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Effects of Amitriptyline and Nortriptyline on Time of Death Estimations in the Later Postmortem Interval Using Insect Development

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Glossary and Abbreviations

Please note that this glossary includes terms from both the present report and the report entitled "Method Validation for Amitriptyline and Nortriptyline in Artificial Foodstuff."

Acceptance Criteria: Acceptance criteria specify the acceptance/rejection thresholds for parameters, such as accuracy and precision, which are described for a given analytical method during the process of method validation.

Accuracy: How closely the measurement result agrees with the true value (EURACHEM Working Group, 1998).

Amitriptyline: Tricyclic antidepressant. The chemical formula for amitriptyline is $C_{20}H_{23}N$. The molecular weight of amitriptyline is 277.41 amu (Budavari *et al.*, 1996).

For an illustration of the chemical structure of amitriptyline see Appendix A.

AMT: Amitriptyline.

Analytical Toxicology: An area of scientific inquiry that is concerned with the detection, identification and measurement of drugs and other poisons in biological materials, such as blood, and other relevant substances (e.g. pill residues). The purpose of such investigation is to assist in the diagnosis and treatment of poisoning. In some situations, information derived from toxicological inquiries may be used to prevent future poisoning (Flanagan, 1993).

Analysis of Variance: In analysis of variance, the mean and variance of two variables are used to determine if the measured difference between the variables is statistically significant (Rutherford, 2001). ANOVA makes several assumptions concerning the data, including that the data compared should be continuous and normally distributed with equal variances.

Analyte: The component or components present in the sample, for which the analysis was conducted (Skoog *et al.*, 1996).

ANOVA: Analysis of Variance.

Apolysis: The separation of the old skin from the new skin. For example, at the beginning of the pupal stage, the outer skin of the larva detaches from the inner wall of the puparium (Greenberg and Kunich, 2002).

Autoinjector: An autoinjector is an instrument that injects programmed sample volumes with a high degree of accuracy and precision (Sadek, 2000).

Band Broadening: In reference to a **chromatographic band**. Band broadening is the increase in the baseline width of a chromatographic peak, as the solute attributed to that peak moves from the point of injection to the detector (Harvey, 2000).

Bartlett's Test: A statistical test that is used to test for unequal variances. Bartlett's test assumes that the data is normally distributed; therefore, Bartlett's test is sensitive to departures from the normal distribution (Sall *et al.*, 2001).

Baseline: The baseline is the chromatographic trace formed when the response of the detector is due only to the injection of the mobile phase into a fully equilibrated chromatographic system (Sadek, 2000). All chromatographic parameters, such as peak height and peak area are dependent on the accuracy of the baseline (Sadek, 2000).

Blank Correction: A blank correction is done when the calibration curve prepared does not accurately take into account the baseline noise (i.e. the curve was not prepared in a matrix of either the same, or similar, composition to the sample matrix. Blank correction involves subtraction of the signal present in the blank (analyte free), at a given retention time, from the signal present at the same retention time in the sample.

Box and Whisker Plot: A box and whisker plot presents the median of a data set as a thick bar, and the interquartile range as a box. The range for the data set is illustrated using two lines extending from the top and bottom of the interquartile box. Outliers, data points that are well outside the range of other data points observed in the data set are illustrated as individual points (Dytham, 2003).

Brown-Forsythe Test: A robust statistical test that is used to detect the existence of unequal variances. The Brown-Forsythe test measures the differences from the median, and then tests those differences using an F-test (Sall *et al.*, 2001).

Calibration Curve: A calibration curve is a plot of detector response *versus* analyte concentration. The ideal calibration curve passes through the origin. Furthermore, in an ideal calibration curve a directly proportional relationship exists between concentration (independent variable) and detector response (dependent variable). In

addition, the ideal calibration curve is constructed from evenly spaced data points that bracket the entire expected analyte range (Sadek, 2000).

Calliphoridae: Family of carrion feeding flies that are commonly referred to as blow flies (Greenberg and Kunich, 2002).

Chromatography: is an analytical method where the components of a mixture are separated based upon the rates at which they are carried through or over a stationary phase by a gaseous or liquid mobile phase (Skoog *et al.*, 1996).

Chromatogram: A chromatogram is a plot of detector response (y-axis) *versus* time (x-axis). In general, time increases from left to right, and the intensity of the response increases from bottom to top (Sadek, 2000).

Chromatographic Peak: Also referred to as a chromatographic band. A chromatographic peak can be defined mathematically as the distribution of a chemical species about a central value (Skoog *et al.*, 1996). Ideally, chromatographic peaks are symmetrical and exhibit a Gaussian distribution (Skoog *et al.*, 1996). In the present thesis, chromatographic peaks are referred to simply as **peaks**.

CI: Confidence Interval.

Coefficient of Variation: See **relative standard deviation**.

Coeluting: Multiple peaks that elute at the same, or nearly the same, retention time. Coeluting peaks can also be referred to as interferences.

Column: The component of the chromatographic system that performs the separation.

Confirmation of Identity: The process of confirming that a peak present in a chromatogram, that has been attributed to be due to the presence of a specific analyte, is in fact, due to the presence of that analyte, and that analyte alone (EURACHEM Working Group, 1998).

Constant Systematic Error: With constant systematic errors, the error is independent of the concentration of the analytes analyzed. The result of the presence of a constant systematic error is the parallel displacement of the calibration curve with respect to the ideal calibration curve (i.e. y-intercept = zero). Constant systematic errors may result from the co-detection of a matrix component, and indicates that the specificity of the method must be improved (Funk *et al.*, 1995).

Crop: A region of the foregut in insects that serves as a food storage organ in fly larvae (Greenberg and Kunich, 2002).

Developmental Maximum: The highest temperature at which insect development will still occur (Higley and Haskell, 2001).

Developmental Minimum: The lowest temperature at which insect development will still occur (Higley and Haskell, 2001).

Discontinuous Batch Extraction: Liquid-liquid extraction where the extracting solvent is added and then removed in discrete steps.

Efficiency: A measure of column suitability or goodness (Sadek, 2000).

Elution: Elution is the process in which solutes are pushed through the stationary phase by movement of the mobile phase (Skoog *et al.*, 1996).

Eluent: An eluent is the solvent that is used to transport the components of a mixture through the stationary phase. Synonymous with mobile phase (Skoog *et al.*, 1996).

Entomotoxicology: Entomotoxicology is a relatively new area of research that can be included under the auspices of forensic entomology. Investigations within the area of entomotoxicology include (Introna *et al.*, 2001):

1. studying the effects of drugs and other toxins on the developmental rate of forensically important insects
2. using insects as alternative toxicological specimens when traditional specimens, such as blood and liver, are no longer available or suitable for analysis.

Exoskeleton: Skeleton outside the body.

Extraction: The process of isolating one or more components from the bulk of the matrix.

Forensic entomology: The application of entomology to law.

Fundamental Analytical Procedure: Procedure in which only analyte standards in pure solvents are analyzed (Funk *et al.*, 1995).

Fundamental Calibration Curve: Calibration curve generated from the **fundamental analytical procedure** (Funk *et al.*, 1995).

Gas chromatography: Chromatographic technique that employs a gaseous mobile phase and a solid or liquid stationary phase (Skoog *et al.*, 1996).

GC-NPD: Gas Chromatography using a nitrogen-phosphorous detector.

Holometabolous: Complete metamorphosis. Immature individuals (e.g. larvae) do not resemble the adults (Elzinga, 2000).

Homogeneous: A material of uniform consistency and composition.

Imaginal discs: Imaginal discs clusters of embryonic cells in larvae that remain viable after the majority of the larval structures are destroyed during the pupal stage. The imaginal discs give rise to adult structures such as legs and wings (Greenberg and Kunich, 2002).

In-House Quality Control: A quality control that is prepared according to strict guidelines within a laboratory. For a specimen to be considered a proper in-house quality control, the control must be prepared by someone other than the person conducting the analyses requiring the quality control.

Injection: The process of introducing a sample of known volume in the chromatographic system (Sadek, 2000).

Instar: An instar is the period of time between larval moults. Fly larvae generally have three larval instars, and therefore two moults (Greenberg and Kunich, 2002).

Integument: Synonymous with **exoskeleton**.

Interference: Components present in the sample that interfere with the signal attributed to the analyte. For example, substances that coelute with the analyte of interest are interferences.

Intermediate Precision: According to Snyder *et al.*, (1997), intermediate precision refers to the agreement between complete measurements, of both standards and samples, when the same method is applied several times in the same laboratory. The evaluation of intermediate precision may require multiple analyses of samples and standards within the same day or on different days, depending on the frequency in which the method will be used.

Internal Standard: A known quantity of a chemical species that is added to the sample under investigation, at the beginning of the sample preparation procedure. The concentration of analyte present in the sample is calculated using the ratio of the analyte response to the internal standard response (Skoog *et al.*, 1996). The chemical species chosen as the internal standard should have chemical and physical properties similar to that of the analyte, and should respond to the chromatographic detection system in a manner similar to that of the analyte(s) (Robards *et al.*, 1994). Further, the internal standard must be well separated from other sample components, but still

be relatively close to the peak(s) attributed to the analyte(s) of interest. In addition, the internal standard must not react with any components of the sample, and must not be found in the sample as a common constituent (Robards *et al.*, 1994). Furthermore, according to Robards *et al.* (1994), the internal standard should be incorporated into the sample in exactly the same way as the analyte(s); however, such an ideal is not normally achieved in analytical practice. Internal standards are commonly used in chromatographic analyses because their use minimizes the uncertainty introduced by variations in the chromatographic system (e.g. variations in sample injection, flow rate, and column condition) (Skoog *et al.*, 1996).

Interquartile Range: The interquartile range is a measure of spread in a data set. The interquartile range is determined by placing the data in rank order, and then selecting the range that encompasses the data from the 25% value to the 75% value (Dytham, 2003).

Isolation Phase: The phase in an analytical procedure that involves the separation of the target analyte from the bulk of the matrix in which it was held. For example, extraction techniques for part of the isolation phase. Chromatographic techniques, which separate the components of a mixture according to physical and chemical properties of the analytes themselves can also be viewed as part of the isolation phase.

Larva: The actively moving and feeding stages of immature flies. The series of stages before the pupa, but after the egg (Greenberg and Kunich, 2002). Synonymous with **maggot**. Plural form is larvae.

Larviparous: Female flies that deposit first instar larvae rather than eggs. These insects can also be referred to as **ovoviviparous**.

Larviposition Media: The food substrate introduced to an insect species to stimulate the deposition of larvae. Liver is a commonly used larviposition media in carrion insects, such as *Sarcophaga bullata*. Often, the substrate used as the larviposition media is the same substance that is used as the rearing media.

Least Squares Method: According to Rutherford (2001), least squares estimation is the most frequently applied method of parameter estimation. The method of least squares minimizes the sum of the squared differences, called **residuals**, between the measured values and predicted values. Proper application of least squares estimation requires that the data be normally distributed (Rutherford, 2001).

Levene's Test: A statistical test that tests for unequal variances. Levene's test estimates the mean of the absolute differences from each group, and then tests the estimates of the means using an F-test (Sall *et al.*, 2001).

Limit of Detection: The limit of detection is the lowest concentration of analyte that can be statistically distinguished from a sample that does not contain the analyte (Wu *et al.*, 1999).

Limit of Quantitation: The limit of quantitation is the lowest concentration of analyte that can be quantitated with a predetermined level of statistical confidence (e.g. 95% confidence) (Wu *et al.*, 1999).

Linearity: The linearity of a method is a measure of how well a plot of signal response *versus* concentration approximates a straight line (Snyder *et al.*, 1997). The magnitude of the coefficient of determination (R^2) is the parameter used to describe the linearity of a method. For most analytical work, an R^2 value of 0.95 or better is required.

Linear Regression: A regression technique that assumes the relationship between two variables is best described by a straight line (Dytham, 2003).

Liquid-liquid Extraction: Liquid-liquid extraction (LLE) is a method of sample preparation in which an excess of inert, water immiscible organic solvent is used to isolate the analytes from an aqueous solution. The extraction of the analytes from the aqueous solvent must be done at an appropriate pH. The organic solvent and the aqueous solution containing the analytes are then mixed together to facilitate the distribution of the analytes to the organic solvent. The two layers are usually separated after mixing by centrifugation. The excess solvent is usually removed under a stream of nitrogen gas, and then the dried extract is reconstituted using a small volume of an appropriate solvent (Flanagan, 1993).

LOD: Limit of Detection.

LOQ: Limit of Quantitation.

Maggot: See **larva**.

Maprotyline: Tricyclic antidepressant. The chemical formula for maprotyline is $C_{20}H_{23}N$. The molecular weight of maprotyline is 277.41 amu (Budavari *et al.*, 1996).

For an illustration of the chemical structure of maprotyline see Appendix A.

Matrix: The matrix is the material that encases the analytes.

Measurement Phase: The phase in an analytical procedure where the analyte(s) isolated from the bulk of the matrix are measured either qualitatively (i.e. identified) or quantitatively (i.e. identified and quantitated).

Metamorphosis: The process by which an organism changes from one shape to another during the life cycle (Elzinga, 2000).

Method Validation: The process by which it is established, by laboratory studies, that the performance characteristics of a particular analytical method are described and are confirmed to be appropriate for the intended purpose of the method (EURACHEM Working Group, 1998).

Mobile phase: The chromatographic phase that moves over or through the stationary phase. The mobile phase carries the analyte through column or across the surface of the stationary phase (Skoog *et al.*, 1996).

Moulting: The process by which insects and other arthropods shed their exoskeleton (Elzinga, 2000).

Necrophagous:An organism, such as an insect, that feeds on that on carrion or other decomposing animal material.

Nitrogen-phosphorous Detector: The nitrogen-phosphorous detector consists of a heated ceramic bead that is coated with an alkali metal such as rubidium or cesium. The alkali metal, when heated, promotes the selective ionization of compounds containing nitrogen or phosphorous (Stafford, 1992). The use of a specific detector, such as the nitrogen-phosphorous detector can significantly increase the sensitivity of the chromatographic system to compounds containing nitrogen or phosphorus. However, the use of a selective detector cannot replace selective sample preparation techniques; components that are present in the sample, but are not detected because of their chemical composition may still interfere with the chromatographic separation (Stafford, 1992).

Noise: Any disturbance in the system that results in a detector response that is not generated by either components of the matrix or by the analytes themselves (e.g. electronic noise and random noise) (Sadek, 2000).

Nortriptyline: Tricyclic antidepressant. The chemical formula for nortriptyline is $C_{19}H_{21}N$. The molecular weight of amitriptyline is 263.38 amu (Budavari *et al.*, 1996).

For an illustration of the chemical structure of nortriptyline see Appendix A.

NPD: Nitrogen-phosphorous detector.

O'Briens Test: A statistical test that is used to test for unequal variances. In O'Brien's test, the variances are treated as means, and then evaluates the variances using an *F*-test (Sall *et al.*, 2001).

Outlier: An anomalous observation that lies well away from the rest of the observations in a given data set (Dytham, 2003).

Ovoviviparous: Insects in which the eggs hatch within the body of the female fly (Elzinga, 2000).

Peak: See **Chromatographic Peak**.

Peak Area: The area under a chromatographic peak, determined by integration.

Peak Height: The height of a chromatographic peak, calculated from the baseline to the apex of the peak.

pA: A unit of detector response (picoamps).

pH: A unit of hydrogen activity.

Poikilotherm: An organism that lacks a temperature regulating system, and as a result has a body temperature that varies with the temperature of its surroundings (Elzinga, 2000).

Postmortem Interval: The length of time between the death of an individual or animal and the discovery of the corpse by humans (Catts and Goff, 1992). Synonymous with time since death, and elapsed time since death.

Postfeeding Larva: Behaviourally distinct stage in the third instar of a fly. During the postfeeding stage the fly larvae ceases to feed, and migrates away from the food source. During the postfeeding stage, the gut contents are digested, and a single layer of fat cells is deposited underneath the cuticle (Greenberg and Kunich, 2002).

Precision: Precision refers to the level of agreement among individual test results, where each result is generated by repeatedly applying the same procedure, from sampling to analysis, to a homogeneous sample (Snyder *et al.*, 1997).

Prepupa: A relatively short, behaviourally distinct stage within the third instar during which the puparium begins to harden and darken. Plural form is prepupae.

Proportional Systematic Error: With proportional systematic errors, the error is dependent on the concentration of the analytes analyzed. The result of the presence of a proportional systematic error is the deviation of the slope from the ideal (i.e. slope = 1). Proportional systematic errors may result from problems caused during individual sample preparation steps, such as matrix digestion (Funk *et al.*, 1995).

Pupa: The intermediate stage between larva and adult in holometabulous fly species

(Elzinga, 2000). During this stage the majority of the larval tissues are destroyed in order to form adult structures, such as legs (Greenberg and Kunich, 2002). Plural form is pupae.

Pupal Stage: The intermediate stage between larva and adult in holometabulous fly species (Elzinga, 2000).

Pupariation: The process that shrinks the postfeeding maggot, and makes it skin dark shiny and brittle (Greenberg and Kunich, 2002).

Puparium: The puparium is the last larval exoskeleton. The pupa detaches from the interior of the puparium during pupariation. In addition, during pupariation, the puparium becomes hard and brittle, and changes from white to dark brown in colour (Elzinga, 2000).

Pupation: The formation of the pupa within the fly puparium (Greenberg and Kunich, 2002).

Quadratic Fit: Also referred to as second order polynomial regression. Regression technique where the relationship between the two variables is assumed to be best described by a quadratic equation (Dytham, 2003).

Range: The range, in terms of analyte concentration, is defined as the lower and upper analyte concentrations for which the analytical method has satisfactory precision, linearity, and accuracy (Snyder *et al.*, 1997).

Recovery Function: Plot of extracted (recovered) concentration *versus* spiked (actual) concentration. Used to investigate constant systematic errors and proportional systematic errors in sample preparation procedures (Funk *et al.*, 1995).

Reference Standard: Most reference standards are obtained from the *National Institute of Standards and Technology* (NIST) (Skoog *et al.*, 1996). Reference standards are materials of known composition that have been analyzed extensively by a variety of different analytical methods, and are certified to contain certain levels of analyte (Skoog *et al.*, 1996).

Regression Analysis: According to Rutherford (2001), regression analysis is a statistical technique that attempts to explain the dependent variable(s) in terms of the independent variable. In regression analysis, the calculated relationship between two or more independent variables and a dependent variable is referred to as the model, and the portion of the data that the model does not explain is referred to as the residual component, or error (Rutherford, 2001). In analytical work, regression analysis is commonly used to compute the line of best fit for a set calibration data (Skoog *et al.*, 1996).

Relative Retention Time: The retention time of the analyte(s) with respect to the retention time of the internal standard. The relative retention times should remain constant even if the actual retention times for each analyte drift slightly with variations in the chromatographic system.

Relative Standard Deviation: Synonymous with **coefficient of variation** when the relative standard deviation is expressed as a percentage. The percent relative standard deviation (%RSD) is computed by dividing the standard deviation by the mean, and then expressing that value as a percentage (Skoog *et al.*, 1996). The %RSD is useful because it incorporates both the standard deviation and the mean of a data set in a single measure. However, the %RSD can only be used to compare normally distributed data (Lang and Secic, 1997).

Repeatability: Repeatability describes the precision of an analytical method over a short period of time (Snyder *et al.*, 1997)

Reproducibility: Reproducibility measures the precision of an analytical method between different laboratories (Snyder *et al.*, 1997).

Residual: The variation in the data left over after a statistical model has been applied to the data set. The model exhibiting the best fit for a given data set is the model that produces the smallest amount of residual variation (Dytham, 2003).

Resolution: The degree of separation between two adjacent chromatographic peaks (Harvey, 2000).

Retention Time: The time required for the mobile phase to move the analyte from the time of injection onto the stationary phase, through the stationary phase, and to the detector. The retention time is determined from the apex (signal maximum) of the analyte peak (Dean, 1995).

Sample: (1) The material containing the analytes of interest (Sadek, 2000), (2) The solution, ready to be introduced into the chromatographic system that contains the analytes of interest (Sadek, 2000), (3) The portion of the population taken for statistical analysis (Dytham, 2003).

Sample Preparation: The steps required to ready a sample for analysis.

Sarcophagidae: Family of carrion feeding flies that are commonly referred to as flesh flies (Greenberg and Kunich, 2002).

Selectivity: 100% specificity.

Silylation: Derivatization process that uses a silane as the derivatizing agent. Sily-

lation is commonly used to deactivate the silanol groups present on chromatographic system components such as the glass liner in the injector.

Silanol: The terminal functional group on a silica stationary phase or other silica containing sorbent. Si-OH (Sadek, 2000). Free silanol groups are responsible for silanophilic interactions.

Silanophilic Interaction: Interactions that result from hydrogen bonding between the solute and surface silanol groups. Silanophilic interactions are a major cause of tailing peaks in the chromatographic separation of basic compounds, such as amines (Sadek, 2000).

Slope: A value that describes how a trend line deviates from zero (Dytham, 2003).

Specificity: A term that describes the ability of a method to accurately measure the concentration of analyte in a sample in the presence of other sample components (Snyder *et al.*, 1997).

Spiking: Addition of a known amount of a chemical species.

Spiracle: An opening in the rear-end of a larva's body through which air passes into the tracheae or breathing tubes of the insect (Greenberg and Kunich, 2002).

SD: Standard Deviation

Standard Operating Procedure: A written authorized procedure which gives instructions for performing an analytical procedure.

Stationary Phase: the chromatographic phase the remains fixed either in a column or on a planar surface (Skoog *et al.*, 1996).

Tailing Peaks: A peak with the tail at the end of it is referred to as a tailing peak. Tailing peaks usually result from the presence of active sites in the stationary phase (Harvey, 2000).

Thermal History: Term used by Greenberg and Kunich (2002) to describe the temperatures experienced by larvae feeding on animal carrion prior to discovery of the carrion by humans. Accurate knowledge of an insect's thermal history is essential to the accurate and precise estimation of the postmortem interval.

Volume of Distribution: The volume of distribution is the apparent volume in which a substance (e.g. a drug) is distributed, following absorption and allocation to different tissues within the body (Klaassen and Watkins, 1999). The volume of distribution is an apparent volume that is calculated based on the concentration of

the substance in the blood. The volume of distribution does not correspond to a real value; therefore it is not directly meaningful from a physiological perspective (Medinsky and Klaassen, 1996). A large volume of distribution, sometimes even greater than the total volume of the body, is a well-established characteristic of a substance with a high tissue affinity (Medinsky and Klaassen 1996). Chemicals with high tissue affinity are likely to accumulate within tissues with high blood-flow to mass ratios, such as the liver, heart and lungs (Stine and Brown 1996). Examples of substances with high volumes of distribution are amitriptyline and nortriptyline.

Chapter 1

Introduction

1.1 Forensic Entomology

The accurate estimation of the **postmortem interval** is extremely critical to the successful completion of death investigations, both criminal and noncriminal. For example, knowledge of the postmortem interval may reduce the number of suspects in a homicide investigation, or in cases where the identity of the deceased is not known, knowledge of the time of death may aid in the identification of the deceased. At the present time, there are several methods available for estimating the postmortem interval. Most of these methods are based on changes that occur to the corpse after death. These changes can be collectively termed postmortem changes (Buchan and Anderson, 2001). In the early postmortem period[1] these processes include *livor mortis*, *algor mortis*, *rigor mortis*, autolysis and putrefaction (Kashyap and Pillay, 1989). In the later postmortem interval, which begins approximately 72 hours after death, animal carrion passes through a series of decompositional stages, including: fresh, bloat, active decay, advanced decay, dry decay and remains (Payne 1965). These stages are easily recognizable, but the boundaries between each stage are diffuse and overlap considerably (Campobasso *et al.*, 2001). Furthermore, their rate of progress can be affected by a number of factors, including humidity, temperature, the presence or absence of clothing, burial and depth of burial (Buchan and Anderson, 2001). As a result, the accurate determination of the onset and duration of these stages may be considerably difficult, thus hindering the accurate and precise estimation of the postmortem interval.

Forensic entomology is the most well-researched method of determining the time since death in the later postmortem interval (Buchan and Anderson, 2001), and can be defined as the application of the study of insects and other arthropods to investigations of violent crimes such as rape, physical abuse, and murder (Keh, 1985; Catts and Goff, 1992). In forensic entomology, the arthropod evidence associated with the corpse is most often used to estimate the elapsed time since death, or postmortem

[1]The early postmortem period lasts up to 72 hours after death.

interval (Catts and Goff, 1992).

Numerous living organisms contribute to the decomposition of animal carrion, including insects, fungi, bacteria, and other scavengers such as rodents. Human or other animal carrion, when viewed from a biological perspective is a rich source of proteins, lipids, carbohydrates and other nutrients (Erzinclioglu, 1992). However, insects remove soft tissue from animal carrion with remarkable speed and completeness (Haskell *et al.*, 1997). As a result, in terms of carrion decomposition, insects are considered to be the most influential group of living organisms (Haskell *et al.*, 1997). For instance, animal carcasses left exposed and uncovered can lose up to 90% of their body weight within seven days during the summer depending on the location and treatment of the corpse[2] (Lord and Rodriguez, 1989).

At present, there are two approaches available for estimating the postmortem interval using insect evidence, and the application of either one largely depends on the state of decomposition of the corpse at the time of discovery by humans. The first method involves the analysis of the pattern of colonization of the carrion by successive waves of insects and other arthropods (Haskell *et al.*, 1997). The second method relies on the development of immature flies that are deposited on the carrion shortly after death. Insect succession can generally be used from about one month after death to one year after death, or until the carrion is completely skeletonized (Anderson, 1999). In contrast, insect development can be used up to one month after death, but rarely any longer than one month. After this time period, the immature stages that were deposited shortly after death would have already completed their development, and therefore, would no longer be useful for estimating the postmortem interval (Anderson, 1999). However, the times given for each method are general; and are based on the assumption that the insects arrived at the corpse within a few hours after death. The use of either approach will also depend on factors such as season, climate, location of the corpse[3] and treatment of the corpse[4].

