, preliminary studies support the idea that blow fly development and larval behavior includes significant intrinsic variation. We see this variation manifested in stage-

specific development times, larval movement, anoxia tolerance, and, particularly, migration at the L3m stage. If confirmed, this variation is itself an important phenomenon of interest from genetic, physiological, ecological, and evolutionary perspectives. Moreover, accounting for this variation will likely place an absolute limit on the potential accuracy of PMI estimates from insect development.

The confirmation from field and laboratory observations that blow fly larvae feed at night is helpful in establishing that developmental regulatory processes do not alter feeding behaviors, at least as pertains to day/night cycles. Similarly, our observations that stage transitions occurred throughout a 24-h cycle disproves hypotheses that stage transitions were set by light or circadian rhythms in blow flies.

Development data for *L. sericata* and *P. regina* both demonstrate that development in the migratory third stage does not increase with temperature. Instead, both species show a threshold temperature of activity (ca. 17.5° C) below which development is serious delayed. Consequently, the application of any temperature driven development model for this larval will result in errors in the resulting development time and PMI estimates. To the best of our knowledge this is the first demonstration of temperature insensitive development, and application of this information will improve PMI estimates in virtually all situations involving the L3m stage.

Regarding maggot survival in maggot masses, our experiments on anoxia tolerance indicate that blow flies have relatively low tolerance of anoxic conditions. However, the LT50's for all species tested were in the 1-6 hour range, therefore it seems more likely that larval movement within a mass is associated with temperature regulation than oxygen availability. Figure 70 shows strong linear relationships between survival times and temperature. These data) 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. 70).

Additional data analysis is being conducted on survivorship and temperature, development at high temperatures and potential involvement of heat shock proteins, and field and historical validation of development models. Most direct comparisons of our results with literature data are not possible (because investigators used different sampling procedures, reported values (like modes) that are not directly comparable, or have too few data points to allow a meaningful comparison.) Fortunately, data from Kamal (1958) do allow a comparison as shown in Fig. 71. We were able to take data from Kamal's 1958 publication, calculate means and transition times, and then calculate accumulated degree days using developmental minima we determined. We don't have data from Kamal's individual replications, so we can't do a statistical comparison, however, values are remarkably similar, and this agreement occurs in both *L. sericata* and *P. regina*. The greatest variation with the L3m stage, which isn't surprising in that L3m has the most variability of any stage. Overall, the general agreement of our data with that of Kamal has potentially important implications. Kamal's

data were taken over 60 years ago and from a population (in Washington) that was over 1300 or 2000 miles distance from the blow fly populations we used to establish our colonies. These observations strongly imply that blow fly development is not as geographically variable as some workers suggest. Coupled with our data documenting the underlying variation in development within (what should be) genetically uniform individuals, we are increasingly skeptical that levels of reported geographical variation make sense. Consequently, the question of population differences in development merits a critical reexamination, with particular focus on experimental methods (given the difficulty in accurately determining developmental times for specific developmental events).

Implications for policy and practice.

In any death investigation, the time of death is an important element. In cases where the victim's remains have decomposed, however, time of death assumes an even greater role. Without direct observation of the crime, timing becomes critical for everything from identifying potential suspects and examining alibis to distinguishing primary and secondary crime scenes. Because blow flies are one of the key biological agents of decomposition, and because it is possible to relate their development closely to time of death, insects often are the only potential indicator of time of death.

The datasets for *Lucilia sericata* and *Phormia regina* provide the most comprehensive data on development of any forensically important insect species. Without question development calculations using these data will produce more accurate and precise PMI estimates. Additionally, these estimates can now be made with statistically valid estimates of variability. Of potentially greater importance are our findings regarding L3m temperature-insensitive development, normal distributions of larvae during stage transitions, durations of transitions, and intrinsic variability in development. We have already used these data in one on-going civil case (myiasis) and will be using data in homicide cases in the near future. The analysis of confirmed time of death cases is underway, to further validate both our data and approach.

Implications for further research.

Our on-going work, not reported here, includes validation of our developmental models with field and known time-of-death case data, development of *C. vicina*, *C. megacephala*, and *C. macellaria*, examinations of genetic variation and underlying variability in development, larval competition, detailing sampling procedures, and the role of heat shock proteins in development at high temperatures.

The most obvious need is to develop comparable datasets on development of other forensically important blow flies. Our results highlight the importance of measuring stage transitions precisely, but our experience also highlights the difficulties in making such measurements. Hundreds of hours of research time taking measurements literally around the clock presents a significant barrier in obtaining data for other species. One approach we are beginning to explore is the potential use of video for observing individual maggots and stage transitions through time. While obtaining sufficient video equipment might be one problem, the current issues are having sufficient resolution to see and identify larvae, accommodating larval movement, and distinguishing individual larvae in images. One potential solution is to develop single larva experimental units, but this requires developing rearing procedures so that larval development is unaffected by larval numbers (if this is possible).

Many other research areas are indicated from our results, some of which we mentioned previously. Work on genetic variability and intrinsic variation in development, temperature and L3m development, and whether or not geographic variation in develop exists are obvious topics. As our understanding and data on temperature and insect development become more detailed, we suspect

new opportunities for applications of thermal modeling (perhaps of soil or surface bacteria, or of autolytic processes) may be possible. Our results indicate that it is possible to greatly improve our precision and accuracy of insect development estimates which necessarily improves the associated PMI estimates. If we can apply some of these same procedures to other taxa associated with decomposition, we think greater improvements in PMI estimation are possible.

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Dissemination of Research Findings

Publications

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Roe, A. L. 2014. Development modeling of *Lucilia sericata* and *Phormia regina* (Diptera: Calliphoridae). Ph.D. dissertation. University of Nebraska-Lincoln, Lincoln, NE.

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