Postmortem interval estimates, calculated using insect development data, are generally more precise than the estimates generated using insect succession data (Haskell *et al.*, 1997). The differences in the precision of the two methods is largely due to the difference in time frames over which the two methods are applied. According to Haskell *et al.* (1997), estimates of the postmortem interval can be within 12 hours or less of the actual time of death when the remains have been exposed for at least 15-20 days. Even so, the calculation of accurate and precise estimates of the postmortem interval requires extensive knowledge of the life histories of the insects present, the ability of the insect species to arrive at the corpse shortly after death, and the ability of the insect species to then deposit their offspring on the corpse (Haskell *et al.*, 1997).

According to Smith (1986), the insects associated with decomposing animal carrion can be divided into four distinct groups:

[2]e.g. buried or exposed

[3]e.g. indoors or outdoors

[4]e.g. buried, wrapped, submerged, etc.

1. Necrophagous species.
2. Parasites and predators of the necrophagous species.
3. Omnivorous species.
4. Adventive species.

From a forensic perspective, adventive species, which are transient arthropods[5] are not useful for estimating the postmortem interval. The time of arrival of adventive species in the pattern of insect succession cannot be predicted with certainty, because it is not associated with a particular stage of decomposition. Adventive species do not rely on the carrion for sustenance, but rather use the carrion as an extension of their habitat (Smith, 1986).

Omnivorous insects[6] are also, in general, not useful for estimating the postmortem interval because they feed on both the carrion and its inhabitants. However, data on omnivorous insects such as ants may prove useful in conjunction with data from other insects species present on the carrion. For example, the time required for the establishment of an ant colony, specifically a colony of *Anoplolepsis longipes* (Jerdon) (Hymenoptera: Formicidae), was used in conjunction with development data for *Hermetia illucens* (Linnaeus) (Diptera: Stratiomyidae) to estimate the postmortem interval for human remains that were discovered inside a toolbox (Goff and Win, 1997). However, omnivorous insect species usually pose the same problem as adventive insect species in that the time of their arrival in the pattern of insect succession cannot be reliably associated with a particular stage of decomposition (Smith, 1986).

In terms of estimating the postmortem interval, the **necrophagous** insects, and their predators are the most valuable. These species are the carrion feeders, and include members of the Orders Diptera (true flies) and Coleoptera (beetles). Of the many species of Diptera, the blow flies (Family Calliphoridae) and the flesh flies (Family Sarcophagidae) are usually the first colonizers of carrion (Smith, 1986). These insects generally arrive within hours of death depending on the season, weather conditions and location of the corpse (Erzinclioglu, 1983). In situations where blood, body fluids, or both, are present, the insects may arrive within minutes of death (Nuorteva, 1977). In general, species of the Family Calliphoridae arrive first, within hours after death, followed by insects of the Family Sarcophagidae, which generally arrive a few days after death (Turner, 1991). However, there have been some instances where in inclement weather, the Sarcophagidae, which are stronger fliers than the Calliphoridae, have arrived before the Calliphoridae (Erzinclioglu, 1983).

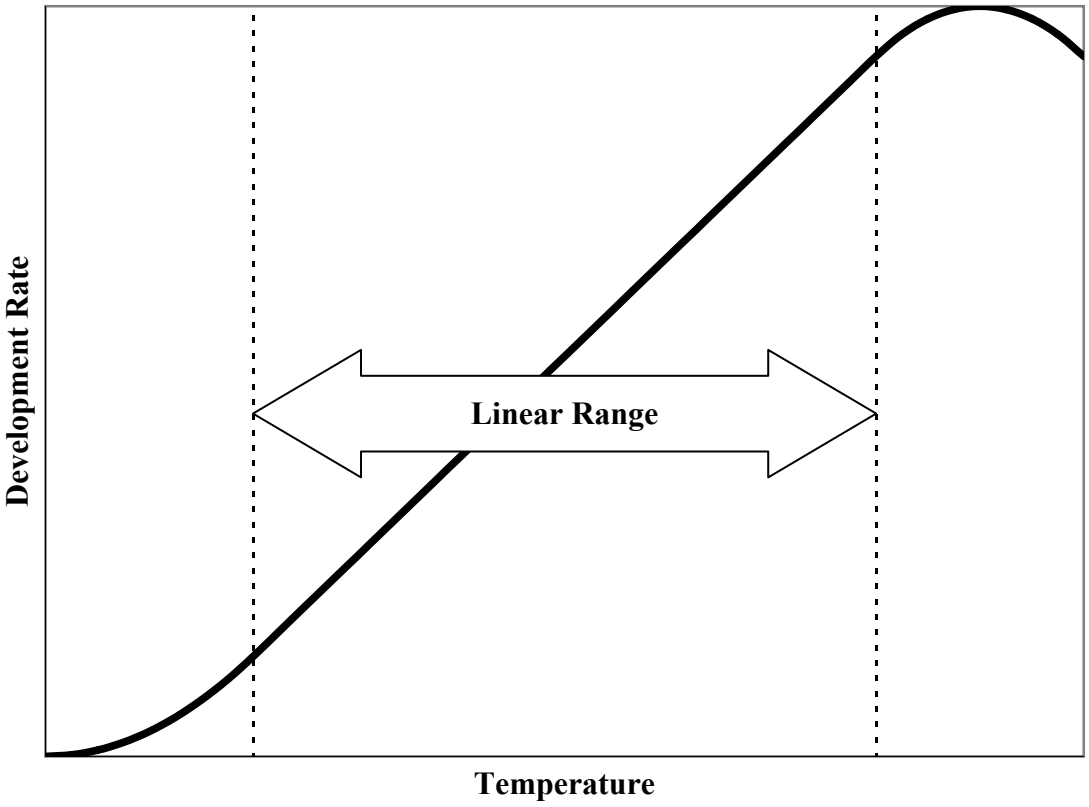


Figure 1.1: Generalized relationship between the rate of insect development and temperature.

1.2 Insect Development

Insects are **poikilotherms**, and therefore they lack a temperature regulating system. As a result, their development is largely dependent on the ambient temperature (Higley and Haskell, 2001). The general relationship between insect development rate and temperature is illustrated in Figure 1.1.

As illustrated in Figure 1.1, the relationship between the rate of insect development and temperature is linear in the middle and nonlinear at the ends (Higley and Haskell, 2001). The curved areas at either end of the generalized development curve represent developmental thresholds, which are the minimum and maximum temperatures at which development will still occur. (Higley and Haskell, 2001). The peak on the right side of the curve represents the highest temperature at which development will occur, and is referred to as the **developmental maximum** (Higley and Haskell, 2001). The trough on the left side of the curve represents the lowest temperature at which development will occur, and is referred to as the **developmental minimum** (Higley and Haskell, 2001). Estimating these developmental thresholds can be very difficult because development is either extremely slow at these thresholds, or occurs at the lethal limits for the species. As a result, maintaining colonies at or near the developmental thresholds is considerably difficult because of high mortality rates at these extreme temperatures (Higley and Haskell, 2001). Although the details of the curve (e.g. the developmental thresholds and the slope of the linear portion of the curve) are species dependent, the development curve presented in Figure 1.1 is similar for all species of Diptera (Higley and Haskell, 2001). Variation within a species further complicates the estimation of the developmental thresholds; therefore, describing the developmental curve for a given species is highly time consuming and labour intensive. Description of the developmental curve for a given species requires developmental data from multiple temperatures, multiple generations and multiple individuals (replicates) (Higley and Haskell, 2001). The accurate and precise estimation of the postmortem interval, by insect development, requires accurate and detailed elucidation of the specific relationship between the external temperature and rate of development for the insect species of forensic interest.

1.3 Insect Development and its Use in Forensic Entomology

Insect evidence discovered at a death scene is primarily used to provide an estimate of the postmortem interval, and the insect evidence most commonly collected, particularly during the early stages of carrion decomposition, are fly larvae (Nuorteva, 1977). According to Catts and Goff (1992), the deposition of offspring on decomposing animal carrion by female flies can be viewed as the activation of a biological

[5]e.g. spiders
[6]e.g. wasps, ants

clock, which can be used to estimate the postmortem interval. Four major requirements govern the use of this biological clock as a means to estimate the postmortem interval, specifically:

1. The species present must be correctly identified.

2. Accurate and precise developmental data must be available for that species. In addition, the development data available must encompass a variety of the temperatures expected for the region where the corpse was discovered.

3. The climatic conditions (e.g. temperature, amount of rainfall) of the location where the corpse was found must be obtained for the time period between colonization and human discovery of the remains.

4. The age of the larvae found at the time of human discovery of the remains must be accurately determined.

Furthermore, accurately establishing the age of the larvae collected from the corpse at the time of discovery is dependent on the accurate determination of the first three requirements listed above.

The generalized life cycle of a flesh fly (Sarcophagidae) is presented in Figure 1.2. Unlike blow flies (Diptera: Calliphoridae), most flesh fly species are **larviparous**, and deposit live first instar larvae rather than eggs. Other than differences in the stage of offspring initially deposited, the development of all species of Diptera is the same and consists of two moults, three feeding stages called **instars**, a **pupal stage** and an adult stage (Greenberg and Kunich, 2002).

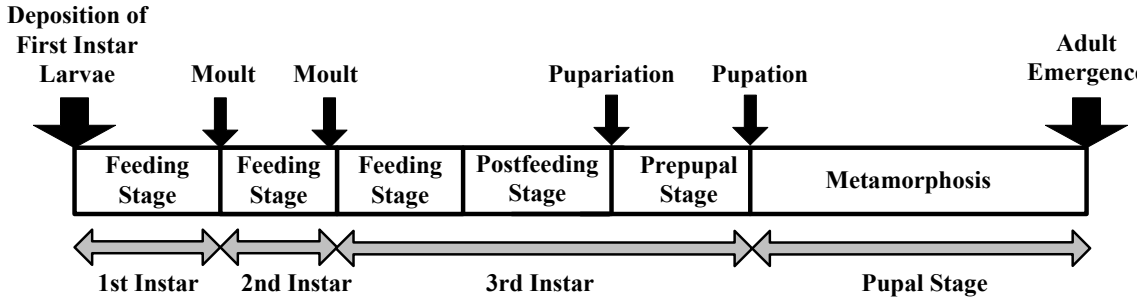


Figure 1.2: Generalized flesh fly (Diptera: Sarcophagidae) life cycle. The size of the boxes representing each stage are not drawn to scale (i.e. the size of the box does not indicate the relative length of the developmental stage).

Adult females generally deposit their offspring in wounds or orifices, such as the mouth and nose, first, because the external tissues in those areas are softer, and, as in the case of wounds, the presence of blood and other body fluids provides a readily accessible source of nourishment to young first instar larvae. First instar larvae are extremely small, and their mouthparts are not strong enough to puncture

hard external tissues such as unbroken skin. The first instar is the first of three feeding stages, and during the first instar the larvae usually increase in size from about 2 mm to 4 mm (Greenberg and Kunich, 2002).

The cuticle, or outer skin of the larva, is quite flexible, and expands considerably within a given stage to accommodate the rapid increase in size that accompanies the voracious feeding of the larva. However, further increases in size require that the larva shed its outer skin in a process called **moulting**. Once a first instar larva moults, it becomes a second instar larva.

The second instar is the second of three feeding stages, and during this stage, the larva grows to about 8 mm in length (Greenberg and Kunich, 2002). More feeding is accomplished in this stage compared to the first instar, largely due to the increased size of the larvae (Haskel *et al.*, 1997). In addition, the pH of the carrion tissue has usually changed by this time, becoming more alkaline. The increased alkalinity of the tissues facilitates the breakdown of connective tissue and muscle, which in turn facilitates the penetration of the tougher tissues by the insects' mouthparts (Haskell *et al.*, 1997). The second instar is typically the shortest in duration of the three feeding stages, lasting approximately 8 to 12 hours in most species at moderate temperatures (Haskell *et al.*, 1997). During the second instar, the **crop**, a food storage organ, becomes visible and starts to increase in size as the rate of food ingested starts to exceed the rate at which food is digested (Greenberg and Kunich, 2002). The cuticle continues to expand to accommodate the rapid growth of the second instar larva, until further increases in size can no longer be accommodated. At this point, the second instar larva moults, and becomes a third instar larva. During the third instar, the larva grows from about 8 mm to between 15 mm and 22 mm, depending on the species (Greenberg and Kunich, 2002).

The third instar can be divided into two, behaviourally distinct stages: (1) the feeding stage, and (2) the postfeeding stage (Anderson, 2000). The feeding stage of the third instar is the last of the three feeding stages, and in this stage, the larvae feed rapaciously. Both the larvae themselves, and their crops increase in size until a maximum size[7] is attained (Anderson, 2000; Greenberg and Kunich, 2002). Once this maximum size has been attained, the third instar larvae stop feeding and wander away from the food source in search of a safe place to pupate (Anderson, 2000). This wandering stage is called the postfeeding stage, and it can last for several days. During this postfeeding stage, the larvae begin to use the food stored within their crops (Greenberg and Kunich, 2002). The size of the crop gradually decreases as the contents are consumed until it is no longer visible. At the same time, an opaque layer of fat bodies are produced under the surface of the **integument** (Anderson, 2000). The formation of opaque fat bodies serves to block the internal structures, including cephalic structures, such as the mouthparts, from view (Catts, 1990). As a result, the white colour of the larval cuticle is transformed to an opaque, creamy whitish yellow.

After several days of wandering, the larvae begin to pupariate (Greenberg and

[7]i.e. the maximum larval size attained is both species dependent and nutrition dependent

Kunich, 2002). **Pupariation** is a developmental process that transforms the pliable whitish-yellow larval cuticle into a hard, brownish-black protective shell (Greenberg and Kunich, 2002). The process of pupariation includes several morphological and physiological changes, including, but not limited to (Greenberg and Kunich, 2002):

1. reduction of the speed of locomotion
2. contraction of longitudinal muscles
3. longitudinal shrinkage of the cuticle
4. hardening and darkening (tanning) of the cuticle

The stage at the start of pupariation is called the **prepupal stage**, and it is relatively short in duration (Greenberg and Kunich, 2002). The prepupal stage should not be confused with the **postfeeding stage**. Pupariation and the formation of the pupa are actually discrete events. In addition, a prepupal larva is still considered to be a third instar larva (Greenberg and Kunich, 2002). The skin of the prepupal third instar larva is called the **puparium**, and after the puparium hardens and darkens, **pupation** begins (Greenberg and Kunich, 2002). During pupation, the insect detaches from the interior surface of the puparium, in a process that is called larval-pupal **apolysis**. At the end of apolysis, the insect, or pupa, is encased in a shell created from its own hardened skin (Greenberg and Kunich, 2002). Approximately 40% of the total development time for a fly is taken up by pupariation and pupation (Catts, 1990).

Since flies are **holometabolous** insects, they undergo complete metamorphosis while in the pupal stage. During the pupal stage, the majority of the larval structures are broken down by **histolysis** (Elzinga, 2000). Adult structures are formed within the hardened puparium at the same time in a process called **histogenesis**. The adult structures are formed from **imaginal discs**, which are regions of embryonic tissue that remain after histolysis (Elzinga, 2000). When metamorphosis is complete, the fully formed adults emerge from the puparia. Newly emerged adults have soft cuticles, very small abdomens, and are unable to fly because their wings have not yet expanded. Within a few hours of emergence, the cuticle will have hardened and the wings and abdomen will have expanded (Greenberg and Kunich, 2002). At this point, the adult is referred to as the teneral adult. Teneral adults are not reproductively mature (Greenberg and Kunich, 2002). In most fly species, protein consumption by both males and females is usually required to initiate the maturation of their respective reproductive organs (Rasso and Fraenkel, 1954; Avancini, 1988; Stoffolano *et al.*, 1995).

1.4 Accuracy, Precision and Reliability of the Entomological Method with Emphasis on Postmortem Interval Estimations Using Insect Development

As noted previously, the deposition of offspring by female flies can be regarded as the start of a biological clock, which can be used to estimate the postmortem interval (Catts and Goff, 1992). However, in order to estimate the postmortem interval using insect development, the age of the developing larvae present on the corpse at the time of discovery must first be established (Catts and Goff, 1992). The age of the oldest larvae present at the time of discovery by humans will give a minimum time since death; for example, if the oldest larvae discovered on the corpse are five days old, then the decedent has been dead for at least five days (Anderson, 1995). The age of the oldest insects collected at the time of discovery give a minimum estimate of the postmortem interval because if the insects are of a given age, the duration of the postmortem interval cannot be less than the number of days required for the insects discovered to have reached that age (Erzinclioglu, 1992). Therefore, accurate estimation of the age of the larvae at the time of discovery of the corpse is absolutely essential for the calculation of a realistic and justifiable estimate of the postmortem interval (Catts, 1992). Furthermore, the calculated estimate of the postmortem interval must be defendable on both scientific and legal[8] grounds (Catts, 1992).

The age of a fly larva is determined primarily by its thermal history[9], species, length or weight and developmental stage (instar) (Greenberg and Kunich, 2002). Insect development studies typically report only one variable of insect size[10] versus time, and the developmental curves created from these studies then serve as the reference material for determining the age of the larvae collected from a corpse.

Larval length, on its own, is not a reliable indicator of larval age. Considerable overlap in larval length exists between the developmental stages, particularly between the second and third instars (Anderson, 2000). The overall size of the larvae discovered, especially for larvae in the second and third instars, largely depends on the nutritional quality of the food source (Greenberg and Kunich, 2002). In addition, during pupariation, the larvae shrink in length as the longitudinal muscles and cuticle contracts. However, the degree of shrinkage experienced by a given larva during pupariation varies both between and within species (Greenberg and Kunich, 2002). Once the larva has entered the wandering stage of the third instar the length of the larva can no longer be used to determine the age of the larva (Greenberg, 1991). Therefore, the length of a larva should never be used as the sole determinant of the age of the larva.

[8] i.e. in court

[9] i.e. the temperatures that it was exposed to during development

[10] e.g. length or weight

Likewise, weight should never be used as the sole determinant of larval age. Considerable overlap exists in the weight distributions for larvae of different ages (Wells and LaMotte, 1995; Williams, 1984). As with larval length, the nutritional quality of the food source will impact the weight of the larvae that were feeding on the substrate, especially during the feeding stages of the second and third instars. In addition, the larval weight decreases sharply during the postfeeding stage of the third instar because the contents of the crop are being consumed in preparation for pupation.

The estimation of larval age usually involves the comparison of preserved larvae that were collected from the corpse with development data generated from larvae of known ages (Wells and LaMotte, 1995). Since a comparative approach is used, the larvae of unknown age must be measured in the same way as the larvae used to generate the reference data (Wells and LaMotte, 1995). Two types of larval samples are usually collected from the corpse, either at the death scene or at autopsy. In some cases, larvae are collected at both the death scene and at autopsy[11]. One of the two samples of larvae is kept alive and reared to adulthood in order to facilitate species identification, and the other sample is preserved for later age estimation, and for possible presentation in court.

Considerable attention must be given to the preservation method used to collect and store the larval evidence, as the method chosen may lead to further error if the sample insects were preserved using a method different from that used to preserve the reference insects.

For example, Tantawi and Greenberg (1993) determined that live peak-feeding third instar larvae of *Protophormia terranovae* (Robineau-Desvoidy) (Diptera: Calliphoridae) shrink when they are placed in San Veino, a surface disinfectant commonly used at autopsy to kill any arthropods still present on the corpse. The degree of larval shrinkage due to storage in San Veino was sufficient to reduce the apparent age of the larvae by 11 hours (Tantawi and Greenberg, 1993). A similar effect was also observed when *P. terranovae* larvae of the same age were killed using formalin. However, in the case of the formalin preserved insects, the under-age error was considerably greater than for the *P. terranovae* larvae preserved in San Veino; *P. terranovae* larvae appear approximately 17 hours younger when they are killed in formalin (Tantawi and Greenberg, 1993). A solution of 70% alcohol was also determined to shrink live peak-feeding third instar *P. terranovae* larvae, and even though the degree of shrinkage was less, it was still significant enough to reduce the apparent age of the larvae by ten hours (Tantawi and Greenberg, 1993). In addition, the degree of shrinkage is not constant over the larval lifespan; larvae of different ages shrink by different amounts. For instance, Tantawi and Greenberg (1993) determined that the shrinkage exhibited by young third instar larvae was greater than the shrinkage exhibited by old third instar larvae. Greater shrinkage was observed in the young third instar larvae because the cuticle in younger larvae is thinner and has greater plasticity (Tantawi and

[11]Preferably, the insects are collected at the death scene, or at both the death scene and at the autopsy, rather than just at autopsy.

Greenberg, 1993).

These errors can significantly undermine the accuracy and precision of postmortem interval estimates generated by analysis of preserved insect specimens. The potential problems associated with larval shrinkage can be circumvented by ensuring that the preservation method used to collect and store insects from the crime scene is the same as the method used to generate the reference curves for the development of the insect species of forensic interest.

Another way to circumvent the shrinkage problem is to kill the live larvae in hot water. According to Greenberg and Kunich (2002) larvae killed in this manner will remain extended regardless of the preservative used to store them. Even though this does circumvent the problem of shrinkage due to the preservative used, it does not eliminate the requirement that the insect development curves used to determine the age of the larvae collected should be generated using the same preservation method, particularly when length is used as a determinant of larval age.

In forensic situations, the length or weight of the largest larvae collected are usually used to determine the postmortem interval (Greenberg and Kunich, 2002). Therefore, in addition to preserving the insects obtained from the scene in the same manner as those used to generate the reference curves, the reference curves should be generated using the largest larvae present at each sampling period (Greenberg and Kunich, 2002). Some entomologists, such as Erzinclioglu (1990) do not advocate the use of only the largest insects collected from a corpse. Instead, Erzinclioglu (1990) advocates the use of the largest insects from the developmental stage present on the corpse in the greatest abundance.

1.5 Factors confounding use of insect development for estimating postmortem interval

To date, forensic entomology is the most well-researched method of estimating the elapsed time since death in the later postmortem interval (Buchan and Anderson, 2001). As a result, it is considered to be the most accurate, reliable and precise method in use world-wide (Buchan and Anderson, 2001), and extensive research has been conducted to support this claim. For instance, in a study conducted by Kashyap and Pillay (1989), the entomological evidence provided an estimate of the postmortem interval that was both more accurate and more precise compared to the estimate generated on the basis of changes in decomposition.

However, there are several factors that, if left unaccounted for, significantly decrease the accuracy and precision of the postmortem estimates calculated using insect development data. Many of these confounding factors relate to temperature fluctuations, which can alter the rate of insect development. In many cases, the exact temperatures to which the insects were exposed to prior to discovery are impossible to determine, and instead they have to be estimated from regional weather reports,

or from consideration of the degree of corpse exposure to sun and or shade. The formation of maggot masses is another factor that can impact the thermal history of the larvae feeding on a corpse. A considerable amount of metabolic heat is generated by a mass of feeding larvae, and may cause an increase in the rate of insect development (Higley and Haskell, 2001).

Forensic entomology is usually used to estimate the postmortem interval in human death investigations, and humans, with the exception of household pets[12], may be viewed as somewhat unique when compared to other animal carrion. While alive, many humans ingest a variety of drugs, both illegal and legal, on a continual basis, and in many cases the drug consumed may accumulate in a variety of human tissues, such as the liver, skeletal muscle and blood. Therefore, when a human dies, the insects deposited on the corpse may be feeding on drug contaminated tissues, and the drugs present may alter the rate of insect development. If the impact of the drugs present in human corpses on the development of the insects is not accounted for, the estimate of the postmortem interval calculated from insect development data may be inaccurate. As a result, the reliability of the post-mortem interval estimate obtained using developmental data from insects reared on drug-contaminated tissue may be highly questionable, and therefore a matter of primary concern.

1.6 Entomotoxicology

Entomotoxicology is a relatively new area of research that can be included under the auspices of forensic entomology. Investigations within the area of entomotoxicology include (Introna *et al.*, 2001):

1. studying the effects of drugs and other toxins on the developmental rate of forensically important insects

2. using insects as alternative toxicological specimens when traditional specimens, such as blood and liver, are no longer available or suitable for analysis.

As discussed earlier in the previous section, the use of entomological evidence to determine the postmortem interval has been shown to be extremely accurate, reliable and precise (Kashyap and Pillay, 1989; Buchan and Anderson, 2001). However, previous research indicates that the developmental patterns of forensically important insects may be altered if the insects are feeding on drug contaminated tissues. The impact of several commonly abused drugs, both legal and illegal, on the developmental patterns of forensically important insects have been researched in a number of countries, including Brazil, France, South Africa and the United States.

Ideally, the effects of drugs on the development of forensically important fly species would be conducted using human tissue contaminated with a known concentration of a particular drug or combination of drugs, as this would eliminate problems associated with the use of non-human animal models. However, the use of human tissues in

[12]e.g. cats and dogs

entomotoxicological research would be problematic for a number of reasons, such as ethical restrictions, health restrictions, problems with producing replicable results[13] and lack of proper experimental control[14].

For this reason, most of the research done to date, regarding the impact of drugs on insect development, has been conducted using rabbits as the experimental model. Typically, live rabbits are infused via ear artery perfusion (e.g. Bourel *et al.*, 1999) or injected via cardiac puncture (e.g. Goff *et al.*, 1989) with a known quantity of drug. The experimental rabbits are then allowed to metabolize the drug for a specified period of time, and after that time are then euthanised. Their carcasses, or a portion of their carcasses[15] then serve as the drug-contaminated food source used to rear the insects. Any alterations in the development of the insects reared on the drug-contaminated tissues are recorded by measuring the length, weight or developmental stage of insects sampled at specified sampling periods.

To date, the impact of several commonly abused drugs, such as cocaine, morphine, phencyclidine, diazepam and amitriptyline, on the development of a forensically important fly species (Diptera) has been investigated. For example, a substantial amount of research has been conducted on *Parasarcophaga ruficornis* (Fabricius) (Diptera: Sarcophagidae) and *Boettcherisca peregrina* (Robineau-Desvoidy) (Diptera: Sarcophagidae), both of which are species of considerable importance in Hawaii (Goff *et al.*, 1986). Other researchers have focused on fly species from the Family Calliphoridae, including *Lucilia sericata* (Meigen), which is a prominent necrophagous species in Europe, and flies of the species Chrysomya, such as *Chrysomya albiceps* (Wiedemann) and *Chrysomya putoria* (Wiedemann), which are species of forensic importance in southeastern Brazil.

The impact of cocaine and its major metabolite benzoylecgonine on the development of *Boettcherisca peregrina* (Diptera: Sarcophagidae) was investigated by Goff *et al.* (1989). The larvae were reared on tissue derived from three different rabbits that were injected with 35 mg, 69 mg or 137 mg of cocaine. These doses were calculated to represent median sublethal, sublethal, median lethal and twice median lethal doses, respectively. The second and third instar larvae reared on rabbit tissues containing the metabolic products of the median lethal and twice median lethal doses of cocaine developed 12 to 18 hours more rapidly than either the larvae reared on the cocaine-free rabbit tissue, or the rabbit tissues containing the metabolic products of the sublethal dose (Goff *et al.*, 1989). In addition, the onset of pupariation occurred earlier in the larvae reared on the median lethal and twice median lethal dosed rabbit tissues. However, the duration of pupation, when compared with the control colony,

[13]The likelihood of obtaining several samples of human tissues with the same concentration of a specific drug, or combination of specific drugs is highly unlikely.

[14]For the same reason as before, the likelihood of receiving human tissue samples that are free of drugs or contaminants is highly unlikely, simply due to the fact that the majority of the human population consumes a wide variety of different drugs or medicaments on a regular basis. The disease state of the tissues obtained would have to be matched as well.

[15]e.g. the liver

was not altered in any of the colonies reared on tissues containing cocaine and its major metabolite (Goff *et al.*, 1989).

In another study conducted by Goff *et al.* (1991), the effects of heroin, as morphine[16], on the development of *Boettcherisca peregrina* (Diptera: Sarcophagidae) were investigated by injecting rabbits with 6 mg, 12 mg, 18 mg and 24 mg of heroin. The levels of heroin injected were calculated to represent the morphine levels found in heroin related human fatalities. The rate of larval growth was accelerated in the colonies reared on the rabbit tissues containing morphine up to 29 hours. In contrast, the duration of the pupal period was extended by an average of 18-36 hours for the four experimental colonies; however, in the case of the 6 mg dose of heroin, the extended duration of the pupal period was not significant. Furthermore, the difference in mean pupal weight between the control colony and the four experimental colonies was also not significant (Goff *et al.*, 1991).

Bourel *et al.* (1999) also investigated the effects of morphine on the development of a forensically important insect species, specifically *Lucilia sericata* (Diptera: Calliphoridae). However, in contrast to the research conducted by Goff *et al.* (1991), Bourel *et al.* (1999) reared the *L. sericata* larvae on the entire rabbit carcass, and not just on rabbit liver. With this in mind, the research conducted by Bourel *et al.* (1999) is likely more applicable to human cases. In their investigation, Bourel *et al.* (1999), dosed experimental rabbits with 12.5 mg, 25 mg, and 50 mg of morphine by ear artery infusion. The use of ear artery infusion allowed for greater control over the resulting blood and tissues levels of morphine in the rabbits. In addition, the use of ear artery perfusion enabled the researchers to achieve end morphine concentrations in the experimental rabbits that more closely matched the concentrations found in morphine related human fatalities (Goff and Lord, 2001). The presence of morphine in the tissues used to rear *L. sericata* decreased the rate of larval growth, in a dose dependent manner; larvae reared on the rabbit tissues that received the largest dose of morphine developed more slowly that those reared on the rabbit tissues containing less morphine. These results are opposite of those found by Goff *et al.* (1991) in their investigation on the effects of heroin, as morphine, on the development of *B. peregrina*. The contrasting results obtained by Goff *et al.* (1991) and Bourel *et al.* (1999) indicate the possible existence of Family specific differences in physiological response to the presence of morphine in the rearing media. Clearly, additional studies with different forensically important species of Calliphoridae and Sarcophagidae are required.

The preceding paragraphs have discussed only a small portion of the research that has been conducted regarding the effects of commonly used or abused drugs on insect development. Although the use of experimental animals, such as rabbits, incorporates aspects of drug metabolism, its major drawback is, in fact, the extensive use of experimental animals. Given the number of drugs, drug combinations, and

[16]Morphine is a major metabolite of heroin, and intravenously administered heroin is rapidly metabolized to morphine. For instance, the half-life of heroin is 2-6 minutes (Baselt, 2000).

insect species to be studied, it is not feasible based on financial and ethical grounds, to rely solely on the use of animals as the experimental model.

1.7 Research Rationale and Objectives

Entomotoxicological data for Canada is limited, and if forensic entomology is to be used to its fullest extent in Canada, this situation must change. The presence of drugs, including alcohol, in British Columbian deaths is not unusual; nor is it unusual for such death investigations to require the techniques of forensic entomology to establish time of death. For instance, in an analysis of 42 death investigations that used insect evidence in British Columbia between 1988 and 1994, 48% (20) of the cases required toxicological analyses. Of the cases that required toxicological analyses, 80% (16) of the cases tested positive for drugs and/or alcohol (Anderson 1995). Therefore, investigating the effects of drugs on the development of forensically significant insects in British Columbia is extremely important. Such knowledge is required to ensure that the estimate of elapsed time since death is as accurate as the quality of evidence obtained will allow.

Therefore, the present research had two objectives:

1. To investigate the impact of a commonly used or abused drug in British Columbia on the development of a forensically important insect species in British Columbia.

2. To investigate the use of a non-live animal model for use in entomotoxicological investigations.

The tricyclic antidepressant amitriptyline was the drug chosen for study in the present study for several reasons, including its relatively low cost, and the existence of a large amount of peer-reviewed literature available on amitriptyline pharmacokinetics, postmortem tissue concentrations and chromatographic analysis. Furthermore, given the fact that the experimental research necessary for this project was conducted at three different locations[17], it was important to pick a drug that did not require special facilities or licenses to have in the laboratory. Since amitriptyline is not an illegal drug, regular laboratory facilities were appropriate for its storage, and therefore amitriptyline was more appropriate for the present study than an illegal substance such as cocaine. In addition, tricyclic antidepressants such as amitriptyline are commonly prescribed antidepressants in British Columbia. During the six year period of 1997-2002, 1,388,166 prescriptions for tricyclic antidepressants were received by BC pharmacies, and of this number 57.6% (793,429) of the prescriptions

[17]The method validation experiments were conducted at the Provincial Toxicology Centre, the artificial foodstuff was prepared at the British Columbia Institute of Technology, and the insect development studies were conducted in the Forensic Entomology laboratory at Simon Fraser University.

were for amitriptyline (BC Ministry of Health, 2003)[18]. Furthermore, a review of the Judgment of Inquiry reports produced by the BC Coroners' Service indicated that 213 deaths during the six year period of 1997-2002 were the result of poisoning by tricyclic antidepressants, and of those 213 deaths, 156 (73.2%) were due to poisoning by amitriptyline. Given these results, amitriptyline was a reasonable choice for an entomotoxicological investigation[19].

Since the second objective of the present project was to evaluate the use of a non-live animal model for investigating the effects of commonly used or abused drugs on the development of forensically important insects, it was also necessary, for comparative purposes, to choose a drug on which entomotoxicological research had already been conducted. In an earlier study, Goff *et al.* (1993) investigated the impact of amitriptyline on the development of *Parasarcophaga ruficornis* (Diptera: Sarcophagidae). Therefore, it was important that either the same species, or a closely related species, was used in the present experiment. Unfortunately, the species used by Goff *et al.* (1991) is not native to Canada, and as a result a different, albeit closely related, species had to be chosen[20].

Insects of the Family Sarcophagidae are insects of considerable forensic importance in tropical areas, such as Hawaii or the southern mainland of the United States (Byrd and Castner, 2001). Of the insect species recovered from corpses located indoors, during the summer months in the southeastern United States, the majority of the species belong to the Family Sarcophagidae (Byrd and Castner, 2001). In regions where they are commonly associated with animal carrion, flies from the Family Sarcophagidae normally arrive at an animal carcass at the same time, or slightly after blow flies (Family Calliphoridae) (Byrd and Castner, 2001). In comparison, insects from the Family Sarcophagidae are rarely recovered from decomposing carrion in British Columbia (Anderson, 1995). In British Columbia, the majority of the insect species collected from decomposing remains are species from the Family Calliphoridae. For example, immature insects from the Family Calliphoridae, during the period of 1988 to 1994, were associated with 60 cases involving human remains, compared to only four cases with immature insects of the Family Sarcophagidae (Anderson, personal communication, 2003). This is somewhat unfortunate, since the results from the present project may not be immediately applicable to forensic case work in British

[18]Neglecting the fact that certain age groups do not typically receive prescriptions for tricyclic antidepressants, and based on the 1996 Census population of 3,724,500 people for British Columbia, this amounts to 0.37 amitriptyline prescriptions per person over the six year period (BC Stats: Ministry of Management Services, no date).

[19]The data obtained from both the BC Ministry of Health and the BC Coroners' Service was obtained under SFU ethics approval for a previous project entitled "The effects of commonly abused drugs in Canada on time of death determinations in the later postmortem interval, using insect development." See Appendix B.

[20]For the purposes of the present study, obtaining a closely related fly species to the one used by Goff *et al.* (1993), for comparative purposes, was viewed to be more important than obtaining a fly species that was of great forensic importance in British Columbia.

Columbia. Even so, this does not negate the validity of the present project since the second objective of the present project was to investigate the suitability of a non-live animal model for use in entomotoxicological investigations.

With this in mind, another fly species from the Family Sarcophagidae was chosen, namely the species *Sarcophaga bullata* (Parker). The Family Sarcophagidae is a large family, consisting of over 2000 species world-wide, with the majority of the species found in tropical or warm temperate regions (Byrd and Castner, 2001). Approximately 327 of these species can be found in the United States and Canada (Byrd and Castner 2001). *S. bullata* is most commonly found in the southern United States, but the species can also be found in Canada. *S. bullata* is closely related to another species commonly found in Canada, *Sarcophaga haemorrhoidalis* (Fallén), and the two species exhibit similar behaviour and habitat preferences (Byrd and Castner 2001). In addition, *Sarcophaga bullata* was a readily available species from the Family Sarcophagidae. Furthermore, the behaviour and biology of *S. bullata*, in the absence of drugs, has been researched by a number of researchers, from both a forensic and a biological perspective (e.g. Mitchell and Soucie, 1992; Christopherson and Gibo, 1997).

Previous research has already indicated that the developmental responses of blow flies (Diptera: Calliphoridae) and flesh flies (Diptera: Sarcophagidae) to some commonly abused drugs are not the same (Goff *et al.*, 1991; Bourel *et al.*, 1999). Therefore, the results obtained from the present study can be used to determine if a given drug response are consistent among insects of the Family Sarcophagidae.

The non-live animal model, or **rearing media**, used in the present study was previously used in an entomotoxicological investigation on the accumulation and elimination of amitriptyline *Calliphora vicina* (Robineau-Desvoidy) (Diptera: Calliphoridae) larvae (Sadler *et al.*, 1997). The artificial food medium was prepared using homogenized beef liver, powdered whole egg and agar. Amitriptyline and its major metabolite, nortriptyline, were added to the artificial rearing media in order to evaluate the impact of the drugs on *S. bullata* development. For comparative purposes, the amitriptyline and nortriptyline were added to the artificial foodstuff according to the levels of amitriptyline and nortriptyline quantitated in the rabbit livers used by Goff *et al.* (1993) to evaluate the impact of amitriptyline and nortriptyline on the development by *Parasarcophaga ruficornis* (Diptera: Sarcophagidae).

Spiking artificial food media with chemicals to test the effects of those chemicals on insect development is not a new technique in itself; the technique is commonly employed in insecticide investigations (Brown, 1960). However, in insecticide studies, only the parent compound is normally under investigation, and not the parent compound in combination with the metabolite(s) specifically produced by the metabolism of the parent compound by humans.

Therefore, the present project expanded the traditional food-spiking technique by adding both the parent compound and the primary human metabolite to the artificial food media. The major metabolite of amitriptyline, nortriptyline, was added to the

artificial rearing media in order to simulate the metabolism of the drug by humans. The benefits of a validated non-live animal model for use in entomotoxicological investigations are considerable. For instance, research costs would be lower; beef liver, agar and powdered whole egg are relatively inexpensive and readily obtainable, especially when compared to the costs associated with experimental animals. In addition, the artificial foodstuff could be custom spiked according to the toxicology results obtained for a case requiring forensic entomology to estimate the postmortem interval. For example, if the toxicology results indicated that a specific amount of amitriptyline and codeine were present in the tissues sampled from the deceased, the same concentrations of each drug could be easily added to the artificial foodstuff. The custom prepared artificial foodstuff could then be used to rear insects of the same species, and any changes in development observed could be directly incorporated into the estimate of the postmortem interval, rather than extrapolating from research that at this time is still fairly limited in scope.

1.8 Report Overview

This chapter has introduced insect development and its relationship to forensic entomology. A look at a few of the limitations of forensic entomology explained the motivation for this project. The method validation is covered in a separate report, "Method Validation for Amitriptyline and Nortriptyline in Artificial Foodstuff." This report will present the methods and results of the rearing experiments conducted with insects of the species *Sarcophaga bullata* (Diptera: Sarcophagidae).

Chapter 2

Insect Development Experiment

2.1 Introduction

As discussed in the introduction, the present project had two objectives; the first was to determine the effects of amitriptyline and nortriptyline on the development of an insect species of forensic importance in British Columbia, and the second was to evaluate the use of a non-live animal model. However, in order to meet the requirements of the second objective, an insect species closely related to the one used by Goff *et al.* (1993) had to be chosen. With this in mind, *Sarcophaga bullata* (Parker) (Diptera: Sarcophagidae) was chosen as the test species.

In order to evaluate the effects of amitriptyline and nortriptyline on the development of *S. bullata*, five different batches of artificial foodstuff, prepared from homogenized beef liver, powdered whole egg, and agar were each spiked with different concentrations of amitriptyline and nortriptyline. The proportions of amitriptyline and nortriptyline added were obtained from a previous entomotoxicological experiment conducted by Goff *et al.* (1993), and were selected to represent levels of the drugs commonly found in human deaths involving amitriptyline.

The artificial foodstuff used to determine the impact of a specific drug, or drugs, on insect development must be homogeneous with respect to the drugs of interest. As a result, each batch of amitriptyline and nortriptyline spiked artificial foodstuff prepared was analyzed to confirm both its amitriptyline and nortriptyline content, and homogeneity using a previously developed GC-NPD method (Refer to "Method Validation for Amitriptyline and Nortriptyline in Artificial Foodstuff"). The method used to analyze the artificial foodstuff was modified from the method used at the Provincial Toxicology Centre, Riverview Hospital, Port Coquitlam, British Columbia; therefore, before this method could be used to analyze the actual artificial foodstuff used in the development experiment for *S. bullata*, the modified analytical method had to be validated.

Now that the performance characteristics of the analytical method have been described the results of the insect development experiment can be presented and

discussed. Therefore, the following chapter presents and discusses the results of the insect development experiment completed for the present project.

2.2 Experimental Methods

2.2.1 Maintenance of the Adult *Sarcophaga bullata* Colony

One hundred and fifty *Sarcophaga bullata* (Parker) (Diptera: Sarcophagidae) pupae were obtained from WARD's Biology (Ontario, Canada), and separated into three groups of approximately 50 pupae. Each group of 50 pupae was placed in the lid portion of a Petri dish on top of a slightly water dampened piece of filter paper. The Petri dishes were then placed into a 75 x 75 x 75 cm rearing cage at room temperature and allowed to emerge. The emerging adult *S. bullata* flies were given water, sugar and skim milk powder *ad libidium*. Three days after emergence, beef liver was added to the rearing cage to allow for ovarian and testes development, and to serve as the larviposition media. Fresh liver was added each day until sufficient larvae had been laid. These larvae were transferred to a rearing jar containing fresh beef liver and were reared until the insects had pupated. The pupae were then collected and placed into another 75 x 75 x 75 cm rearing cage. This F1 generation colony served as the stock colony for the experimental colonies.

Once the pupae of the F1 generation had emerged, they were provided with water, sugar and skim milk *ad libidium*, followed by fresh beef liver as the larviposition media. When sufficient larvae were deposited, they were transferred to rearing jars and reared until the insects had pupated. The pupae of the F2 generation was then divided into three groups of approximately 50 pupae. Each set of 50 pupae was then placed into separate 75 x 75 x 75 cm rearing cages. Each of the three cages was provided with water, sugar and skim milk powder *ad libidium*. These three F2 colonies provided all the insects used in the experiments and are referred to as the experimental or working colonies.

2.2.2 Experimental Design

Three pieces of fresh beef liver[1] were introduced into each of the three experimental colonies, and checked every 20 minutes for larvae. The experimental colonies did not deposit a sufficient quantity of larvae for the experiment until three hours after the liver was introduced into the rearing cages. After three hours, the liver pieces were removed from the rearing cages. The time of larviposition was recorded as 1.5 hours after the initial introduction of the larviposition media.

Eighteen rearing jars, three per type of rearing media, were set up with sawdust and labelled. One hundred grams of the appropriate rearing media was placed in each of the 18 rearing jars.

[1] larviposition media

Approximately 400 larvae were placed in each of the 18 rearing jars. The larvae were transferred from the larviposition media to the rearing media using a blunt-tipped metal spatula. Only clumps of larvae were selected for transfer from the larviposition media to the rearing jars. Although the larvae on the perimeter of the larval mass may be damaged during transfer, larvae in the middle of the mass should be protected from damage caused during transfer. Therefore, only a subsection of the larvae deposited on each of the nine[2] pieces of liver used as larviposition media were used in the present experiment. In addition, preferentially selecting clumps of larvae resulted in the selection of more larvae from one of the three experimental colonies compared with the other two. This was simply due to the fact that one experimental colony deposited substantially more larvae compared with the other two. Therefore larval selection was non-random. To alleviate the non-random selection of the insects, each clump of insects was transferred to a randomly selected rearing jar. As a result, the order in which the insects were transferred to each of the eighteen rearing jars was randomized through the use of a random number table.

Overall, three replicates were set up for each of the six types of rearing media. Figure 2.1 outlines the experimental design for the insect development experiment. Batch A refers to the artificial foodstuff containing 168.41 mg/kg amitriptyline and 7.65 mg/kg nortriptyline. Batch B refers to the artificial foodstuff containing 9.55 mg/kg amitriptyline and 3.21 mg/kg nortriptyline. Batch C refers to the artificial foodstuff containing 41.38 mg/kg amitriptyline and 1.92 mg/kg nortriptyline. Batch D refers to the artificial foodstuff containing 42.59 mg.kg amitriptyline and 1.22 mg/kg nortriptyline.

2.2.3 Sampling

As indicated in Figure 2.1, ten individual larvae, from each of the 18 jars, were to be sampled every eight hours. The larvae from each sample were then killed by placing them in hot water (approximately 90°C) for 30 seconds. After removal from the hot water, the larvae were placed in 75% ethanol for storage until the length of each larvae could be measured and its developmental stage determined.

During the first instar and second instar, the first ten larvae observed at each sampling period were collected. In the case of the liver control, selecting the first larvae observed was not a problem, for most of the larvae were still present on the outer surface of the liver. However, the artificial foodstuff was soft enough that the young first instar larvae could burrow into it, and as a result, the majority of the larvae were concealed from view.

When the larvae reached the third instar, the sampling of larvae from the rearing jars containing artificial foodstuff became difficult because the larvae were concealed by undigestable[3] portions of the foodstuff. Rather than pick through the undigested

[2]Recall that three pieces of liver were placed in each rearing cage to serve as the larviposition media.

[3]post-consumption by larvae

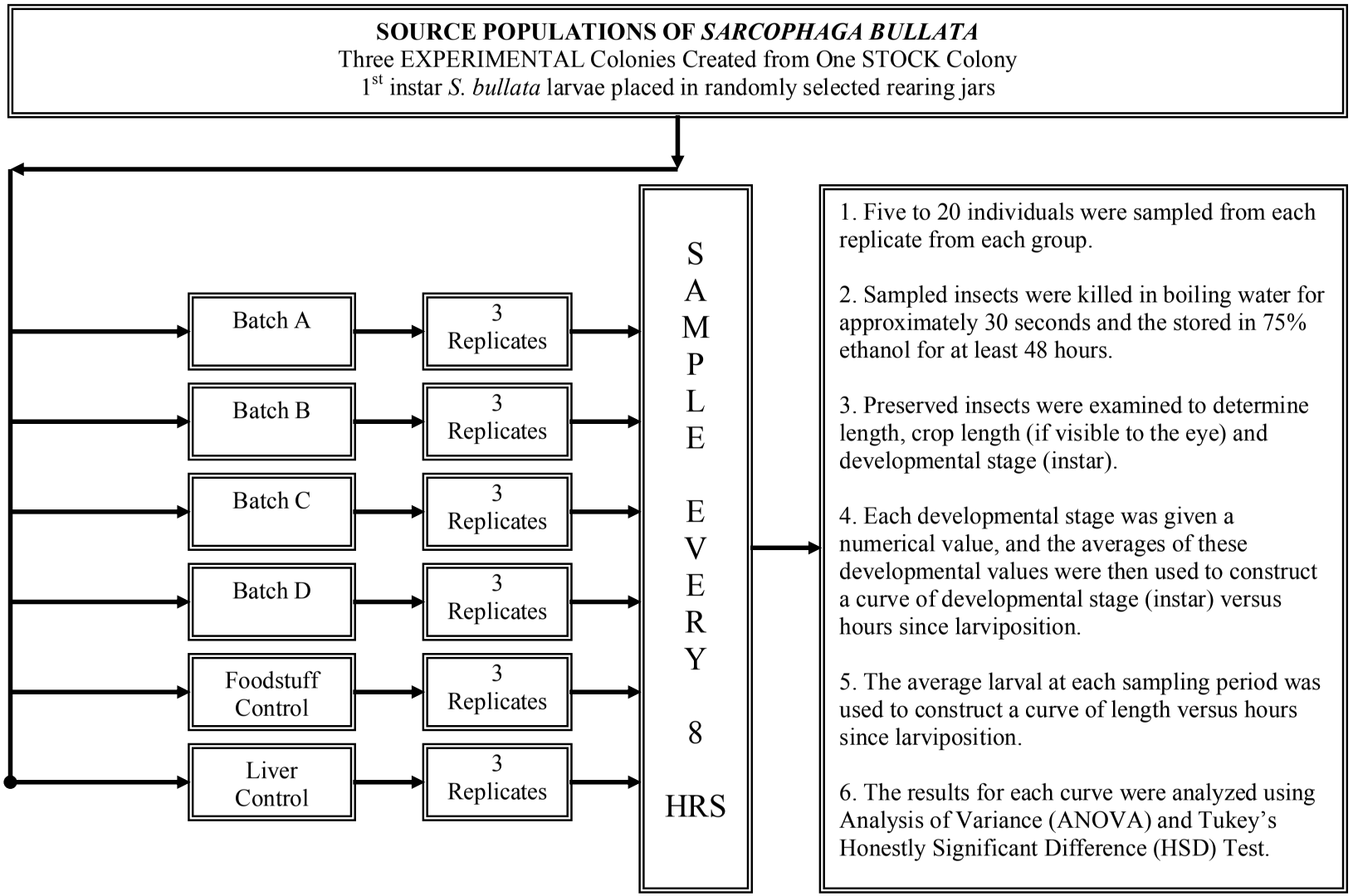


Figure 2.1: Outline of the experimental design used to investigate the effects of amitriptyline and nortriptyline on the development of *Sarcophaga bullata*. Batch A = 168.41 mg/kg AMT + 7.65 mg/kg nortriptyline; Batch B = 9.55 mg/kg AMT + 3.21 mg/kg NOR; Batch C = 41.38 mg/kg AMT + 1.92 mg/kg NOR; Batch D = 42.59 mg.kg AMT + 1.22 mg/kg NOR. (AMT = amitriptyline; NOR = nortriptyline).

portions of the foodstuff, a small handful of the undigested portions were removed at each sampling period and placed into the hot water. The hot water killed larvae present in the handful of indigestible foodstuff were collected and placed in 75% ethanol. Sampling of the third instar larvae from the liver proceeded in much the same way, except that the larvae reared one the liver could be removed directly from the surface of the liver, killed with hot water and then placed in the preservative.

Although the behaviour of the larvae and the condition of the rearing media made random sampling of the larvae difficult, no attempt was made to sample only the largest larvae observed in each rearing jar because the statistical tests used in the present project require normally distributed data in order to appropriately apply them to a data set. Sampling larvae of only a certain size has the potential to skew the distribution.

However, it should be noted that it is common practice in forensic entomology to use the largest larva present in the sample collected from the corpse to estimate the postmortem interval. Even so, some researchers, as discussed in the introduction, consider this method to be problematic, and advocate that the age of the largest larva from the most abundant larval stage collected from the corpse should be used to estimate the postmortem interval (Erzinclioglu, 1990). With this in mind, a substantial amount of larvae, of various sizes, should be collected from the corpse. The sampling procedure used in the present project was intended to more closely resemble the collection of a large number of larvae of various sizes, and possibly, ages from the corpse.

2.2.4 Rearing Jars

The rearing jars used throughout the experiment consisted of 4.5 L wide-mouthed glass jars that were lined with 10 cm of slightly water dampened sawdust, followed by a piece of folded paper towel. Beef liver or foodstuff, depending on the experimental treatment, was then placed on top of the paper towel. First instar larvae were then transferred from the larviposition media to the liver or foodstuff in the rearing jar using a blunt-tipped spatula. The jar was closed using a piece of 2-ply cotton netting approximately 30 cm^2, followed by two layers of paper towel. Both the cloth and the paper towel were secured with several elastic bands.

The cotton netting was necessary to prevent the *S. bullata* larvae from escaping. Previous rearings by the author demonstrated that the third instar *S. bullata* larvae could chew through two layers of paper towel but not the combination of paper towel and cotton netting.

2.2.5 Rearing Conditions

The larvae were reared in a Conviron E7A incubator (Controlled Environments Limited, Winnipeg, Manitoba, Canada) at 26°C, 75% relative humidity with a photoperiod of 16 hours light and 8 hours dark.

2.2.6 Data Analysis

The rate of *S. bullata* development was evaluated in the present project by two different techniques: (1) larval length, (2) developmental stage. All statistical analyses were performed in JMP IN® (SAS Institute, Inc., Cary, NC, USA).

One-way analysis of variance (ANOVA) was used to compare both the pooled mean maximum lengths, and the mean time required for the larvae reared on each of the six types of foodstuff to reach the mean maximum length. ANOVA assumes that the data is normally distributed, and that the variances associated with each of the groups are equal. JMP IN® (SAS Institute, Inc., Cary, NC, USA) provides four different tests for determining if the variance between the groups is unequal: Levene's test, the Brown-Forsythe test, O'Brien's test and Bartlett's test. If, at an alpha level of 0.05, any of these four tests revealed that the variances between the groups were unequal, the results of the Welch ANOVA for unequal variances was reported instead. Furthermore, if ANOVA indicated that there was a difference present between the larvae reared on the six types of rearing media, Tukey-Kramer's Honestly Significant Difference (HSD) test was used to determine which of the six groups were different from each other.

The development of *S. bullata* larvae was divided into two major stages, the larval stage and the pupal stage, and one-way analysis of variance was also used to compare the mean duration of both the larval stage and pupal stage between the insects reared on the six different types of rearing media. The development of *S. bullata* larvae was further divided into three feeding stages (first, second and feeding third instars), and a prepupal stage. One way analysis of variance was then used to compare the mean duration of the each of these stages between the six groups of insects.

As with the mean maximum length comparisons, Levene's test, O'Brien's test, Bartlett's test, and the Brown-Forsythe test were used to test for the presence of unequal variances, and if, at an alpha level of 0.05, any of the four tests revealed that the variances between the groups were unequal, the results of the Welch ANOVA for unequal variances was reported. Further, if the results of the one-way ANOVA indicated that there was a difference present between the larvae reared on the six types of rearing media, Tukey-Kramer's Honestly Significant Difference (HSD) test was used to determine which groups were significantly different.

The mean pupal weight, and adult weight for the insects reared on the six different types of rearing media were also compared using one-way analysis of variance. Unequal variances were determined using the procedures described earlier in the present section.

2.2.7 Larval Length as an Indicator of Larval Age

The total length and crop length (if visible) of each larva preserved at each sampling period was measured using a 10 x 10 mm sheet of laminated graph paper under a binocular microscope. The larvae were measured crop side up, with their posterior end lined up with the edge of one of the thickened gradations. The length was mea-

sured to the nearest 0.1 mm. All of the insects sampled were measured over a period of two months (July and August 2003), after storage in 75% ethanol for at least 24 hours. Preservation of the killed larvae was required because of the large number of larvae sampled at each sampling period, and because of the length of time required to sample the insects from each of the 18 rearing jars. Length measurements were not taken from any of the preserved larvae until after they had been in the preservative for at least 24 hours because research conducted by Adams and Hall (2003) indicated that the majority of the postmortem changes in the length of hot water killed larvae occurred within the first 12 hours of placing the larvae in the preservative. However, it should be noted that Adams and Hall (2003), investigated the effects of 80% ethanol on hot water killed postfeeding third instar larvae of the species *Calliphora vomitoria* (L.). Therefore, these results may not be directly transferable to the larvae of the species *Sarcophaga bullata*. At the present time data on the postmortem changes in the length of hot water killed larvae are not available for species of the Family Sarcophagidae.

The average maggot length from each sampling period, for each experimental set and control was plotted against the hours since larviposition using Microsoft®Excel 2002 (Microsoft Corporation, 1981-2001). The larvae at each sampling period were not separated according to developmental stage (i.e. instar). Instead, the length of each maggot was measured, and then an average larval length was computed for each sampling period. This is a commonly used technique for investigating the rate of insect development, and has been use by a number of researchers, including Goff *et al.* (1993) and Bourel *et al.* (1999).

Larvae increase in length over time until a maximum length is reached. The maximum length is generally attained at peak feeding during the third instar. Length can be used as a measure of insect growth until the onset of pupariation. When pupariation begins, the length of the larvae decrease in length until pupation.

With this in mind, the time required for the larvae to reach the mean maximum length can be used as a comparative measure for evaluating differences in the rate of development between sets of larvae reared under different experimental conditions.

In this research project both the time required to reach the mean maximum length, and the mean maximum length, were compared for each type of rearing media. The statistical significance of any observed differences in mean maximum length, and the time required to reach the mean maximum length, were evaluated using one-way analysis of variance (ANOVA). The Tukey-Kramer Honestly Significant Difference (HSD) test for multiple comparisons was used to identify which groups were significantly different from each other. All statistical tests were performed in the statistical program JMP IN®(SAS Institute, Inc., Cary, NC, USA).

In addition, because the measurement process extended over a fairly long period of time, ten third instar larvae were selected for a measurement test. These ten insects were numbered and stored in separate vials of 75% ethanol. Both the body length and the crop length for each of the ten larvae were recorded at five different times

during the two month measurement period. The same ten insects were measured once at the beginning of the measurement period, once at the end, and three times in the middle. Overall, the length measurements for the measurement test larvae were conducted at roughly two week intervals. The purpose of this test was to estimate the amount of intra-observer error present in the insect measurement process.

2.2.8 Developmental Stage as an Indicator of Maggot Age

The development of *Sarcophaga bullata* was divided into six stages. Stages 1-3 represented the feeding instars, including the feeding phase of the third instar, stage 4 represented the prepupal stage, stage 5 represented the pupal stage, and stage 6 represented the adult stage. Each developmental stage was assigned a numerical value, with the value increasing according to increasing insect age (Table 2.1).

Developmental Stage	**Developmental Marker**	**Numerical Value**
First Instar	Two sets of poorly defined spiracular slits.	1
Second Instar	Two sets of two well-defined, chitinized spiracular slits	2
Feeding and Postfeeding Stage (Third Instar)	Two sets of three well-defined spiracular slits	3
Prepupal Stage (Third Instar)	No visible crop	4
Pupal Stage	Formation of pupa	5
Adult	Emergence of adult fly	6

Table 2.1: The developmental marker for each developmental stage, and the numerical value assigned to each development stage of *Sarcophaga bullata* larvae in the insect development study.

First, second and third instar larvae were distinguished primarily on the basis of the number of posterior spiracular slits. Third instar larvae have two sets of three well-differentiated posterior spiracular slits, second instar larvae have two sets of well-differentiated spiracular slits, and first instar larvae have two sets of two poorly differentiated spiracular slits that are attached at the bottom. The posterior **spiracles** of the flesh flies (Family Sarcophagidae), including *S. bullata*, are located at the bottom of a depression at the tip of the abdomen, the edges of which are rimmed with fleshy tubercles (Byrd and Castner 2001).

When the crop was no longer visible under the dissecting microscope, the larva was identified as a prepupal larva. Even though the crop was not visible to the eye, it may not be completely empty at this point. Therefore, it is important to note that the definition used in the present project likely overestimated the duration of the prepupal stage. As mentioned in the introduction, it is important that the prepupal stage not be confused with the postfeeding stage, because during the prepupal stage,

which is relatively short in duration, the larvae start to irreversibly shrink in size (Greenberg and Kunich, 2002)

One other drawback to the developmental stage classification scheme used in the present project, was the fact that the scheme was unable to distinguish between the feeding stage of the third instar and the postfeeding stage of the third instar. The differentiation between the third instar and the prepupal stage was defined as the point in which the crop was no longer visible to the eye. Previous to this point in time, the crop would have reached a maximum length, and then would have started to shrink in size. However, given the changes that were observed during the measurement test, changes in crop length would not have been a consistent age marker for *S. bullata* larvae. Therefore, decreases in visible crop length were not used to determine the stage of the developing larvae during this investigation. This is unfortunate, because for many species (e.g. *Calliphora vicina*), decreases in crop length can indicate the age of a postfeeding larva at forensically useful intervals, such as three hours, nine hours, etc. (Greenberg and Kunich, 2002).

Insects that were in the middle of a moult were given a value half way between the insect's previous stage and the insect's next stage. Moulting, for the purposes of this experiment was considered to be imminent when the spiracular slits of the upcoming developmental stage were visible behind the spiracular slits of the preceding stage. For example, if three spiracular slits were visible behind two spiracular slits, the larvae was given a numerical value of 2.5. If the larvae were shaped like a pupa, and displayed a corresponding reduction in length, but had yet to tan[4], the insect was given a numerical value of 4.5. There were two drawbacks to this method of assigning developmental values. First, a value of 3.5 could not be accurately assigned because the assignment would have been too subjective. For this experiment, the goal was to assign developmental values based on simple objective criteria, such as the number of spiracular slits. Similarly, a value of 5.5 could not be assigned because pupae would have to be sacrificed in order to do so.

In the present research project, the onset of the next developmental stage is defined as the time at which 50% of the sample had entered the next developmental stage. Therefore, the duration of the larval period was defined as the time from larviposition to the time at which 50% of the sampled population had pupated. Likewise, the duration of the pupal period was defined as time at which 50% of the sampled population had pupated to the time at which 50% of the sampled population had emerged as adults. With this in mind, when the larval development was divided into instars, the duration of an instar was defined as the time at which 50% of the sample had entered the next instar. For example, given a sample of ten maggots, if five of the maggots were second instar and the other five third instars, then onset of the third instar would be taken as the time that this sample was collected.

[4]During pupation, the puparium hardens and darken in colour. Tanning occurs over a period of approximately 10 hours, and the puparium changes from white to dark brown in colour.

2.3 Insect Development Experiment

2.3.1 Measurement Test Results: Estimation of Intra-observer Error

The mean body length (±SD) and the mean crop length (±SD) for the ten third instar *Sarcophaga bullata* larvae set aside for the measurement test are presented in Table 2.2. The same 10 larvae were measured five times over a period of two months (July and August, 2003). The relative standard deviation (RSD), expressed as a percentage, associated with the five measurements of both total length and crop length, on each of the ten larvae are also presented in Table 2.2.

Insect #	**Total Length**		**Crop Length**	
	Mean ±SD (mm)	**RSD (%)**	**Mean ±SD (mm)**	**RSD (%)**
1	16.6 ± 0.15	0.92	6.8 ± 0.27	4.0
2	16.4 ± 0.08	0.51	7.5 ± 0.15	2.0
3	16.3 ± 0.13	0.80	7.6 ± 0.22	2.8
4	16.0 ± 0.05	0.34	6.5 ± 0.46	7.0
5	16.4 ± 0.19	1.1	7.7 ± 0.31	4.1
6	18.0 ± 0.04	0.25	9.2 ± 0.18	1.9
7	17.1 ± 0.09	0.52	6.7 ± 0.27	4.1
8	15 ± 0	0	5 ± 0	0
9	17.2 ± 0.1	0.58	7.2 ± 0.43	6.0
10	16.6 ± 0.05	0.33	7.1 ± 0.08	1.2

Table 2.2: The mean total length ±SD and the mean crop length ±SD for the ten third instar *Sarcophaga bullata* larvae, measured five times over a period of two months (July and August, 2003). The relative standard deviation, RSD, (%) of the five measurements made for both total length and crop length, on each of the ten larvae are presented as well.

Table 2.2 indicates that the measurement of total larval length as a function of time is quite precise. In fact, the highest coefficient of variation is only 1.1%. Therefore, the precision is known to be better than 1.1%. In comparison, a lower degree of precision was obtained for the crop length measurements. The precision of the crop length measurements were only known to be better than 7.0%. In addition, a trend of decreasing crop length for each insect over time was observed. This may be an indication that the crop becomes less visible after storage in 75% ethanol, and as a result, the decrease in the visibility of the crop over time may be a storage artefact. This has important implications for age estimations based on the ratio of crop length to total body length, particularly when the larvae have been stored in a preservative such as 75% ethanol for a considerable period of time. Therefore,

because the measurement of visible crop length was shown to be unreliable due to the preservation technique, visible crop length was not used in the present project as an indicator of larval age. Furthermore, since crops are also visible in the second instar, the effect of preservation and storage time on the visible crop length of second instar larvae should also be investigated.

In addition, it should be noted that this measurement test does not provide a measure of intra-observer measurement accuracy. A determination of intra-observer measurement accuracy would require the comparison of larval lengths obtained with several different methods. In addition, the above method only measured the precision of the measurement process for fully elongated third instar larvae. Given the fact that the same technique for determining both total length and crop length was used for all three stages of larval development, it was assumed that measurement precision would not change as a function of developmental stage. This assumption should be tested in future work.

2.3.2 Larval Length as an Indicator of Larval Age: Results

Mean Time Required to Reach the Mean Maximum Length

The mean, the standard deviation (SD), and the relative standard deviation (RSD), for the time required for the larvae in each experimental group to reach the mean maximum length is presented in Table 2.4. The larvae reared on foodstuff containing 168.41 mg/kg amitriptyline and 7.65 mg/kg nortriptyline (Batch A), at a mean of 83.3 hours, required the greatest amount of time to reach the mean maximum length (Table 2.4). In contrast, the larvae reared on the foodstuff control[5], at a mean of 53.5 hours, required the least amount of time to reach their mean maximum length. Overall, the order, in terms of decreasing mean time required reach mean maximum length, was as follows (Table 2.4):

Batch A > Liver Control > Batch C > Batch B > Batch D > Foodstuff Control

One-way analysis of variance (ANOVA) of the data presented in Table 2.3 indicated that the differences between each group of foodstuff, in terms of the time required for the larvae to reach the mean maximum length, was not statistically significant at an alpha level of 0.05 ($F_{5,12} = 1.90$; $P = 0.17$; Table 2.3).

Given the high variation, measured in terms of relative standard deviation, observed between the three replicates within each type of rearing media, the differences in the mean time required to reach the mean maximum length for each type of rearing media was not unexpected. For instance, the variation between the three replicates in Batch A, at 28.8%, was substantial (Table 2.4). Considerable variation was also observed between the replicates within each of the remaining groups of drug-spiked artificial foodstuff. For instance, the variation in the time required to reach the mean maximum length between the replicates was 23.4% for Batch C, 17.7% for Batch B, and 17.5 % for Batch D (Table 2.4). In contrast, the variation in the times required to reach the mean maximum length, between the three foodstuff control replicates, at 3.0%, was considerably lower compared to the variation observed within the other groups of artificial foodstuff. Overall, the smallest variation between replicates, in terms of the time required for larvae reared on a particular type of foodstuff to reach their mean maximum length, was observed for the Liver Control replicates, as the variation between the three Liver Control replicates was only 1.3% (Table 2.4).

[5]i.e. drug-free foodstuff

Source of Variation	**DF**	**Sums of Squares**	**Mean Square**	***F***	***P***
Rearing Media	5	1721.30	344.26	1.90	0.17
Error	12	2177.10	181.43	-	-

Table 2.3: Results of one-way ANOVA for the mean time required for the larvae reared on six different types of rearing media to reach the mean maximum length, at 26°C.

Foodstuff ($n = 3$)	**Mean Time ±SD Required to Reach Mean Maximum Length (hr.)**	**Relative Standard Deviation (%)**
Liver Control	71.8 ± 0.9	1.3
Foodstuff Control	53.5 ± 1.6	3.0
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	83.3 ± 24.0	28.8
Batch B (9.55 mg/kg AMT; 3.21 mg/kg NOR)	63.5 ± 11.2	17.7
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	71.4 ± 16.7	23.4
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	58.5 ± 10.2	17.5

Table 2.4: Mean, standard deviation (SD), and relative standard deviation for the time (hrs) required for *Sarcophaga bullata* larvae, for each type of rearing media, at 26°C to reach their respective mean maximum length (AMT = amitriptyline; NOR = nortriptyline).

Mean Maximum Larval Length

The mean maximum length, standard deviation and relative standard deviation (RSD) for each replicate, within each type of rearing media, are summarized in Table 2.5. The number of larvae sampled in each replicate varied from a minimum of ten to a maximum of 34. The difference in sample size was due to the problems associated with extracting the larvae from the undigestable portion of the artificial foodstuff.

Considerable variation in larval length was observed for insects of relatively the same age (± 1.5 hours). This variation is illustrated by the rather large relative standard deviations associated with each replicate, within each type of rearing media (Table 2.5). The maggots used to generate the mean maximum lengths were mostly third instar, except for a few postfeeding larvae. Normally, postfeeding larvae, especially those nearing the onset of pupariation, would not be used to generate the mean maximum lengths since the overall maggot length starts to shrink at this stage. However, the lengths of the postfeeding maggots were within the range of maggot lengths observed for the feeding third instar maggots, and as a result, there was no reason to exclude the lengths of the prepupal maggots.

Since each of the three replicates for each type of rearing media were set up in an identical manner, at the start of the experiment, with approximately the same number of larvae, it is reasonable to pool together the larval lengths from each of the three replicates for each type of rearing media. Further, given the fact that only three replicates were set up for each type of rearing media, the removal of outliers revealed through statistical tests is not warranted. The mean pooled length, standard deviation and relative standard deviation for each type of rearing media is also presented in Table 2.5.

The greatest mean maximum length, at 20.4 ± 1.4 mm, was observed in the Liver Control replicates. In comparison, the smallest mean maximum length was observed in the larvae collected from the rearing media containing 55.7 mg/kg amitriptyline and 1.7 mg/kg nortriptyline (Batch C). In fact, the mean maximum lengths observed for the individual replicates of Batch C were considerably different. The mean maximum length observed for Replicate 3 of Batch C was quite different from the mean maximum lengths observed for Replicates 1 and 2 of Batch C. The differences in the mean maximum lengths observed between the Batch C replicates may be the result of several factors, including, but not limited to, genetic differences between the larval groups placed in each of the rearing jars, and differences in the number of insects added to each rearing jar. The effect of these two factors on the experiment were considered in the experimental design, but perhaps more rigorous procedures are necessary in future experiments. For instance, approximately 400 larvae were placed in each rearing jar, and the number of larvae was estimated based on the size of the larval groups being transferred. A more rigorous procedure would be to accurately count the number of larvae placed in each rearing jar. In any event, no statistical tests for outliers were conducted given the fact that there exists only three replicates for each batch of foodstuff.

Rearing Media	Mean Maximum Maggot Length per Replicate (mm)			Pooled Mean Maximum Lengths (mm)	
	Replicate	Mean ± SD (mm)	RSD (%)	Mean ± SD (mm)	RSD (%)
Liver Control	R1 ($n = 10$)	20.1 ± 2.2	11.0	20.4 ± 1.4	6.9
	R2 ($n = 21$)	19.9 ± 1.1	5.4	20.4 ± 1.4	
	R3 ($n = 20$)	21.0 ± 0.9	4.2		
Foodstuff Control	R1 ($n = 21$)	16.3 ± 1.5	8.9	16.9 ± 1.6	9.2
	R2 ($n = 21$)	16.6 ± 0.8	5.0		
	R3 ($n = 23$)	17.6 ± 1.9	10.5		
Batch A (168.41 mg/kg AMT 7.65 mg/kg NOR)	R1 ($n = 20$)	16.9 ± 0.8	4.9	17.2 ± 1.1	6.1
	R2 ($n = 20$)	17.9 ± 1.0	5.6		
	R3 ($n = 10$)	16.3 ± 0.6	3.6		
Batch B (22.0 mg/kg AMT; 3.21 mg/kg NOR)	R1 ($n = 33$)	17.2 ± 2.0	11.4	16.6 ± 1.8	11.1
	R2 ($n = 34$)	16.7 ± 0.7	4.2		
	R3 ($n = 21$)	15.7 ± 2.4	15.4		
Batch C (55.7 mg/kg AMT; 1.7 mg/kg NOR)	R1 ($n = 13$)	17.3 ±0.9	4.9	15.5 ± 2.0	12.9
	R2 ($n = 17$)	16.0 ± 1.1	6.6		
	R3 ($n = 20$)	14.0 ± 2.0	14.3		
Batch D 1.0 (59.3 mg/kg AMT; mg/NOR)	R1 ($n = 20$)	16.8 ± 0.9	5.1	16.6 ± 0.8	5.1
	R2 ($n = 10$)	16.6 ± 1.0	5.8		
	R3 ($n = 10$)	16.4 ± 0.7	4.3		

Table 2.5: The mean larval length, standard deviation (SD), and relative standard deviation (RSD) for each replicate for each type of rearing media, and the mean pooled larval length, standard deviation, relative standard deviation for each type of rearing media used to rear larvae of the species *Sarcophaga bullata* (Diptera: Sarcophagidae) at 26°C.

One-way ANOVA analysis of the pooled length data indicated that the differences in mean maximum length between the six different forms of rearing media were statistically significant at an alpha level of 0.05 ($F_{5,338} = 58.97$; $P < 0.0001$; Table 2.6). Further analysis with Tukey's Honestly Significant Difference (HSD) pairwise comparison procedure to control for multiple testing, indicated that the mean maximum lengths (±SD) for Batches A, B, C, D, and the Foodstuff Control were significantly less than the mean lengths (±SD) for the Liver Control (Table 2.4; Table 2.5). Furthermore, only the mean maximum lengths from the larvae reared on artificial foodstuff containing 41.38 mg/kg amitriptyline and 1.92 mg/kg nortriptyline (Batch C) were shown to be significantly different from the mean maximum lengths obtained for the larvae reared on the other four types of artificial foodstuff (Table 2.4; Table 2.5). However, given the much lower mean maximum length observed for Replicate 3, Batch C, this result was not unexpected.

The method validation determined that only Batches A and D were determined to be homogeneous in terms of the spiked amitriptyline concentration. With this in mind, it is possible that the inhomogeneity of Batch C resulted in one replicate with a considerably lower mean maximum length compared to the other two replicates. Overall, the mean maximum lengths of the larvae reared on artificial foodstuff were significantly different from the lengths of larvae reared on beef liver (Control). This trend is clearly illustrated in the box and whisker plot of the pooled maximum length data for each type of rearing media (Figure 2.2).

The order, in terms of decreasing mean maximum pooled length was as follows (Table 2.5):

Liver Control > Batch A > Foodstuff Control > Batch B = Batch D > Batch C

Source of Variation	**DF**	**Sums of Squares**	**Mean Square**	***F***	***P***
Rearing Media	5	719.12	143.83	58.97	< 0.0001
Error	338	824.39	2.44	-	-

Table 2.6: The results for the one-way ANOVA analysis of the pooled mean maximum length data for *Sarcophaga bullata* larvae reared on six different types of rearing media.

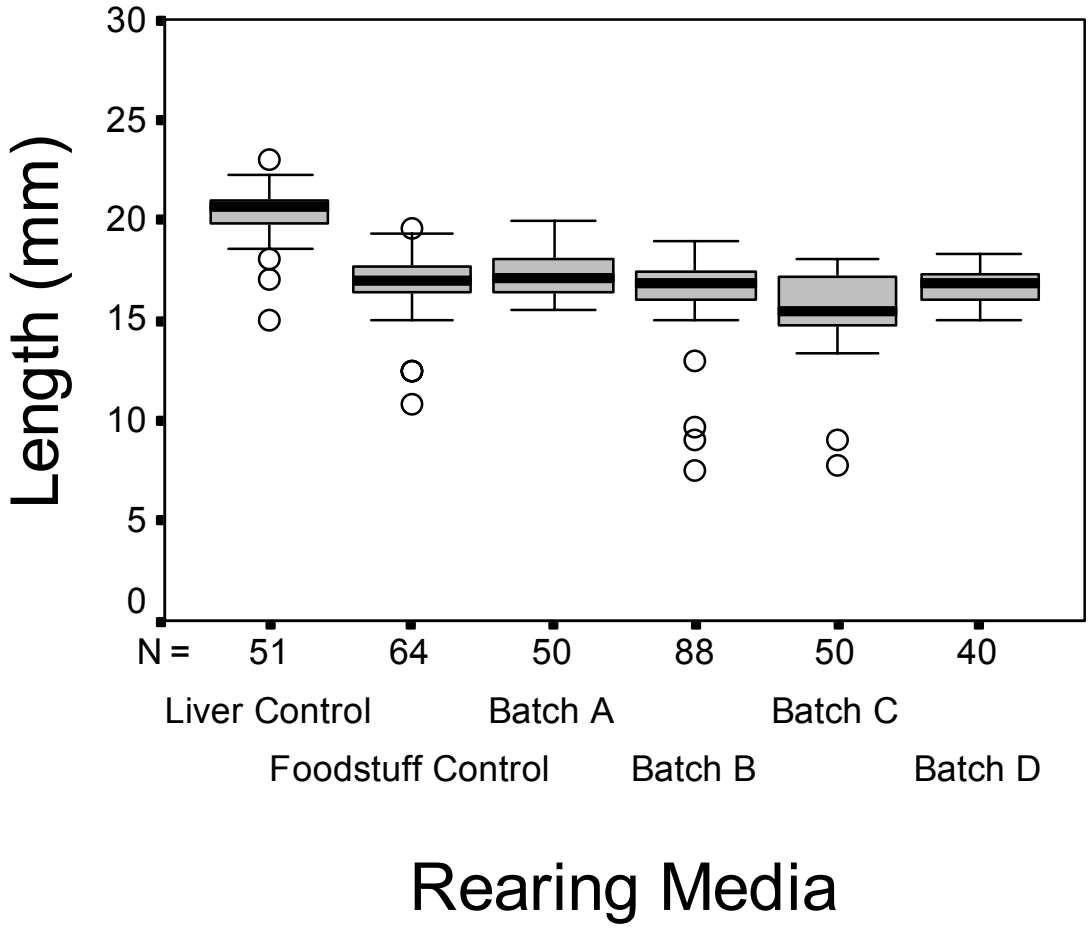


Figure 2.2: Box and whisker plot of the length data used to calculate the mean maximum larval length for larvae reared on each of the six types of rearing media at 26°C. The box and whisker plot was constructed using the length data from all three replicates from each type of rearing media. The plot shows the median length for each type of rearing media as a thick black bar. The interquartile range of the length data for each type of rearing media is shown as a box. The range of the length data for each type of rearing media is illustrated by the thin black bars (whiskers). Data points that are outside the range of the other points observed for the data set are illustrated by circles.

Larval Development Curves: Mean Length *versus* Time

The development curves created from the length data for the first experimental set are illustrated in Figures 2.3 through 2.8. Replicates 1 and 2 from Batch B required a slightly different procedure to obtain the mean maximum lengths. As illustrated in Figure 2.4, the mean maggot lengths for these two replicates of Batch B plateaued for two sampling periods. The mean lengths for the larvae of Replicate 1 plateaued between approximately 70 and 81 hours, and for Replicate 2, the mean lengths plateaued between approximately 53 and 71 hours. Therefore, the time at which the mean maximum length was achieved for Replicates 1 and 2 of Batch B was estimated by calculating the average of their respective plateau times.

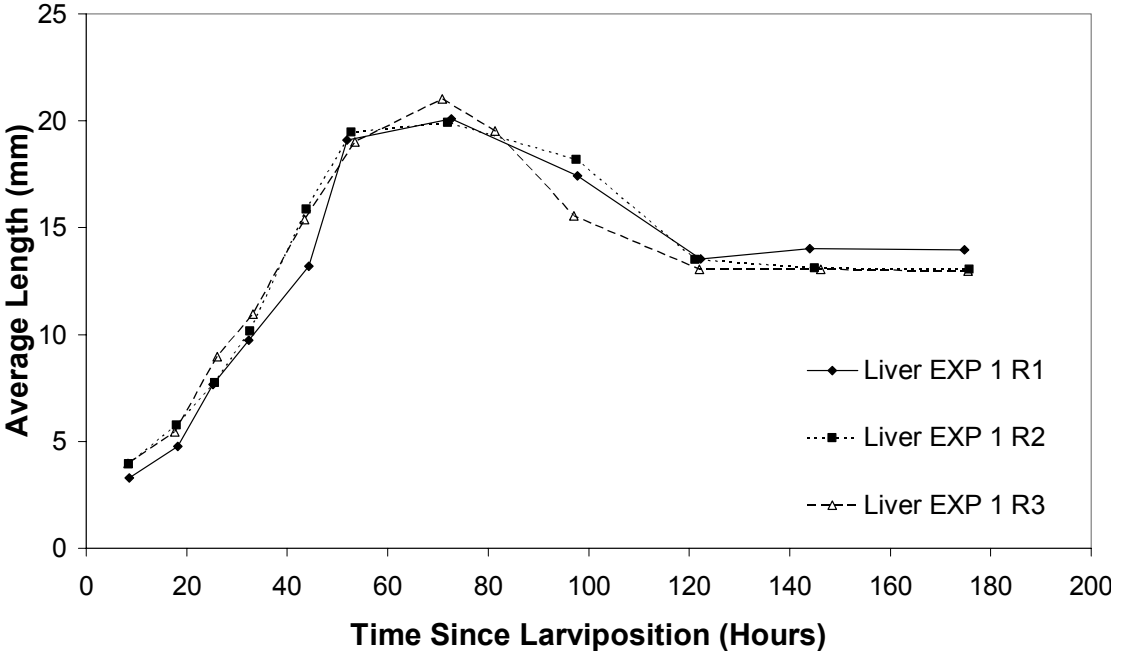


Figure 2.3: Mean larval length vs. time since larviposition (hrs) for *Sarcophaga bullata* larvae reared on the Liver Control at 26°C.

All three replicates of larvae reared on the liver (Liver Control) experienced a relatively smooth and constant increase in mean body length until the mean maximum length was achieved (Figure 2.3). The same relatively smooth and constant increase in mean body length was observed in all three replicates of larvae reared on the blank artificial foodstuff (Foodstuff Control) (Figure 2.4).

In comparison, only one of the three larval replicates reared on the artificial foodstuff containing 22.0 mg/kg amitriptyline and 3.21 mg/kg nortriptyline (Batch B) experienced a relatively smooth increase in length over time. Given the inhomogeneity of Batch B, this result was not surprising. Both Replicates 1 and 3 of Batch B experienced a sharp decrease in mean larval length at approximately 33 hours since

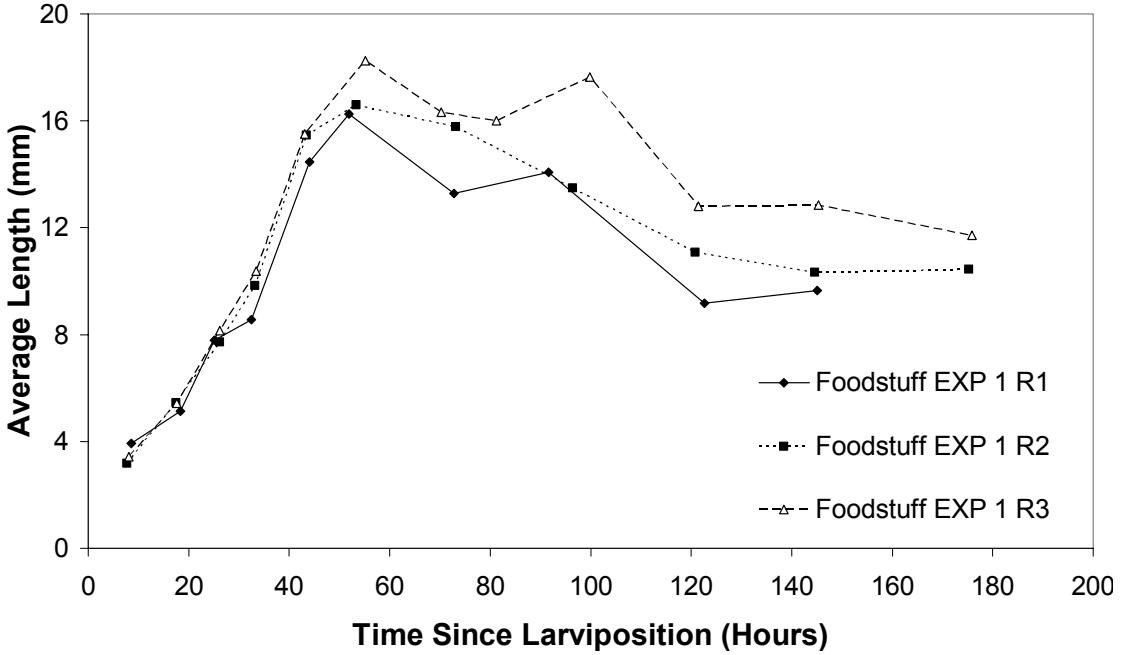


Figure 2.4: Mean larval length vs. time since larviposition (hrs) for *Sarcophaga bullata* larvae reared on the Foodstuff Control at 26°C.

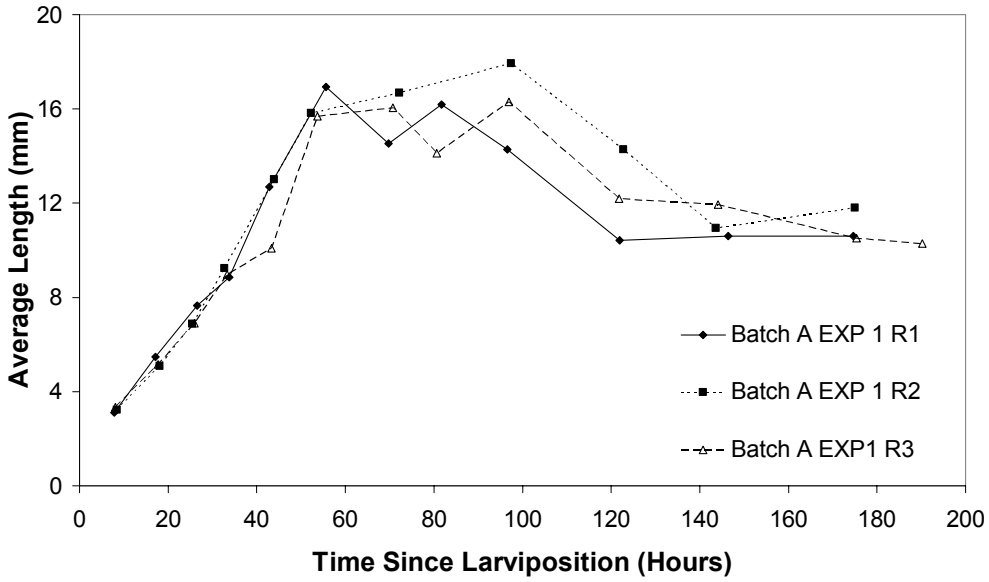


Figure 2.5: Mean maggot lengths vs. time since larviposition (hrs) for *Sarcophaga bullata* larvae reared on artificial foodstuff containing 168.41 mg/kg amitriptyline and 7.65 mg/kg nortriptyline (Batch A) at 26°C.

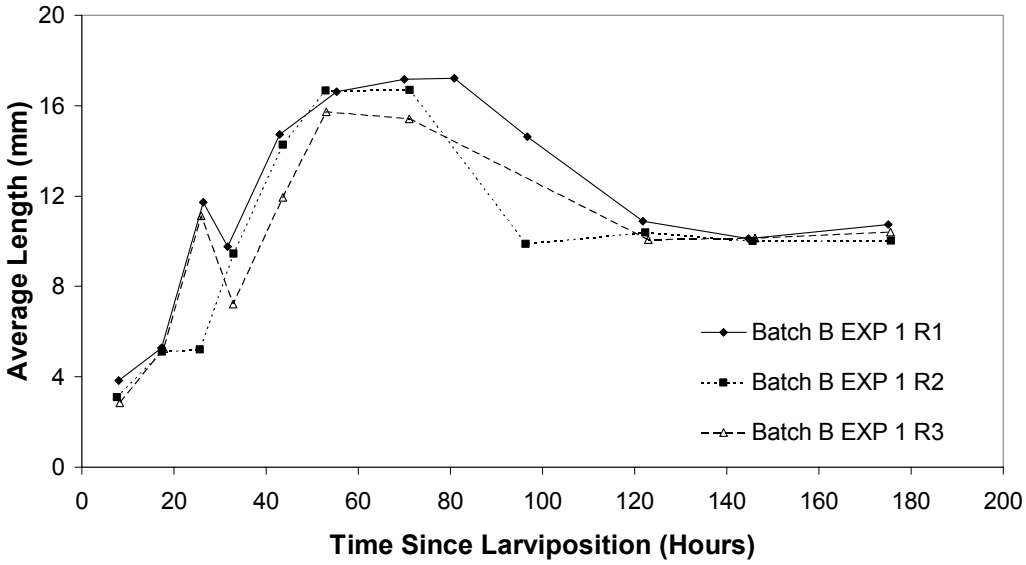


Figure 2.6: Mean maggot lengths vs. time since larviposition (hrs) for *Sarcophaga bullata* larvae reared on artificial foodstuff containing 22.0 mg/kg amitriptyline and 3.21 mg/kg nortriptyline (Batch B) at 26°C.

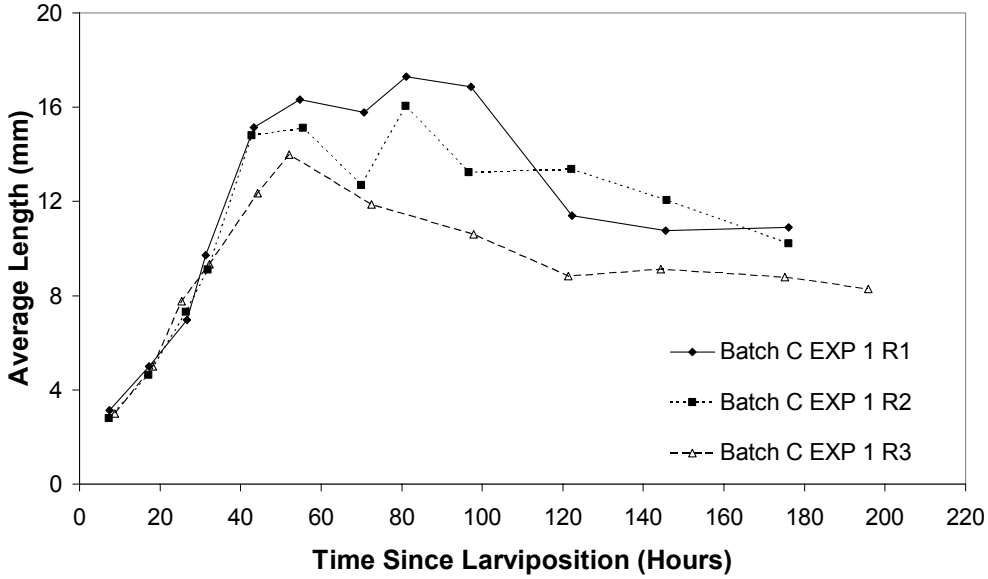


Figure 2.7: Mean maggot lengths vs. time since larviposition (hrs) for *Sarcophaga bullata* larvae reared on artificial foodstuff containing 41.38 mg/kg amitriptyline and 1.92 mg/kg nortriptyline (Batch C) at 26°C.

larviposition (Figure 2.6). The larvae sampled from Batch B Replicate 1 at approximately 33 hours since larviposition were a mix of moulting second instars and feeding third instars. Larval lengths at that time ranged from a minimum of 8.7 mm to a maximum of 10.6 mm. In Batch B Replicate 2, larval lengths ranged from 8.6 mm to 10.1 mm, and all of the insects sampled were second instars. In contrast, the larvae sampled from Batch B Replicate 3 at approximately 33 hours since larviposition were a mix of feeding second instars and moulting second instars. Larval lengths at that time ranged from 2.6 mm to 10.6 mm.

In addition, the variation in larval lengths at 33 hours since larviposition for Batch B Replicate 3 were quite large, as indicated by the magnitude of the error bars in Figure 2.12C. The errors bars in the figure represented the standard deviation about the mean. The variation in larval lengths at 33 hours since larviposition for Batch B Replicate 1 (Figure 2.12A) and Replicate 2 (Figure 2.12B) was considerably lower than the variation in length observed for Batch B Replicate 3 (Figure 2.12C).

The larvae sampled from Replicates 1 and 2 of Batch C also exhibited a sharp decrease in mean body length before the mean maximum length was achieved (Figure 2.13). The sharp decrease in the mean larval length occurred approximately 70 hours after larviposition in both Replicates 1 and 2. In contrast, although Replicate 3 of Batch C exhibited a similar sharp decrease in mean larval length at approximately 70 hours, the following sharp increase in mean larval length, which was observed in both Replicates 1 and 2 of Batch C at approximately 96 hours. In fact, the overall mean maximum length for larvae from Batch C Replicate 3 was achieved at approximately 52 hours after larviposition. Therefore, it appears that the larvae in Replicate 3 of Batch C were developing at faster rate compared to the larvae in Replicates 1 and 2.

Larval lengths for the larvae sampled from Batch C Replicate 1 ranged from 13.2 mm to 18 mm. Furthermore, all of the insects sampled were feeding third instar larvae. In comparison, the larvae sampled from Batch Replicate 2, 70 hours after larviposition, were a mixture of feeding and postfeeding third instar larvae. Larval lengths for Replicate 2 ranged from 5 mm to 15.3 mm. The shortest larva (5 mm) in sample obtained from Replicate 2 was a postfeeding larva. In comparison, the larvae sampled from Batch C Replicate 3, ranged from 9.5mm in length to 13.8 mm in length, and were also a mixture of feeding and postfeeding third instar larvae.

Graphs illustrating the mean length (±SD) *versus* time since larviposition, for each replicate, for each type of rearing media are illustrated in Figures 2.9 through 2.12.

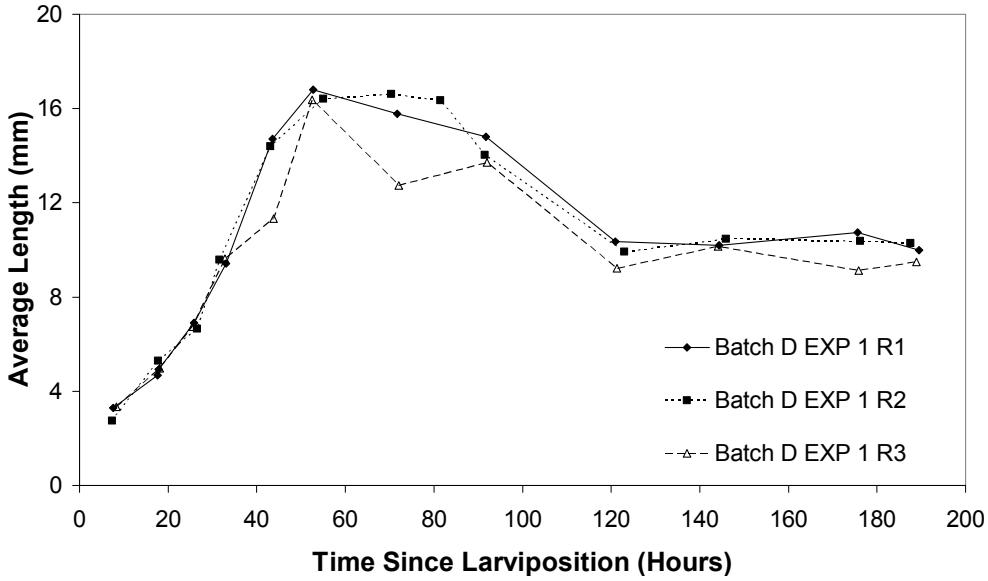
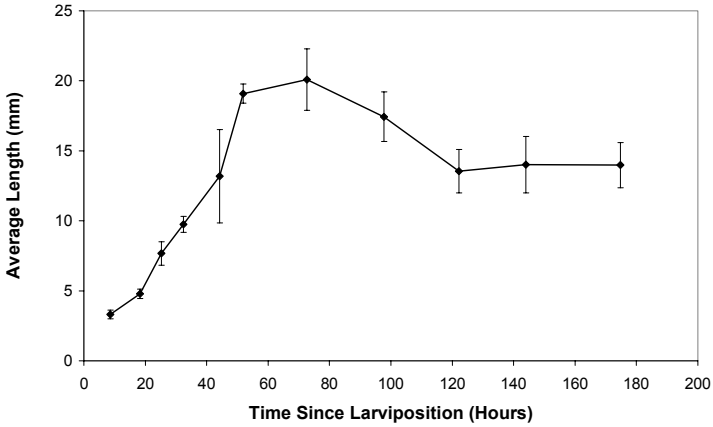
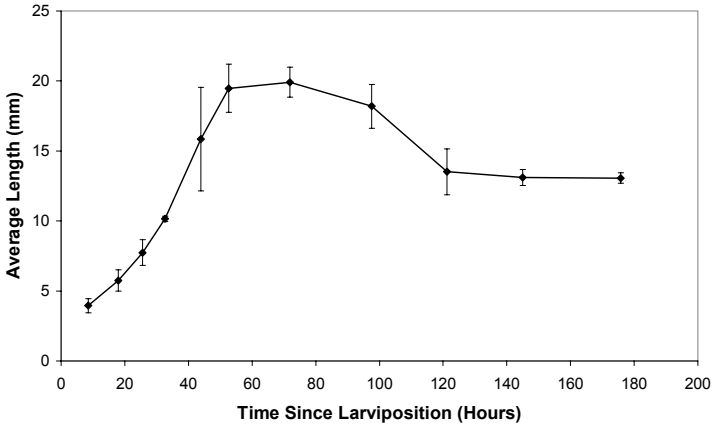


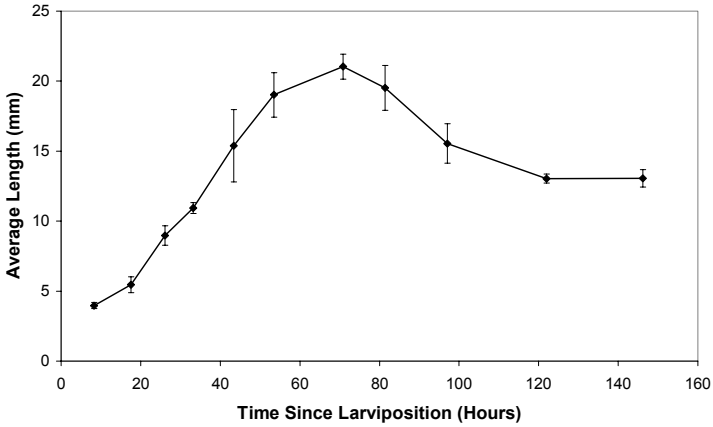
Figure 2.8: Mean maggot lengths vs. time since larviposition (hrs) for *Sarcophaga bullata* larvae reared on artificial foodstuff containing 42.59 mg/kg amitriptyline and 1.22 mg/kg nortriptyline (Batch D) at 26°C.



(A)

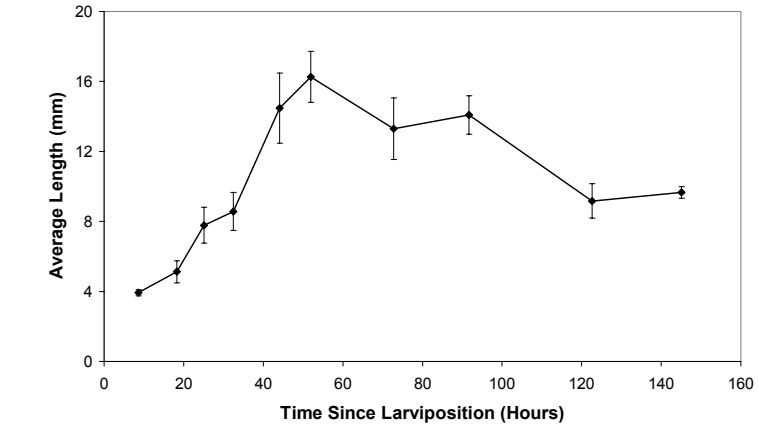


(B)

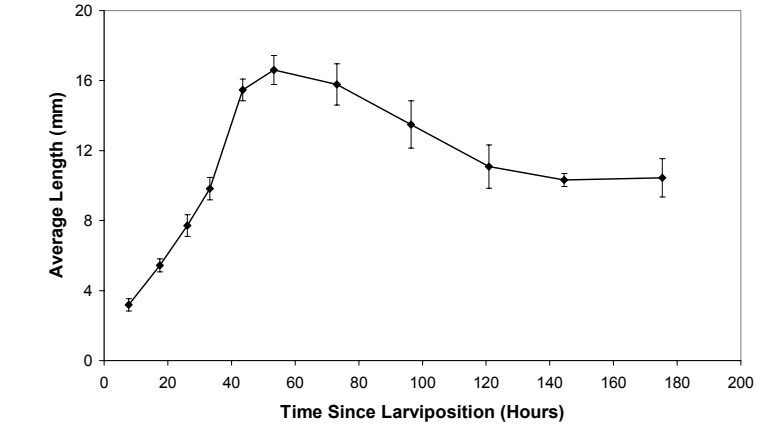


(C)

Figure 2.9: Mean larval length (±SD) *versus* time since larviposition for the three replicates of *Sarcophaga bullata* larvae reared on liver at 26°C. (A) Liver Control Replicate 1, (B) Liver Control Replicate 2 and (C) Liver Control Replicate 3.



(A)



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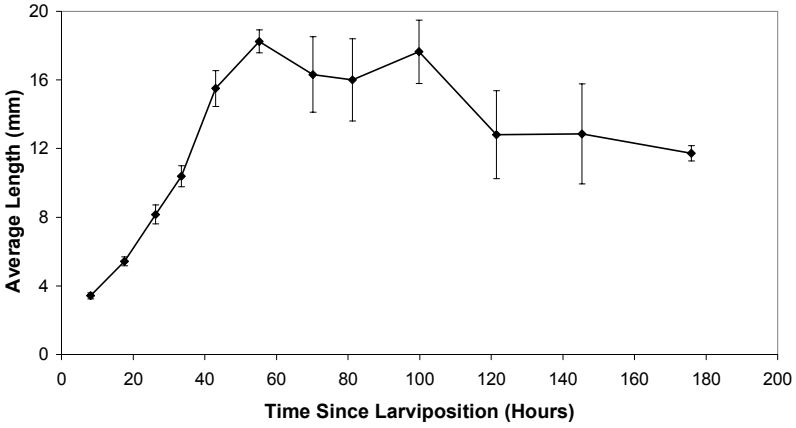
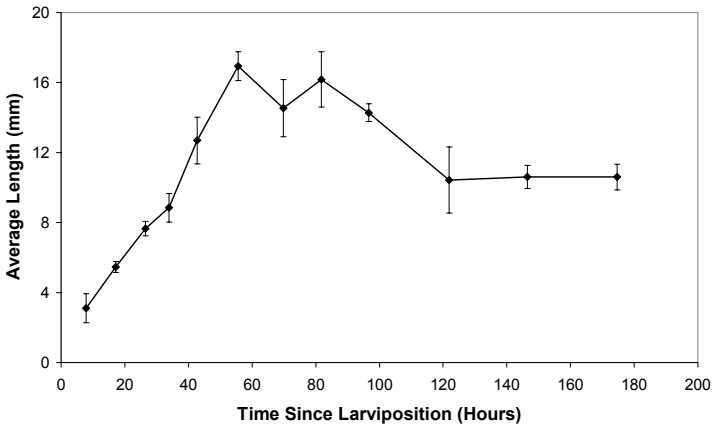
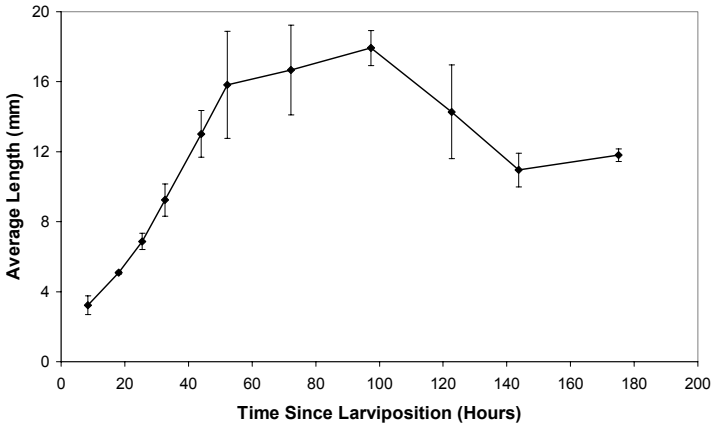


(C)

Figure 2.10: Mean larval length (±SD) *versus* time since larviposition for the three replicates of *Sarcophaga bullata* larvae reared on blank artificial foodstuff (Foodstuff Control) at 26°C. (A) Foodstuff Replicate 1, (B) Foodstuff Replicate 2, and (C) Foodstuff Replicate 3.



(A)



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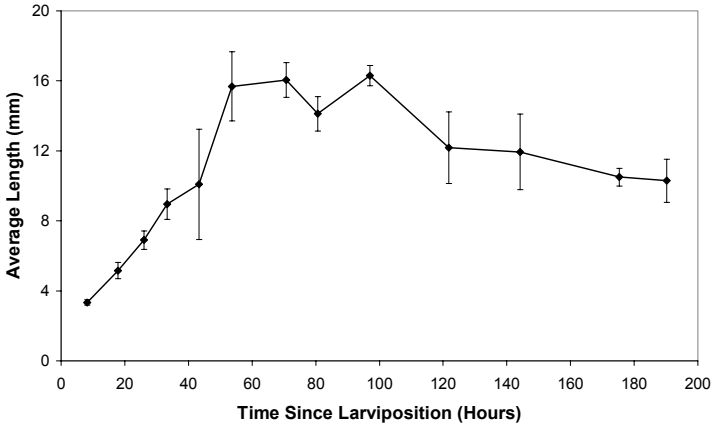
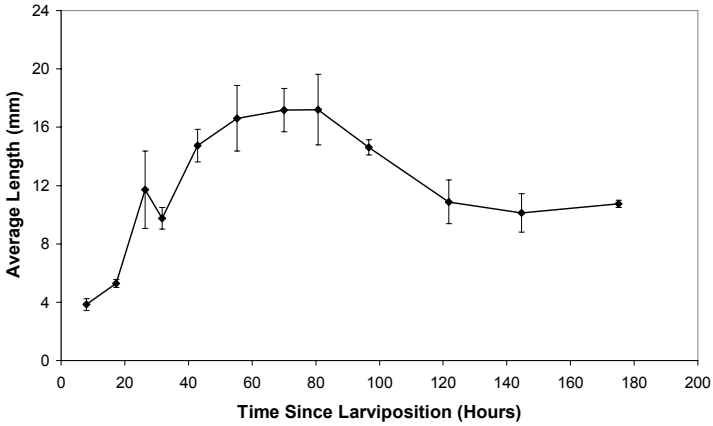
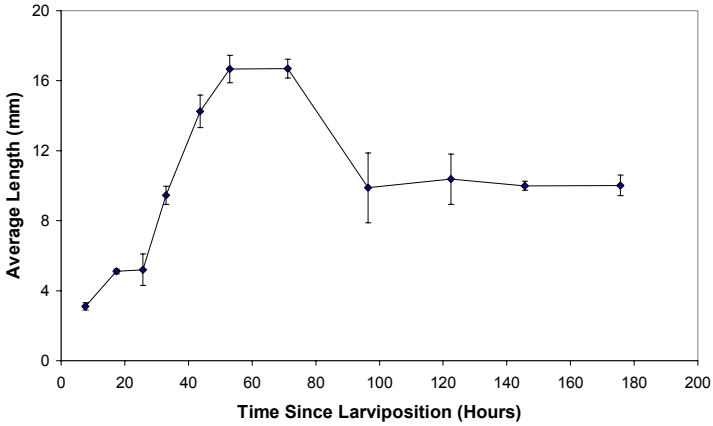


(C)

Figure 2.11: Mean larval length (±SD) *versus* time since larviposition for the three replicates of *Sarcophaga bullata* larvae reared on artificial foodstuff containing 168.41 mg/kg amitriptyline and 7.65 mg/kg nortriptyline (Batch A) at 26°C.(A) Batch A Replicate 1, (B) Batch A Replicate 2, and (C) Batch A Replicate 3.



(A)



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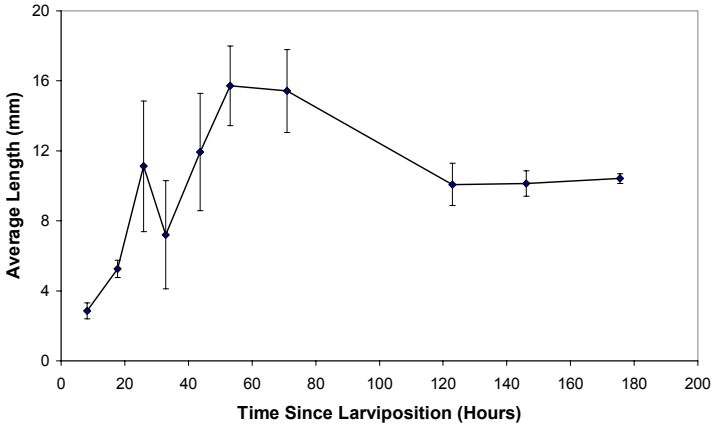
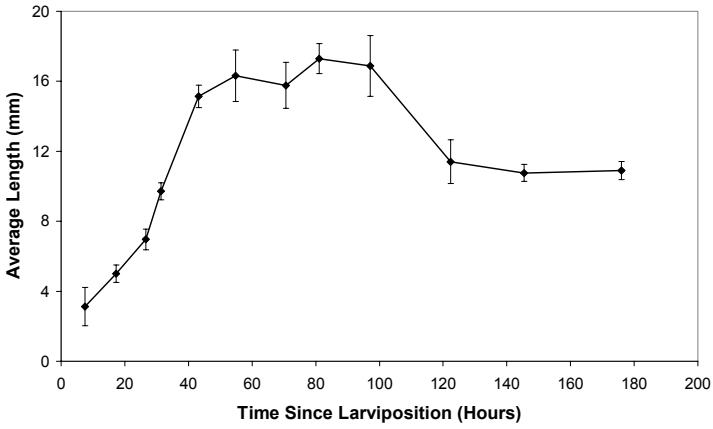
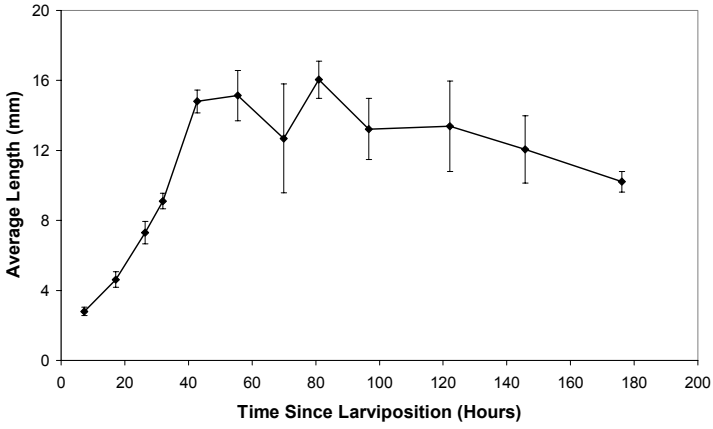


(C)

Figure 2.12: Mean larval length (±SD) *versus* time since larviposition for the three replicates of *Sarcophaga bullata* larvae reared on artificial foodstuff containing 9.55 mg/kg amitriptyline and 3.21 mg/kg nortriptyline (Batch B) at 26°C. (A) Batch B Replicate 1, (B) Batch B Replicate 2, and (C) Batch B Replicate 3.



(A)



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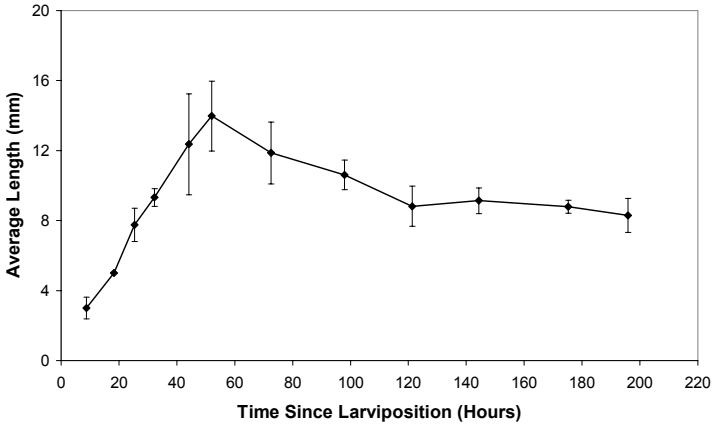
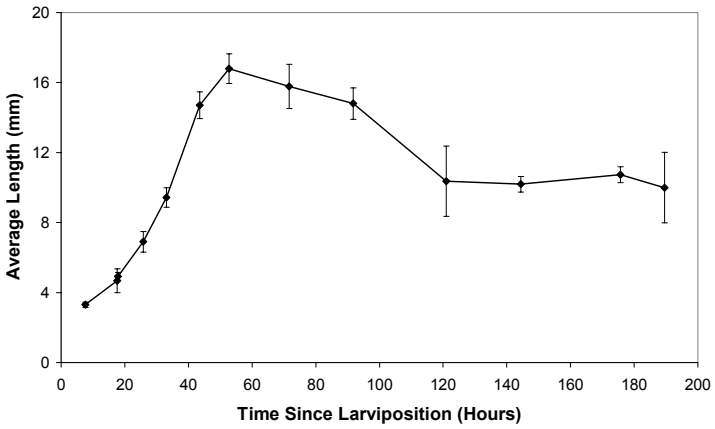
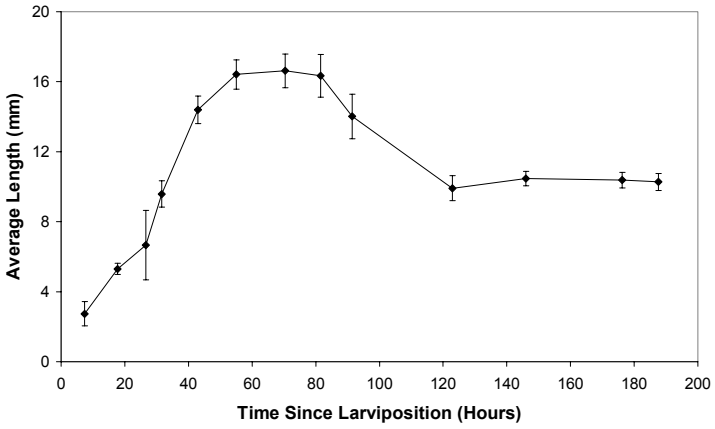


(C)

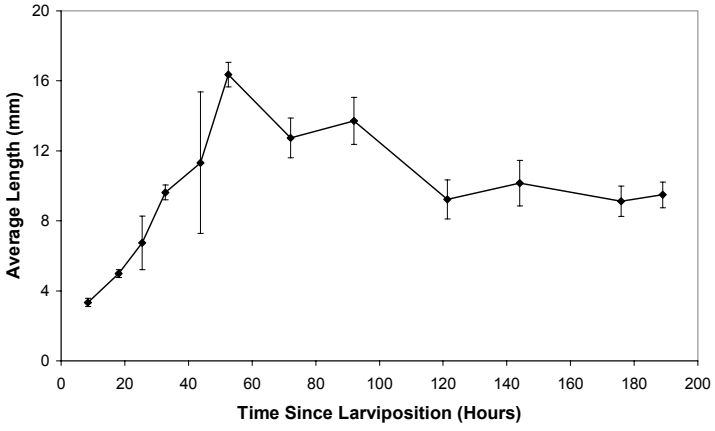
Figure 2.13: Mean larval length (±SD) *versus* time since larviposition for the three replicates of *Sarcophaga bullata* larvae reared on artificial foodstuff containing 55.7 mg/kg amitriptyline and 1.7 mg/kg nortriptyline (Batch C) at 26°C. (A) Batch C Replicate 1, (B) Batch C Replicate 2, and (C) Batch C Replicate 3 for Experiment 1.



(A)



(B)



(C)

Figure 2.14: Mean larval length (±SD) *versus* time since larviposition for the three replicates of *Sarcophaga bullata* larvae reared on artificial foodstuff containing 42.59 mg/kg amitriptyline and 1.22 mg/kg nortriptyline (Batch D) at 26°C. (A) Batch D Replicate 1, (B) Batch D Replicate 2, and (C) Batch D Replicate 3.

2.3.3 Discussion of the Effects of Amitriptyline and Nortriptyline on Larval Length

Based on these results, it appears that while the addition of drugs to the rearing media increased the variability in the time required for the maggots to reach their respective mean maximum lengths, the presence of a specific amount of either amitriptyline or nortriptyline did not appear to significantly alter the time required for the larvae reared on the six different sets of rearing media to reach their respective mean maximum length. In the study conducted by Goff *et al.* (1993), no difference in the time required to reach the mean maximum length was observed among any of the colonies, control or drug-spiked.

Further, no obvious pattern, in terms of amitriptyline batch inhomogeneity was observed for the variability in the time required for the larvae reared on each batch of foodstuff to reach their respective mean maximum length. For example, the RSD associated with the amitriptyline concentration in Batch B was 46.7%, while RSD associated with the time required to reach the mean maximum length for the larvae reared on Batch B foodstuff was only 17.7%. In comparison, the RSD associated with the amitriptyline concentration in Batch A was only 7.9%, while the RSD associated with the time required to reach the mean maximum length was 28.8%.

In the study conducted by Goff *et al.* (1993), the greatest mean lengths were observed for the colony reared on the liver from the rabbit that had received 1000 mg of amitriptyline by ear vein infusion. The concentration of amitriptyline and nortriptyline present in the liver of this rabbit was 49.0 mg/kg and 0.9 mg/kg, respectively. These concentrations were comparable to those found in Batch C of the present experiment. Even so, no statistically significant difference in the mean maximum length was observed across the four different insect colonies studied by Goff *et al.* (1993).

However, in the present experiment, the lowest mean maximum lengths were observed for the larvae reared on the foodstuff containing 41.38 mg/kg amitriptyline and 1.92 mg/kg nortriptyline (Batch C), which was the exact opposite of what was observed by Goff *et al.* (1993). The percent difference between the mean concentration of amitriptyline present in Batch C and the target amitriptyline concentration was -15.5%. Although this difference is substantial, it is unlikely that the difference between the target concentration and the mean measured concentration of amitriptyline was influential enough to have caused a complete reversal of the results for mean maximum length observed by Goff *et al.* (1993).

Three possible explanations for this, and any other differences observed between the results observed by Goff *et al.* (1993), and the results observed by the author, are:

1. Species difference; Goff studied the effects of amitriptyline and nortriptyline on the development of *P. ruficornis*, and the present project studied the effects of amitriptyline and nortriptyline on the development of *S. Bullata*

2. Goff *et al.* (1993), used rabbit tissue for their rearing media. The present project

used beef liver.

3. Goff *et al.* (1993), used a single drug-contaminated rabbit tissue for their experiment. Given the relatively high density of larvae added to the liver, a food shortage may have occurred. In contrast, in the present experiment, fresh food was added on a continual basis by the author.

Therefore, although both the present experiment, and the experiment conducted by Goff *et al.* (1993) used fly species from the Family Sarcophagidae, it is possible that differences, in terms of the physiological response to the presence of amitriptyline and nortriptyline in the rearing media contributed to the different results. Or, the species difference, with respect to the rearing media, may have contributed to the reversal in results observed for *S. bullata* compared with *P. ruficornis*.

In the present study, the mean maximum lengths obtained from the larvae reared on both the drug-spiked artificial foodstuff (Batches A, B, C and D), and the drug-free artificial foodstuff (Foodstuff Control) were significantly different from the mean maximum lengths of the larvae reared on liver (Liver Control). Since a relationship between drug concentration and mean maximum length was not observed in the data, the differences in length are more likely due to the use of the artificial foodstuff, and not the presence of amitriptyline and nortriptyline in the artificial foodstuff.

Overall, the pooled length data for the three replicates of Batch B and the replicates of Batch C exhibited the greatest variability in mean maximum larval length, with RSDs of 11.1% and 12.9%. Given the fact that these two batches were heterogeneous in terms of amitriptyline concentration, the greater variability is not unexpected if amitriptyline and nortriptyline actually have an effect on *S. bullata* development. However, the effects of amitriptyline and nortriptyline on *S. bullata* development, if there are any, are indistinguishable from the effect of the artificial foodstuff.

Overall, the inhomogeneity of the artificial foodstuff with respect to amitriptyline appeared to contribute to the variability observed in mean maximum length, but not to the variability observed in the time required to reach the mean maximum length.

2.3.4 Developmental Stage as an Indicator of Maggot Age: Results

Total Development Time, from Larva to Adult for *Sarcophaga bullata*

The mean total development time for the larvae reared on each of the six types of rearing media is presented in Table 2.7.

Rearing Media	**Mean (±SD) Mean Total Development Time (hrs)**	**Relative Standard Deviation (%)**
Liver Control	438.1 ± 1.48	0.3
Foodstuff Control	409.7 ± 19.29	4.7
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	422.5 ± 21.51	5.1
Batch B (9.55 mg/kg AMT; 3.21 mg/kg NOR)	404.5 ± 23.90	5.9
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	397.5 ± 17.9	4.5
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	384.4 ± 12.23	3.2

Table 2.7: The mean, standard deviation (±SD), and relative standard deviation for the total development time for Sarcophaga bullata reared on six different types of rearing media.

Based on the results presented in Table 2.7, the order, in terms of decreasing mean total development period of *S. bullata* was as follows:

Liver > Batch A > Foodstuff Control > Batch B > Batch C > Batch D

One-way ANOVA analysis of the mean total development time indicated that the differences in the mean total development time for *S. bullata*, from larviposition to adult emergence, were significant ($F_{5,12} = 3.432$; $P = 0.037$; Table 2.8).

Further analysis by Tukey's Honestly significant difference test indicated that the total development time for the insects reared on the liver control differed significantly from the total development time observed for larvae reared on Batch B, Batch C and Batch D. In addition, the total development time for the insects reared on Batch D significantly differed from the total development time for the insects reared on Batch A.

The development curves for each set of *Sarcophaga bullata* larvae reared on the six different types of rearing media are illustrated in Figures 2.15 through 2.20.

Source of Variation	**DF**	**Sums of Squares**	**Mean Square**	***F***	***P***
Rearing Media	5	5373.11	1074.62	3.43	< 0.0372
Error	12	3757.31	313.11	-	-

Table 2.8: One-way ANOVA results for the mean total development time for *S. bullata* reared on six different types of rearing media at 26°C.

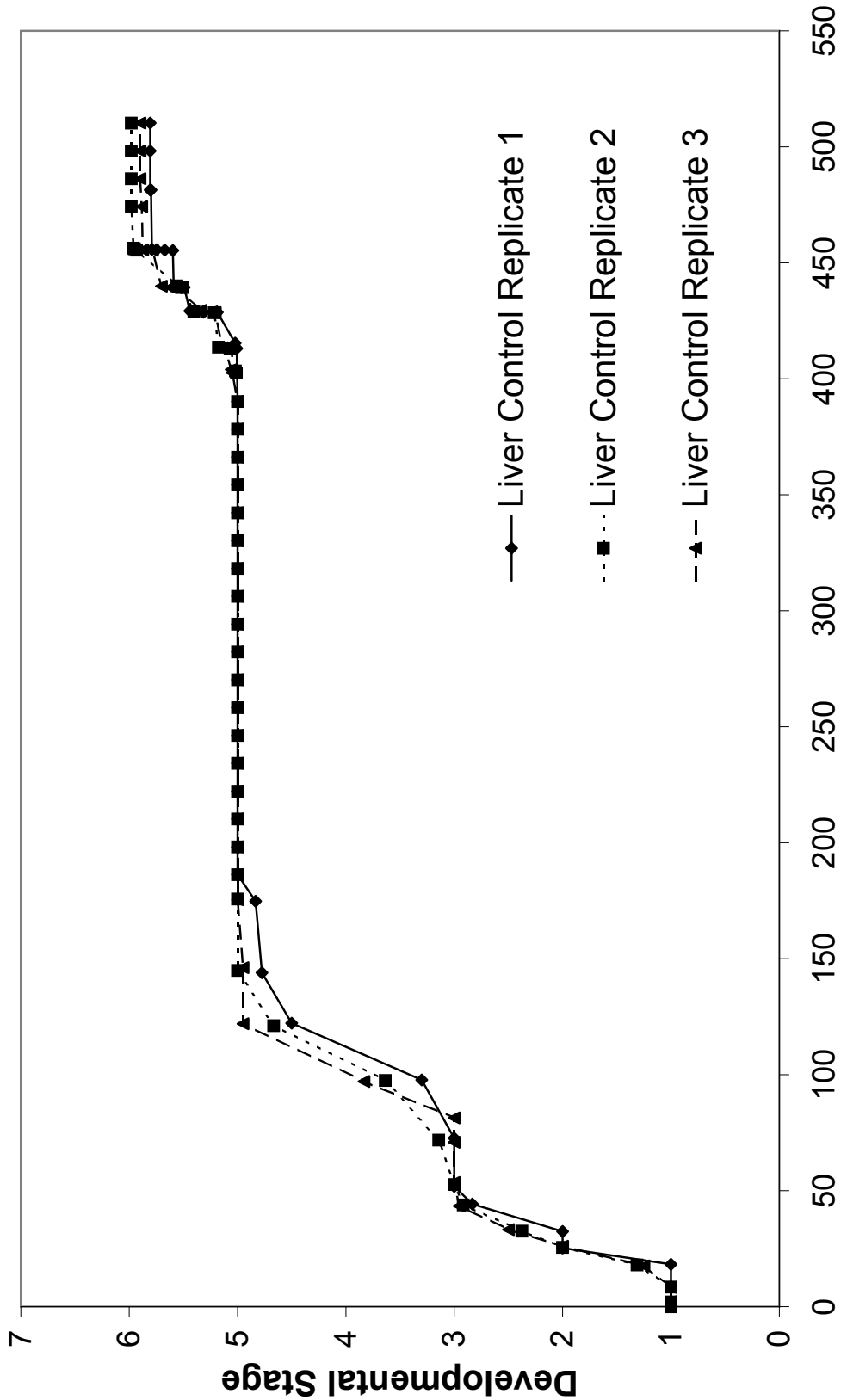


Figure 2.15: Development curve for *Sarcophaga bullata* larvae reared on liver at 26 °C. Stage 1 = first instar, Stage 2 = second instar, Stage 3 = third instar (feeding and postfeeding), Stage 4 = prepupal larvae, Stage 5 = pupal stage and Stage 6 = adult emergence.

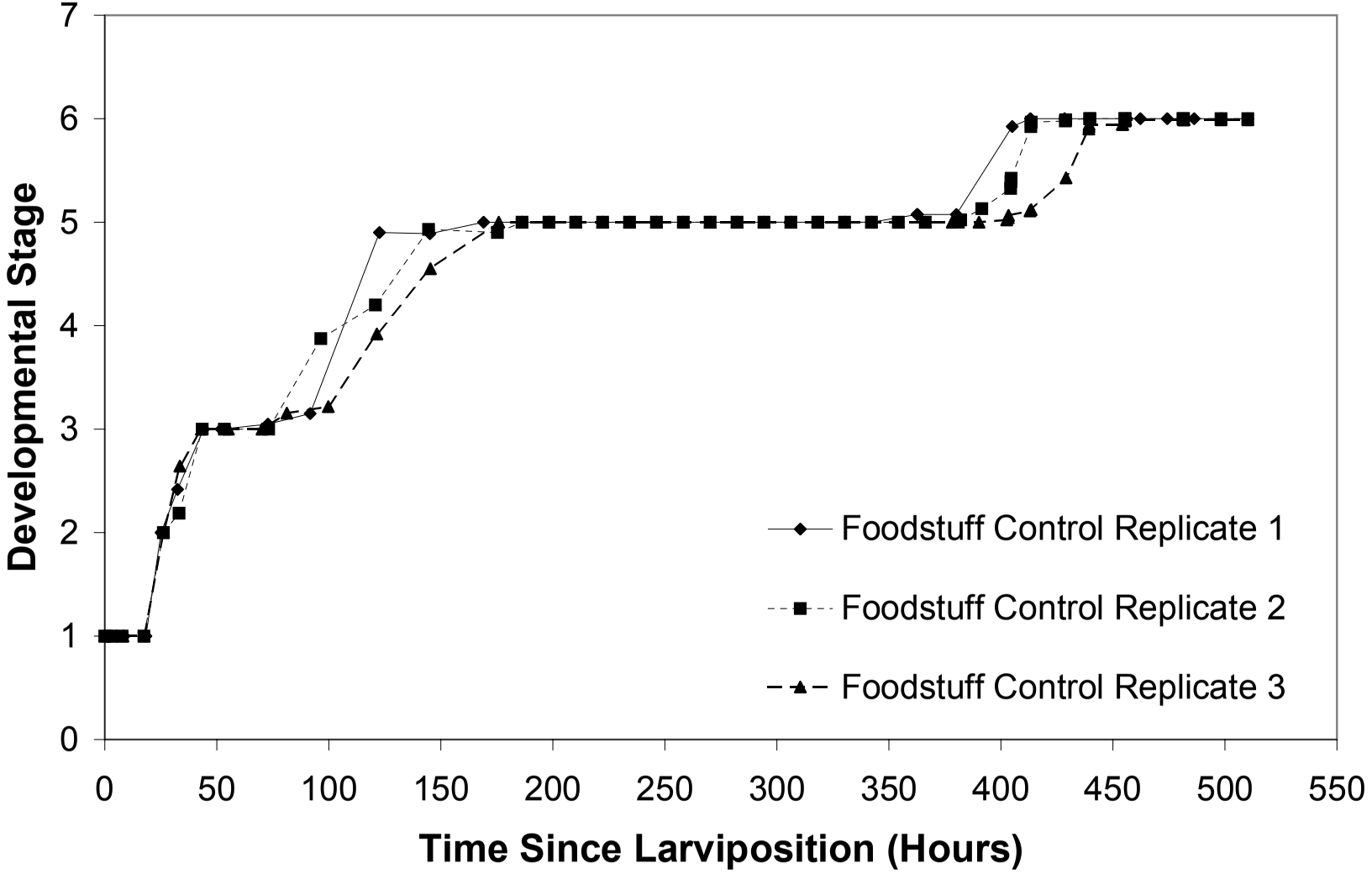


Figure 2.16: Development curve for *Sarcophaga bullata* larvae reared on blank artificial foodstuff (Foodstuff Control) at 26°C. Stage 1 = first instar, Stage 2 = second instar, Stage 3 = third instar (feeding and postfeeding), Stage 4 = prepupal larvae, Stage 5 = pupal stage and Stage 6 = adult emergence.

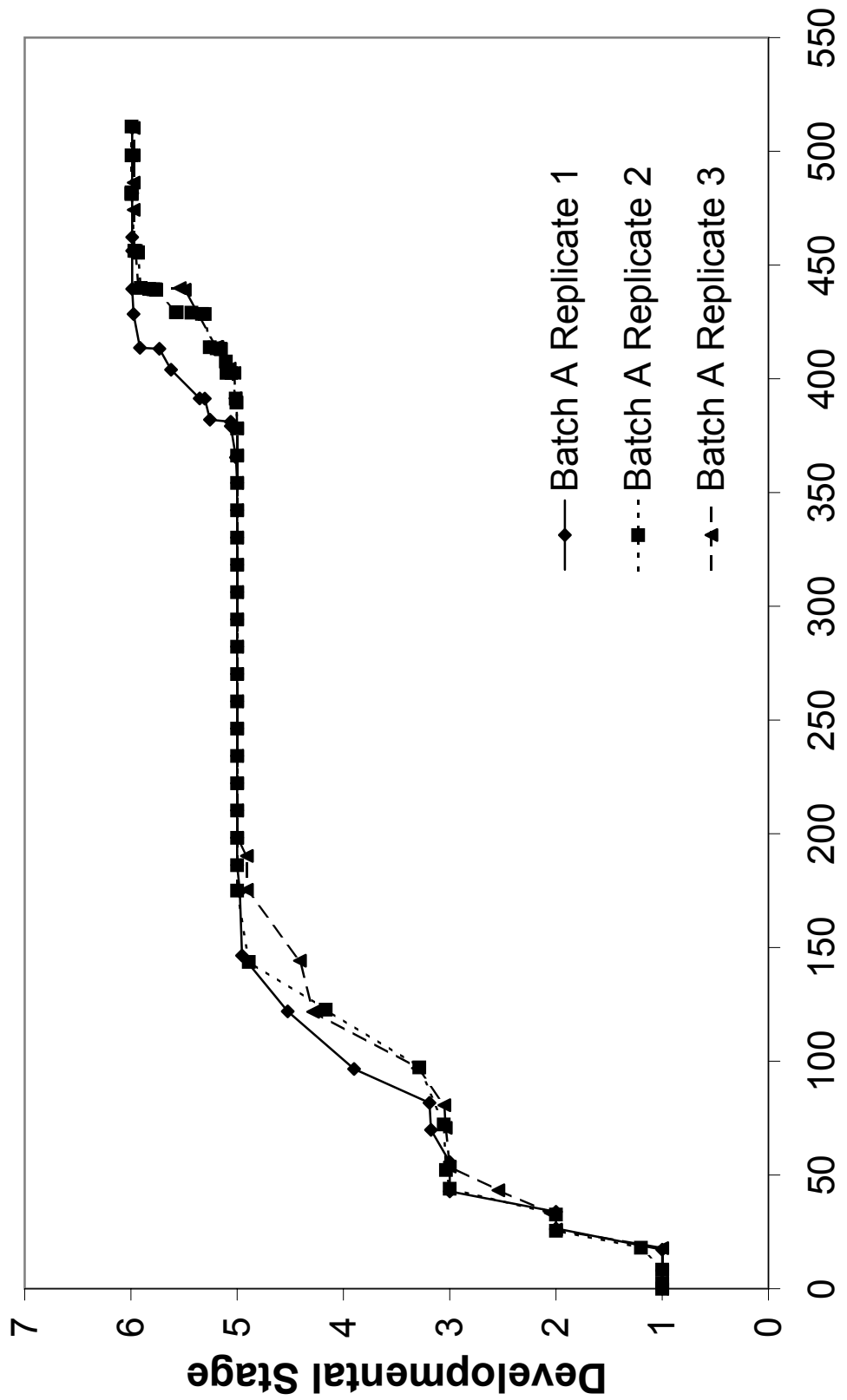


Figure 2.17: Development curve for *Sarcophaga bullata* larvae reared on artificial foodstuff containing 168.41 mg/kg amitriptyline and 7.65 mg/kg nortriptyline (Batch A) at 26°C. Stage 1 = first instar, Stage 2 = second instar, Stage 3 = third instar (feeding and postfeeding), Stage 4 = prepupal larvae, Stage 5 = pupal stage and Stage 6 = adult emergence.

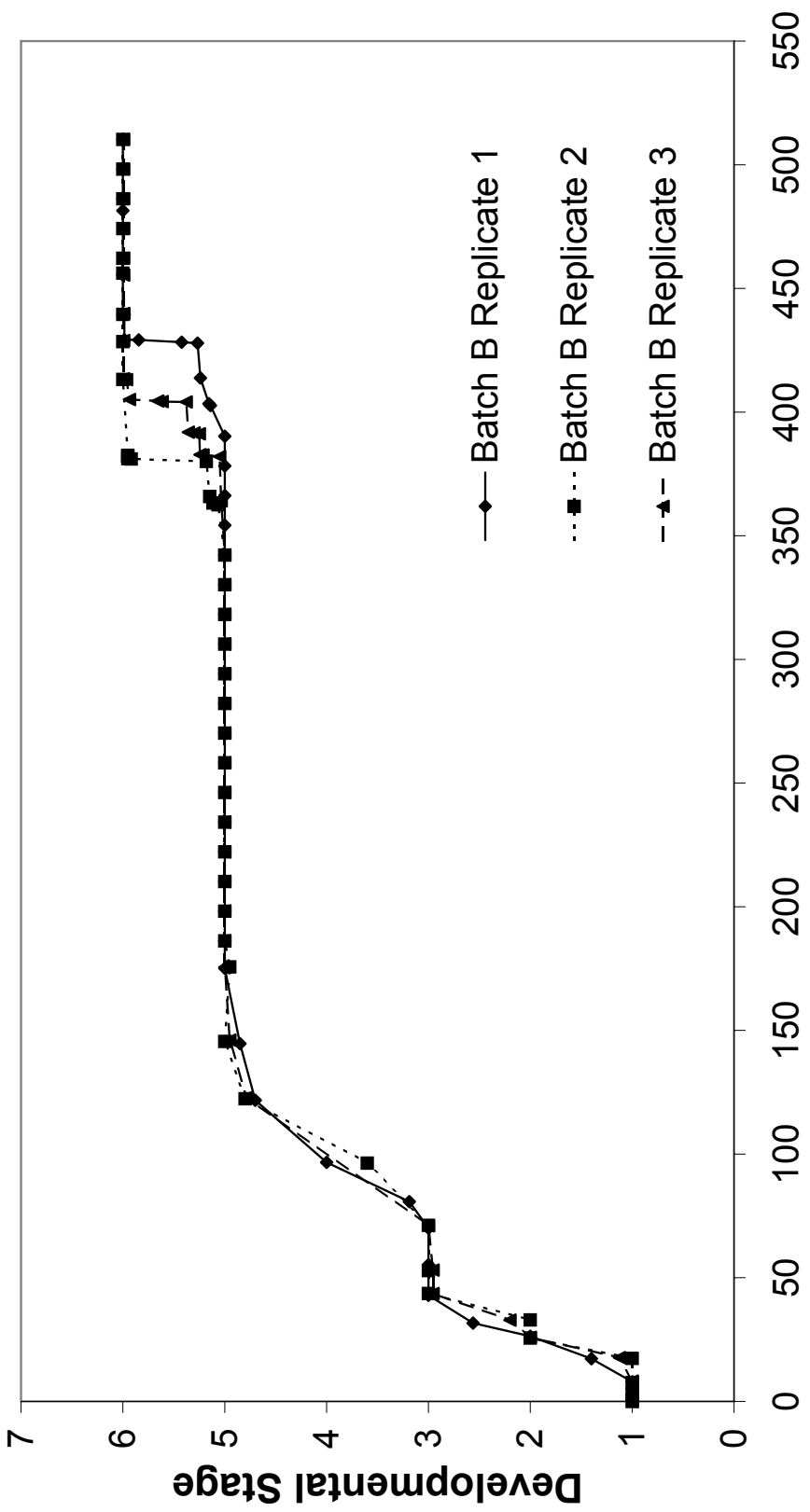


Figure 2.18: Development curve for *Sarcophaga bullata* larvae reared on artificial foodstuff containing 9.55 mg/kg amitriptyline and 3.21 mg/kg nortriptyline (Batch B) at 26°C. Stage 1 = first instar, Stage 2 = second instar, Stage 3 = third instar (feeding and postfeeding), Stage 4 = prepupal larvae, Stage 5 = pupal stage and Stage 6 = adult emergence.

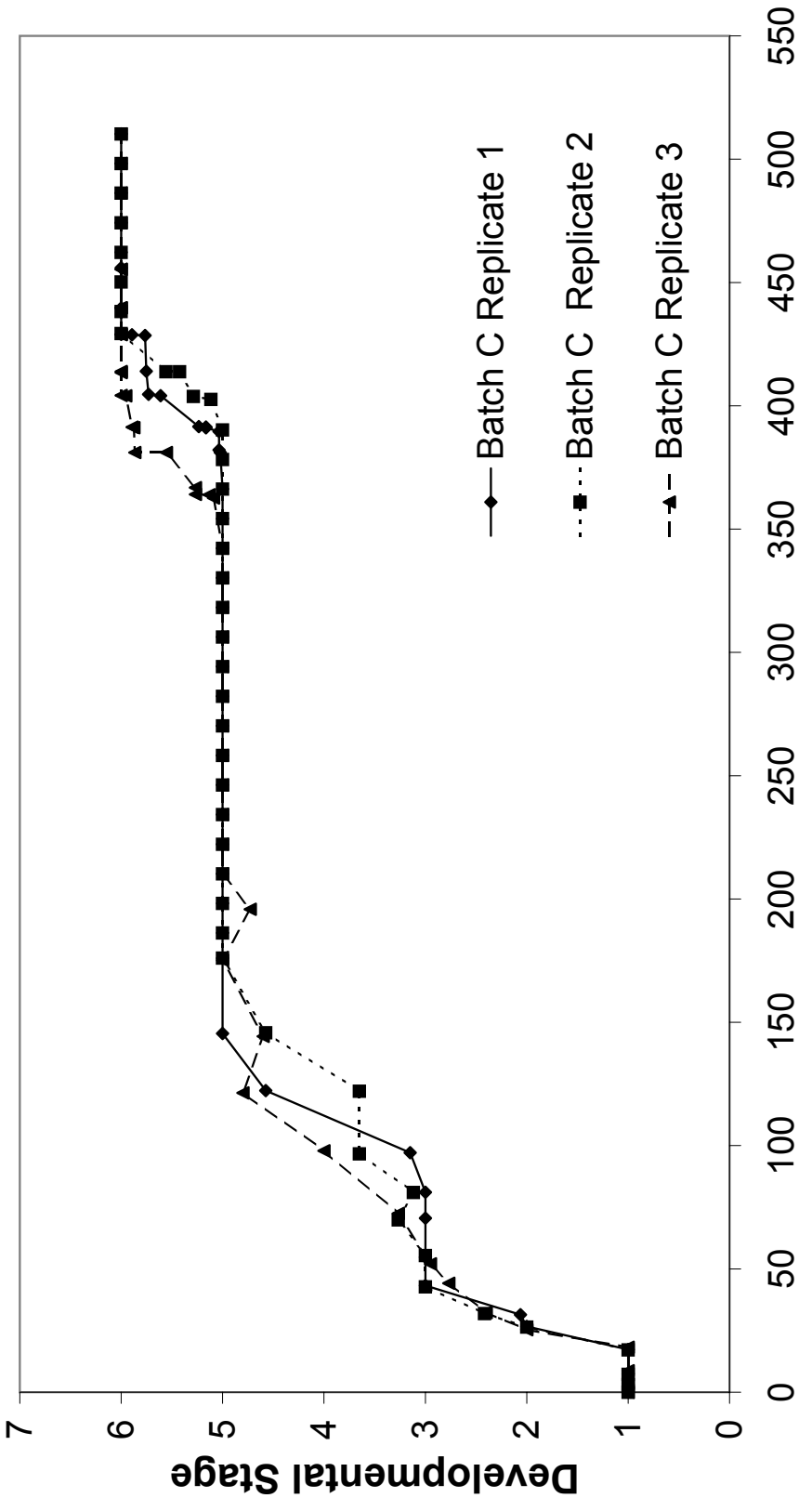


Figure 2.19: Development curve for *Sarcophaga bullata* larvae reared on artificial foodstuff containing 41.38 mg/kg amitriptyline and 1.92 mg/kg nortriptyline (Batch C) at 26°C. Stage 1 = first instar, Stage 2 = second instar, Stage 3 = third instar (feeding and postfeeding), Stage 4 = prepupal larvae, Stage 5 = pupal stage and Stage 6 = adult emergence.

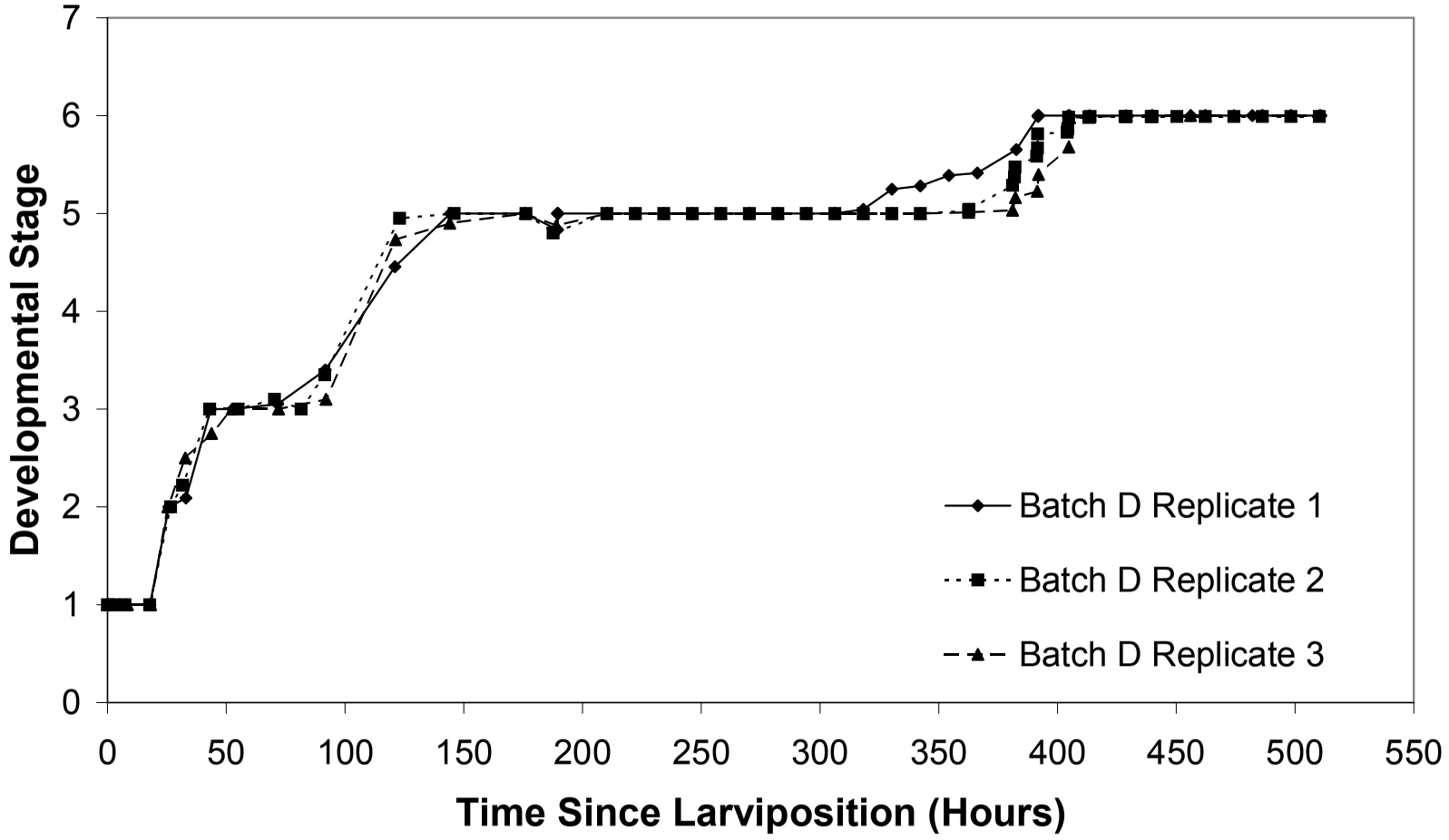


Figure 2.20: Development curve for *Sarcophaga bullata* larvae reared on artificial foodstuff containing 42.59 mg/kg amitriptyline and 1.22 mg/kg nortriptyline (Batch D) at 26°C. Stage 1 = first instar, Stage 2 = second instar, Stage 3 = third instar (feeding and postfeeding), Stage 4 = prepupal larvae, Stage 5 = pupal stage and Stage 6 = adult emergence.

Duration of the Larval Period

Preliminary examination of the results for the total development time indicate that the presence of amitriptyline and nortriptyline in the rearing substrate decreases the time required, from larviposition to adult emergence, for *S. bullata* development at 26°C. However, the effect of amitriptyline and nortriptyline may not be continuous over the entire development period. Flies are holometabolous insects, and as a result drastic physiological changes occur during the transition from larva to adult. With this in mind, the effect of amitriptyline and nortriptyline on the duration of the larval period on its own was also examined.

Rearing Media	**Mean (±SD) Duration of Larval Period (hrs)**	**Relative Standard Deviation (%)**
Liver Control	117.2 ± 5.12	4.4
Foodstuff Control	129.8 ± 13.97	10.8
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	136.2 ± 16.92	12.4
Batch B (9.55 mg/kg AMT; 3.21 mg/kg NOR)	115.2 ± 0.65	0.57
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	125.8 ± 16.21	12.9
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	118.1 ± 4.49	3.8

Table 2.9: The mean, standard deviation (±SD), and relative standard deviation for the mean duration of the larval period for *Sarcophaga bullata* reared on six different types of rearing media.

Based on the results presented in Table 2.9, the order, in terms of decreasing mean duration of the larval period was as follows:

Batch A > Foodstuff Control > Batch C > Batch D ≈ Liver Control > Batch B

One-way ANOVA analysis of the data presented in Table 2.9 indicated that the differences in the duration of the larval period between rearing medias were not significant ($F_{5,12} = 1.56$; $P = 0.24$; Table 2.10).

Duration of the Individual Larval Stages

However, the larval period can also be divided into its individual stages or instars, and then the duration of the each of these stages can be compared across the types of rearing medias. For the purposes of the present experiment, the larval stage was divided into the following four stages:

1. First instar
2. Second instar
3. Third instar
4. Prepupal stage

The mean duration of the first instar, for the larvae reared on each of the six types of rearing media is presented in Table 2.11.

Based on the results presented in Table 2.11, the order, in terms of decreasing mean duration of the first larval instar was as follows:

Batch C ≈ Foodstuff Control = Batch A ≈ Batch D > Batch B = Liver Control

However, a one-way ANOVA analysis of the differences in the mean duration of the first instar across the six different types of rearing media indicated that the differences between the types of rearing medias were not statistically significant ($F_{5,12} = 1.23$; $P = 0.35$; Table 2.12).

The mean duration of the second instar, for the larvae reared on each of the six types of rearing media is presented in Table 2.13).

Based on the results presented in Table 2.13, the order, in terms of decreasing mean duration of the second instar was as follows:

Batch A > Liver Control ≈ Batch B > Batch D ≈ Batch C > Foodstuff Control

However, a one-way ANOVA analysis of the difference in the mean duration of the second instar across the different rearing medias indicates that the mean difference in the duration of the second instar was not statistically significant for the six groups of *Sarcophaga bullata* larvae reared on the different types of rearing media ($F_{5,12} = 2.29$; $P = 0.11$; Table 2.14).

The mean duration of the third instar (feeding stage plus postfeeding stage), for the larvae reared on each of the six types of rearing media is presented in Table 2.15. Based on the results presented in Table 2.15, the order, in terms of decreasing mean duration of the third instar (feeding stage plus postfeeding stage) was as follows:

Foodstuff Control > Batch D > Liver Control ≈ Batch A > Batch C > Batch B

Source of Variation	**DF**	**Sums of Squares**	**Mean Square**	**F**	**P**
Rearing Media	5	719.12	143.83	58.97	< 0.0001
Error	338	824.39	2.44	-	-

Table 2.10: One-way ANOVA results for mean duration of the larval period for *S. bullata* larvae reared on the six different types of rearing media at 26°C

Rearing Media	**Mean (±SD) Duration of the First Instar (hr.)**	**Relative Standard Deviation (%)**
Liver Control	20.7 ± 0.89	4.3
Foodstuff Control	21.8 ± 0.10	0.4
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	21.8 ± 0.06	0.3
Batch B (9.55 mg/kg AMT; 3.21 mg/kg NOR)	20.7 ± 1.68	8.1
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	21.9 ± 0.11	0.5
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	21.5 ± 0.85	4.0

Table 2.11: The mean, standard deviation and relative standard deviation for the duration of the first instar for *Sarcophaga bullata* larvae reared on six different types of rearing media at 26°C.

Source of Variation	**DF**	**Sums of Squares**	**Mean Square**	***F***	***P***
Rearing Media	5	4.475	0.895	1.232	0.353
Error	12	8.716	0.726	-	-

Table 2.12: One-way analysis of variation (ANOVA) for the duration of the first instar of *Sarcophaga bullata* larvae reared in six different types of rearing media at 26°C.

Rearing Media	**Mean (±SD) Duration of Second Instar (hr.)**	**Relative Standard Deviation (%)**
Liver Control	15.2 ± 2.47	16.2
Foodstuff Control	12.6 ± 2.69	21.4
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	18.2 ± 2.11	11.6
Batch B (9.55 mg/kg AMT; 3.21 mg/kg NOR)	15.0 ± 2.44	16.3
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	13.4 ± 1.66	12.4
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	13.9 ± 1.87	13.5

Table 2.13: The mean, standard deviation and relative standard deviation for the duration of the second instar for *Sarcophaga bullata* larvae reared on six different types of rearing media at 26°C.

Source of Variation	**DF**	**Sums of Squares**	**Mean Square**	***F***	***P***
Rearing Media	5	54.471	11.494	2.294	0.111
Error	12	60.125	5.011	-	-

Table 2.14: One-way analysis of variation (ANOVA) for the duration of the second instar of *Sarcophaga bullata* larvae reared in six different types of rearing media at 26°C.

However, one-way ANOVA analysis of the differences in the mean duration of the third instar (feeding stage plus postfeeding stage) across the six different types of rearing media were not statistically significant ($F_{5,12} = 0.618$; $P = 0.690$; Table 2.16).

The mean duration of the prepupal stage of the third instar, for the larvae reared on each of the six types of rearing media is presented in Table 2.17. Based on the results presented in Table 2.17, the order, in terms of decreasing mean duration of the prepupal stage of the third instar was as follows:

Batch A > Batch C > Foodstuff Control > Batch B > Liver Control ≈ Batch D

However, the differences in the mean duration of the prepupal stage of the third instar for *Sarcophaga bullata* larvae were not significant based on a one-way ANOVA analysis ($F_{5,12} = 1.023$; $P = 0.447$; Table 2.18).

Since the third instar can be divided up into three behaviourally and/or physically distinct stages, it is also prudent to compare the mean duration of the third instar, as a whole[6], across the six different types of rearing media (Table 2.19). Based on the results presented in Table 2.19, the order, in terms of decreasing mean duration of the prepupal stage of the third instar was as follows:

Batch A > Foodstuff Control > Batch C > Batch D > Liver Control ≈ Batch B

However, the differences in the mean duration of the third instar, as a whole, for *Sarcophaga bullata* larvae were also not significantly different based on one-way ANOVA analysis ($F_{5,12}1.023$; $P = 0.447$; Table 2.20).

[6]Feeding stage plus postfeeding stage plus prepupal stage

Rearing Media	**Mean (±SD) Duration of the Third Instar: Feeding Stage + Postfeeding Stage (hr.)**	**Relative Standard Deviation (%)**
Liver Control	58.4 ± 3.59	6.1
Foodstuff Control	63.2 ± 13.66	21.1
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	58.2 ± 7.74	13.3
Batch B (9.55 mg/kg AMT; 3.21 mg/kg NOR)	52.7 ± 3.85	7.3
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	55.1 ± 10.73	19.5
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	60.6 ± 5.10	8.4

Table 2.15: The mean, standard deviation and relative standard deviation for the duration of the third instar (feeding stage plus postfeeding stage) for *Sarcophaga bullata* larvae reared on six different types of rearing media.

Source of Variation	**DF**	**Sums of Squares**	**Mean Square**	***F***	***P***
Rearing Media	5	213.519	42.704	0.618	0.690
Error	12	830.380	69.239	-	-

Table 2.16: One-way analysis of variation (ANOVA) for the duration of the third instar (feeding stage plus postfeeding stage) of *Sarcophaga bullata* larvae reared in six different types of rearing media.

Rearing Media	**Mean (±SD) Duration of the Prepupal Stage of the Third Instar (hr.)**	**Relative Standard Deviation (%)**
Liver Control	22.8 ± 3.56	15.6
Foodstuff Control	32.2 ± 13.43	41.2
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	38.0 ± 12.63	33.2
Batch B (9.55 mg/kg AMT; 3.21 mg/kg NOR)	26.8 ± 2.78	10.4
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	35.5 ± 19.76	55.7
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	22.0 ± 5.62	25.6

Table 2.17: The mean, standard deviation and relative standard deviation for the prepupal stage of the third instar for *Sarcophaga bullata* larvae reared on six different types of rearing media at 26°C

Source of Variation	**DF**	**Sums of Squares**	**Mean Square**	***F***	***P***
Rearing Media	5	667.012	133.402	1.023	0.447
Error	12	1564.541	130.378	-	-

Table 2.18: One-way analysis of variation (ANOVA) for the duration of the prepupal stage of the third instar of *Sarcophaga bullata* larvae reared in six different types of rearing media at 26°C

Rearing Media	**Total Mean (±SD) Duration of the Third Instar (hr.)**	**Relative Standard Deviation (%)**
Liver Control	81.2 ± 2.15	2.6
Foodstuff Control	95.5 ± 15.20	15.9
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	96.2 ± 14.79	15.4
Batch B (22.0 mg/kg AMT; 3.21 mg/kg NOR)	79.5 ± 3.48	4.4
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	90.5 ± 17.61	19.4
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	82.7 ± 3.68	4.5

Table 2.19: The mean, standard deviation and relative standard deviation for total duration of the third instar (feeding stage + postfeeding stage + prepupal stage) of *Sarcophaga bullata* larvae reared on six different types of rearing media.

Source of Variation	**DF**	**Sums of Squares**	**Mean Square**	***F***	***P***
Rearing Media	5	824.441	164.822	1.252	0.345
Error	12	1579.670	131.639	-	-

Table 2.20: One-way analysis of variation (ANOVA) for the duration of third instar (feeding stage + postfeeding stage + prepupal stage) of *Sarcophaga bullata* larvae reared in six different types of rearing media at 26°C.

Duration of the Pupal Stage

The duration of the pupal stage of the insects reared on each of the six types of rearing media is presented in Table 2.21. Based on the results presented in Table 2.21, the order, in terms of decreasing mean duration of the pupal stage was as follows:

Liver Control > Batch B > Batch A > Foodstuff Control > Batch C > Batch D

Rearing Media	**Duration of the Pupal Stage (hr.)**	**Relative Standard Deviation (%)**
Liver Control	321.0 ± 3.66	1.1
Foodstuff Control	279.9 ± 6.39	2.3
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	286.4 ± 9.82	3.4
Batch B (9.55 mg/kg AMT; 3.21 mg/kg NOR)	289.3 ± 24.60	8.5
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	271.7 ± 6.94	2.6
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	266.3 ± 15.49	5.8

Table 2.21: The mean, standard deviation and relative standard deviation for the duration of the pupal stage of *Sarcophaga bullata* larvae reared on six different types of rearing media at 26°C

Based on a one-way ANOVA analysis, the differences in the mean duration of the pupal stage, for *Sarcophaga bullata* across the six different types of rearing media larvae, was significantly different ($F_{5,12} = 6.426$; $P = 0.004$; Table 2.23).

Source of Variation	**DF**	**Sums of Squares**	**Mean Square**	***F***	***P***
Rearing Media	5	5591.369	1118.27	6.426	0.004
Error	12	2088.425	174.04	-	-

Table 2.22: One-way analysis of variation (ANOVA) for the duration of the pupal stage of *Sarcophaga bullata* larvae reared in six different types of rearing media at 26°C.

Further analysis by Tukey's HSD test revealed that the duration of the pupal period for the insects reared on the liver control was significantly different from the duration of the pupal period for the insects reared on Batches C, D, and the foodstuff control. However, the duration of the pupal stage for the insects reared on the liver control did not significantly differ from the duration of the pupal stage for the insects reared on Batches A and B. Furthermore, the duration of the pupal period for the

insects reared on Batches A and B significantly differed from the duration of the pupal period for the insects reared on Batches C, D and the foodstuff control. In comparison, the duration of the pupal stage for the insects reared on Batches C, D and the foodstuff control did not significantly differ from each other.

2.3.5 Discussion of the Effects of Amitriptyline and Nortriptyline on the Duration of the Developmental Stages

Based on the results presented above, amitriptyline and nortriptyline do not appear to have an effect on the duration of the larval period, or any effect on the duration of any of the individual larval stages. These results were the same as those observed by Goff *et al.* (1993). In the study conducted by Goff *et al.* (1993), no significant differences in the duration of the larval period were observed for larvae of the species *P. ruficornis*.

In the study conducted by Goff *et al.* (1993), the duration of the pupal period was significantly longer for insects of the species *P. ruficornis* that were reared on tissues representing the administration of 1000 mg[7] and 600 mg[8] of amitriptyline to the experimental rabbits by ear vein infusion.

Similarly, the duration of the pupal stage was shown to be significantly different across the six types of rearing media. Unfortunately, unlike in the study conducted by Goff *et al.* (1993), a clear pattern between the concentrations of amitriptyline and nortriptyline in the rearing media, and alterations in the duration of the pupal stage across the six types of rearing media, was not observed.

Of the four drug-spiked foodstuff batches tested, only Batches A and D were classified as being homogeneous. With this in mind, a tentative conclusion regarding the effects of amitriptyline and nortriptyline on the duration of the pupal stage can be made. It appears that 168.41 mg/kg amitriptyline and 7.65 mg/kg nortriptyline increased the duration of the pupal stage, and therefore increased the total duration of the development period, compared to the insects reared on the foodstuff control, but not compared to the insects reared on the liver control. A similar result was observed in the study conducted by Goff *et al.* (1993). However, in their study, the duration of the pupal period of *P. ruficornis* reared on the rabbit tissue containing 154.0 mg/kg amitriptyline and 7.1 mg/kg nortriptyline significantly differed from the rabbit liver control as well, and not just the other drug-spiked food substrates, as was observed in the present investigation.

[7]The administration of 1000 mg of amitriptyline resulted in tissue concentrations of 49.0 mg/kg amitriptyline and 0.9 mg/kg nortriptyline, roughly equivalent to the concentrations present in Batch C

[8]The administration of 600 mg of amitriptyline resulted in tissue concentrations of 154.0 mg/kg amitriptyline and 7.1 mg/kg nortriptyline, roughly equivalent to the concentrations present in Batch A

The inhomogeneity of Batches B and C, with respect to amitriptyline, did not appear to increase the variability in the duration of the developmental stages between replicates of the same batch. However, three replicates are not enough to make a definitive conclusion in this regard.

Unfortunately, these results do not present any reliable conclusions regarding the effects of amitriptyline and nortriptyline on the duration of the pupal stage of *S. bullata*. The poor drug homogeneity associated with two batches of the artificial foodstuff, and the lower nutritional quality of the foodstuff as a whole, confounds such attempts.

2.4 Pupal Weight Comparison

2.4.1 Introduction

Pupal weight is a common measure used to evaluate the appropriateness of a given type of food source for rearing a given species of insect. An increase or decrease in the mean pupal size of the insects reared on the test substrate, compared to the mean pupal size of insects reared on the controls, provides insight into the relative nutritional quality of the test substance to the control substance. Pupal size, measured in terms of either weight or length, has been used by a number of researchers to evaluate the nutritional quality of a wide variety of food substrates for rearing a wide variety of insect species (e.g. Sherman and Tran, 1995; Okorie and Okeke, 1990; Daniels *et al.*, 1991 and Perotti and Lysyk, 2003).

In the present project, pupal weight was used as the comparative measure because pupal weight was the measure used by Goff *et al.* (1993) in their investigation of the effects of amitriptyline and nortriptyline on the development of *P. ruficornis*.

2.4.2 Results for the Pupal Weight Comparison

Pupation was first observed in the liver control, the foodstuff control and in all the drug-spiked artificial foodstuffs between 121 and 122 hours since larviposition. In the present research project, the first observation of pupae is not likely to be a good comparative measure as the sampling period prior to the time of observed onset of pupation was approximately 24 hours previous. This means that the error in the estimate of the time of first pupation is at least ± 12 hours. This is a significant error, and as such, any difference in first onset of pupation would be masked.

The mean pupal weights for each batch of artificial foodstuff and liver control are shown in Table 2.23. The mean pupal weights for the liver control and the blank foodstuff control were considerably heavier than the weights of the pupae that were reared on drug-spiked foodstuff. Furthermore, the mean weight for the pupae that developed from insects that were reared on liver were considerably heavier than the mean weight for the pupae that developed on artificial foodstuff, regardless of the presence of amitriptyline and nortriptyline. The box and whisker plot presented in Figure 2.21 clearly illustrates this trend.

All four of the tests for unequal variances, indicated that the variances in pupal weights were unequal at an alpha level of 0.05 ($P < 0.0001$), and therefore Welch's ANOVA was used rather than the results of the one-way ANOVA that assumed equal variances. Welch's ANOVA indicated that there was a significant difference between the pupal weights resulting from larvae reared on the six different types of rearing media ($F_{5,163.61} = 232.5112$; $P < 0.0001$).

Further analysis with Tukey's HSD pairwise comparison procedure revealed that the weight of the pupae from the liver control was significantly greater than the weight of pupae from both the foodstuff control and from the drug-spiked artificial foodstuff. In addition, Tukey's HSD test revealed that the mean pupal weight of the insects

Rearing Media	**Mean Pupal Weight per Replicate**			**Pooled Mean Pupal Weight**	
	Replicate ($n = 20$)	**Mean Pupal Weight ±SD (g)**	**Relative Standard Deviation (%)**	**Mean Pupal Weight ±SD (g)**	**Relative Standard Deviation (%)**
Liver Control	R1	0.171 ± 0.015	8.7	0.163 ± 0.015 (9.3 %)	9.3
	R2	0.164 ± 0.018	10.2		
	R3	0.155 ± 0.010	6.3		
Foodstuff Control	R1	0.151 ± 0.013	8.9	0.122 ± 0.028 (23.2 %)	23.2
	R2	0.089 ± 0.008	9.3		
	R3	0.127 ± 0.014	10.8		
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	R1	0.091 ± 0.014	15.1	0.098 ± 0.019 (19.4 %)	19.4
	R2	0.119 ± 0.013	10.9		
	R3	0.085 ± 0.009	10.5		
Batch B (22.0 mg/kg AMT; 3.21 mg/kg NOR)	R1	0.101 ± 0.010	9.7	0.087 ± 0.013 (15.3 %)	15.3
	R2	0.074 ± 0.006	8.8		
	R3	0.086 ± 0.005	5.8		
Batch C (55.7 mg/kg AMT; 1.7 mg/kg NOR)	R1	0.101 ± 0.010	9.9	0.079 ± 0.026 (33.0 %)	33.0
	R2	0.087 ± 0.020	22.8		
	R3	0.048 ± 0.004	7.6		
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	R1	0.099 ± 0.009	9.2	0.082 ± 0.018 (21.3 %)	21.3
	R2	0.085 ± 0.009	10.0		
	R3	0.062 ± 0.009	14.9		

Table 2.23: Mean (±SD) *Sarcophaga bullata* pupal weights (g) for the insect reared on six different types of rearing media at 26°C.

that were reared on the foodstuff control were significantly different from the mean pupal weight of the insects that were reared on Batches A, B, C and D. Furthermore, the mean pupal weight from the insects that were reared on the foodstuff containing 168.41 mg/kg amitriptyline and 7.65 mg/kg nortriptyline (Batch A) were significantly different from the mean pupal weights of the insects that were reared on Batches B, C, and D. Interestingly, Tukey's HSD test revealed that the mean pupal weights of the insects that were reared on Batches B, C and D were not significantly different from each other.

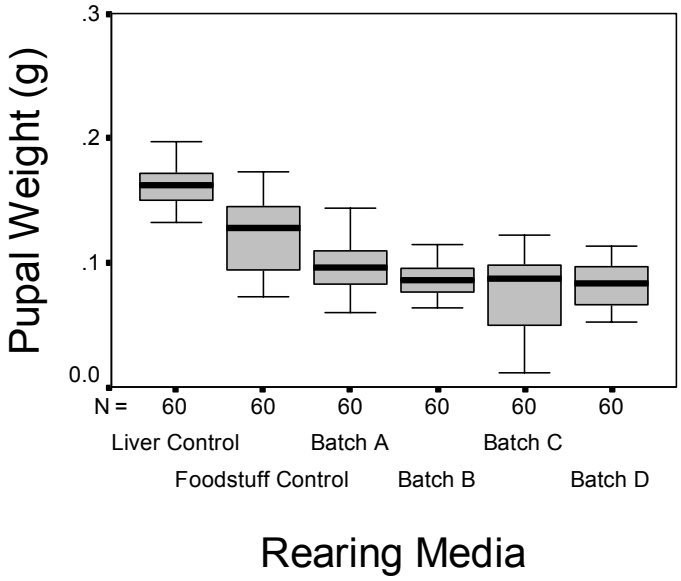


Figure 2.21: Box and whisker plot of the pupal weight data for the insects reared on each of the six types of rearing media at 26°C. The box and whisker plot was constructed using the pupal weight data from all three replicates from each type of rearing media. The plot shows the median pupal weight for each type of rearing media as a thick black bar. The interquartile range of the pupal weight data for each type of rearing media is shown as a box. The range of the pupal weight data for each type of rearing media is illustrated by the thin black bars (whiskers). Data points that are outside the range of other points observed for the data set are illustrated by circles.

2.4.3 Discussion of the Pupal Weight Comparison

The weight of the pupae obtained from the foodstuff control were significantly greater than the weight of the pupae from the drug spiked artificial foodstuff. Based on these results it appears that both the foodstuff and the drugs have an additive affect on pupal weight. In other words, larvae that are reared on liver produce heavier pupae than do insects that are reared on blank artificial foodstuff, and insects that are reared on blank foodstuff produce heavier pupae than do the pupae that are reared on the foodstuff containing amitriptyline and nortriptyline. However, despite the masking effect of the artificial foodstuff, it appears that larvae reared on 168.41 mg/kg amitriptyline and 7.65 mg/kg nortriptyline (Batch A) produce heavier pupae than the larvae that were reared on Batches B, C and D. Even more importantly, the result for Batch A can be viewed as a true reflection of the effects of amitriptyline and nortriptyline on the development of *S. bullata* larvae because Batch A was homogeneous for both amitriptyline and nortriptyline.

A similar result was observed by Goff *et al.* (1993) for *P. ruficornis*. In their study, heavier pupae were produced by the insect colonies that were reared on rabbit tissues containing 154.0 mg/kg amitriptyline and 7.1 mg/kg nortriptyline, concentrations that are roughly equivalent to those found in Batch A. However, in contrast to the present study, the greater pupal weights observed for *P. ruficornis* reared on the rabbit tissues containing 154.0 mg/kg amitriptyline and 7.1 mg/kg nortriptyline were also greater than the pupal weights observed for *P. ruficornis* reared on the drug-free rabbit tissue (Goff *et al.*, 1993).

Another clearly visible trend is the increased variation in pupal weight for the pupae that were reared on artificial foodstuff compared to the variation pupal weight observed for the pupae that were reared on liver (Table 2.23). The relative standard deviation for pupal weight, for Batches A, B, C, D, and the Foodstuff Control were considerably greater than the relative standard deviation observed for the pupae that were reared on liver. However, the inhomogeneity of Batches B and C, with respect to amitriptyline, did not appear to increase the variability in mean pupal weight between replicates of the same batch.

This common trend speaks to the suitability of the artificial foodstuff as a rearing media. The higher the coefficient of variation, the less useful the estimate. Any differences in development caused by the presence of amitriptyline and nortriptyline may be masked due to the high variation in the results. The high variation in the pupal weights for the insects reared on the artificial foodstuff indicates that the artificial foodstuff is of lower nutritional quality than the liver. Therefore, the lower nutritional quality of the artificial foodstuff may be contributing, or masking, the effects of amitriptyline and nortriptyline on the development of *S. bullata*.

2.5 Conclusion

Some similarities between the results from the present study and the study con-

ducted by Goff *et al.* (1993) were observed. However, based on the results for the present study, it appears that nutritional improvements to the artificial foodstuff are required before further entomotoxicological experiments, using it as the experimental model, can be conducted. Furthermore, the poor nutritional quality of the food source and the inhomogeneity of some of the drug-spiked batches of artificial foodstuff made the reliable detection of dose-dependent developmental changes extremely difficult. Therefore, the homogeneity of the artificial foodstuff in terms of the added drugs, must also be improved before further experiments can be conducted.

Chapter 3

Conclusion

3.1 Evaluation of the Artificial Foodstuff for Use in Entomotoxicological Research

An ideal high quality larval diet should be easy to prepare from readily available, low cost materials (Sherman and Tran, 1995). In addition, when rearing necrophagous flies, the food source should be relatively free from offensive odours. Arguably the most important characteristic of a food source, however, is the production of healthy insects (Sherman and Tran, 1995).

The foodstuff used in the present experiment was relatively easy to prepare, and was prepared from low-cost, readily obtained materials. In addition, it proved to be less malodorous than liver. However, certain aspects of the preparation and storage of the foodstuff were problematic and may limit the successful use of the artificial foodstuff as a rearing media for entomotoxicological studies. For instance, in the present experiment, the foodstuff was stored in the freezer and thawed before use. However, the foodstuff exhibited significant water loss during thawing. In the case of amitriptyline and nortriptyline, analysis of the water lost from the foodstuff by GC-NPD indicated that a negligible amount of amitriptyline and nortriptyline were present. Given the fact that amitriptyline and nortriptyline are both drugs with a large volume of distribution, this result was not unexpected. However, if drugs with small volumes of distribution (i.e. water soluble drugs) are investigated using this artificial foodstuff, there is the potential to lose a considerable amount of the drug during thawing if the foodstuff is frozen prior to use.

Furthermore, water loss from the foodstuff after quantitation but before introduction to the larvae indicates that the larvae likely received a higher concentration of both amitriptyline and nortriptyline than originally intended. This will be problematic when trying to attribute a given developmental response to a specific concentration of the drug. A simple solution to this problem would be to vacuum pack the foodstuff and store it at 4°C, rather than at -10°C.

However, the most serious problem encountered with the artificial foodstuff used in the present thesis was the fact that it altered the development of *S. bullata* compared to the beef liver control, and as a result ascribing specific changes in the duration of some of the developmental stages to the presence of amitriptyline and nortriptyline was not possible.

The foodstuff used in the present thesis is very similar the liver-agar rearing media evaluated by Sherman and Tran (1995) for rearing *Lucilia sericata* (Meigen) (Diptera: Calliphoridae). In addition, when the liver-agar foodstuff was prepared in a 1:1 ratio of liver weight (kg) to dissolved agar weight (kg), the development of *L. sericata* reared on the liver-agar foodstuff was observed not to differ from the development of the larvae reared on the liver control (Sherman and Tran, 1995). Furthermore, Sherman and Tran (1995) used a 3% agar solution by weight.

In comparison the artificial foodstuff used in the present thesis contained significantly less liver, and more agar. The artificial foodstuff prepared for the present thesis contained only a 0.5:1 ratio of liver weight (kg) to dissolved agar weight (kg) was used. Furthermore, a 4.5% agar solution was used compared to the 3% solution used by Sherman and Tran (1995).

With this in mind, it is apparent that the artificial foodstuff used in the present thesis to rear *S. bullata* larvae was not of an equivalent nutritional value to the liver control. The artificial foodstuff also contained powdered whole egg but it is unlikely that the protein and nutrient content present in the powdered whole egg was sufficient to make up for the nutritional losses resulting from a reduced liver content. Therefore, for future research, the artificial foodstuff should be modified to more accurately match the nutritional level of the specific type of animal tissue being used as a the control.

Based on the results presented in this thesis, the use of a non-live animal model for use in insect development experiments is feasible, but requires significantly more preliminary research to use effectively and reliably, compared to the use of a live animal model. For instance, density controlled experiments should be conducted to determine if the blank artificial foodstuff alters the development of the insect under investigation compared to the animal tissues used as the control. If a difference is noted, the composition of the rearing media should be altered so that the development of the insects reared on the artificial foodstuff more closely matches the development of the insects reared on the control tissues. However, changing the composition of the foodstuff to any great extent may require revalidation of the analytical method used to quantitate the drug levels present in the foodstuff. The extent of revalidation required would depend on the extent of the alterations made to the artificial foodstuff. In general, significant alteration of the proportion of each component present in the matrix would require revalidation of the following parameters: recovery, accuracy, limit of quantitation, limit of detection and homogeneity.

3.2 Recommendations

1. The degree of shrinkage, caused by storage in 75% ethanol, in the length of *S. bullata* larvae should be investigated. In addition, the investigation of larval shrinkage should not be limited to one larval stage; the degree of shrinkage should be assessed for all larval stages of development.

2. Visible crop length was observed to decrease as a function of time since preservation of the larvae in 75% ethanol. Since in many insect species, the ratio of crop length to body length is an excellent indicator of larval age, future research should perhaps include the dissection of the crops in order to determine crop length.

3. However, given the number of insects usually required for developmental research, the time required to dissect the crops would likely be prohibitive. With this in mind, experiments could be conducted to mathematically describe the degree of visible crop shrinkage as a function of time since preservation. This would then enable the reliable use of the preserved, visible crop length, as an indicator of larval age.

4. Insect mortality was not sufficiently investigated in the present thesis, and therefore, mortality experiments need to be conducted in future research to fully characterize the growth and development of an insect species reared on the artificial foodstuff.

5. In the experiment conducted by Goff *et al.* (1993), no additional food was supplied to the insect colonies, other than the first initial liver sample. In contrast, in the present experiment, fresh food was added on a continual basis. Therefore, the effects of food shortage and food desiccation should be investigated in future experiments.

6. Before use of the artificial foodstuff with a new insect species, density controlled experiments should be conducted using drug-free artificial foodstuff to determine the impact of the foodstuff itself on the development of the insects. If the artificial foodstuff was shown to impact the development of the insects, its composition should be modified to more closely match that of the tissue used as the control.

7. If the composition of the artificial foodstuff (e.g. ratio of liver to agar) has to be altered to suit the specific nutritional requirements of a given fly species, the extent of analytical method revalidation required will have to be assessed, and subsequently conducted.

8. Given the propensity of the foodstuff to lose water, the foodstuff should not be frozen prior to use. Instead, it should be vacuum-sealed and stored at 4°C.

Appendix A

Chemical Structures of Analytes

The following diagrams were created using ISIS™ Draw 2.4 (MDL Information Systems Inc., San Leandro, California).

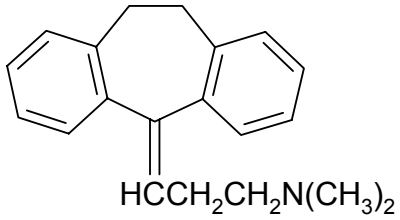


Figure A.1: Chemical structure of Amitriptyline.

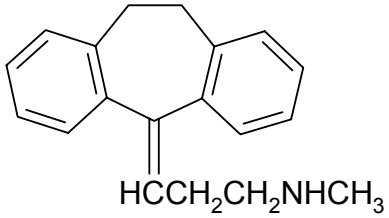


Figure A.2: Chemical structure of Nortriptyline.

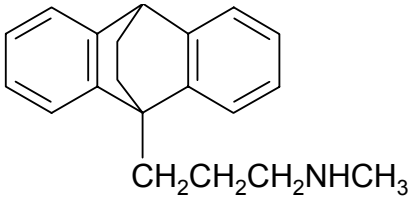


Figure A.3: Chemical structure of Maprotyline.

Appendix B

Ethics Approval

This section presents the ethics approval obtained for the previous research study "The effects of commonly abused drugs in Canada on time of death determinations in the later postmortem interval, using insect development."

The data obtained from this previous research study provided the background information on tricyclic antidepressant use in British Columbia, which was necessary for the present thesis.

Simon Fraser University

Office of Research Ethics

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Duke

Record Number

4 - 195

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Status	Graduate Student
Title	The effects of commonly abused drugs in Canada on time of death determinations in the later postmortem interval, using insect development.
Co Investigator	none
Supervisor	Gail Anderson
Department	Criminology
Application Date	4/11/2002
Date	4/13/2002
Date Created	4/13/2002
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Current Review	
Date Modified	4/13/2002
Start Date	5/1/2002
End Date	12/30/2003
Medical	
Funded	Canadian Police Research Centre
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Approval	Approved

Minimal Risk	X	**Entered intoGrant Track**	**Grant Track Pending**
Non Minimal Risk		X	
Approval Date	4/16/2002	**Grant Track No**	
Code	33337		

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