Effect of Diazepam on the development of *Lucilia cuprina* (Diptera: Calliphoridae)

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Abstract: *Lucilia cuprina* species were collected from the decaying meat in the Aurangabad region. The life cycle of *Lucilia cuprina* species includes egg, three instars, pre-pupa, pupa and adult stages. Changes in the life cycle of *Lucilia cuprina* species was studied after exposure to diazepam. Diazepam lowered the rate of growth at higher concentrations. The prepupation period of *Lucilia cuprina* was delayed in larvae fed on diazepam containing diet. The adults of *Lucilia cuprina* emerged out after 8 days in control while at 8 ppm, 12 ppm and 16 ppm diazepam containing food, the adults emerged out after 8, 9 and 10 days respectively.

Key words: Calliphorid, *Lucilia cuprina*, diazepam, life cycle.

Introduction

Forensic Science can be defined as scientific studies or investigation of crime. Along with the development of science and technology the criminal also often uses different techniques for commission of various crimes within our society. So it has become a problem for the investigative agencies to check the potentiality of crimes. For such checking the need of forensic science becomes an important prerequisite on the part of the investigative agencies. Forensic science is a multidisciplinary subject used for investigation crime scenes and collecting evidence to be used in prosecution of offenders in a court of law. It fulfills the growing demand for expertise in investigatory, enforcement and monitoring work, including incident scene investigation, laboratory analysis of evidence, physical evidence collection and defense of testimony (Lincoln, 2010).

Forensic entomology is legal application of the science of entomology. Forensic entomology is primarily associated with death investigations however it may also be used to detect drugs and poisons, determine the location of an incident and the presence and time of the infliction of wounds. Forensic entomology is the broad field where arthropod science and the judicial system interact. Insects and other arthropods are found in almost every possible type of habitat. The ubiquitous nature of insects, especially with regards to the flies and beetles shall facilitate the search, recognition and collection of insect specimens for evidence (Byrd and Castner, 2001).

The blow flies (Calliphorids) are especially valuable for establishing postmortem interval (PMI) because of their profound association with a corpse soon after death. In addition to estimating the minimum postmortem interval at crime scenes, the larvae of these blow flies are able to reveal other important

information about crimes such as place of death, manner of death and the presence of drugs or toxins in a corpse (Carvalho et al., 2004; Catt's and Goff 1992; Goff 1991; Introna et al., 1998; Lord 1990). Blowflies are usually the first organisms to arrive at a corpse, sometimes within minutes of death, and they are also the species of greatest forensic importance (Goff 2000; Byrd and Castner, 2001; Arnaldos et al., 2005).

Lucilia cuprina is a species of blow fly characterized by a metallic outer appearance and reddish eyes, usually they have a shiny green or greenish/blue abdomen with bronze/coppery reflections. Because of this, *Lucilia* species are recognized as the bronze bottle flies (Drees and Jackman, 1998). Their body shape is round to oval and length varies from 4.5–10 millimeters. They have two pairs of wings, the first pair being membranous wings and second pair being reduced wings known as halteres which are used for flight stabilization (Durden, 1999).

A sedative is a substance that induces sedation by reducing irritability (Johns Hopkins Colon) or excitement (Dorland's). At higher doses it may result in slurred speech, poor judgment, staggering gait and slow, uncertain reflexes. At high doses many of these drugs can cause unconsciousness and even death. All sedatives can cause physiological and psychological dependence when taken regularly over a long period of time, even at therapeutic doses (Yi et al., 2007; Ebert et al., 2006 and Sarrecchia et al., 1998). Dependent users may show symptoms ranging from restlessness and insomnia to convulsions and death. When users become psychologically dependent, they feel as if they need the drug to function.

Diazepam is one of the most frequently prescribed drugs of the benzodiazepine group, used in treatment of anxiety and anxiety related muscle relaxant, anti-epileptic and pro-operative sedative. Regular long-term use of this drug can lead to the psychological and physical dependence. Diazepam and its metabolites are commonly detected in post-mortem sample and often in conjugation with other illicit drug.

Forensic entomology-toxicology includes the study of effects of toxins and drugs on development rate of carrion-feeding insects, but comparatively little research has carried out in the use of larvae in India. The presence of the sedative drugs in the dead tissue can also affect on the longevity of the life cycle stages of blow flies and hence it is essential to study the effect of the sedative drug diazepam on the periods of the developmental stages of *Lucilia cuprina*.

Material and Methods:

The *Lucilia cuprina* (Calliphoridae) flies were used as the biomaterials. The flies does not need the flesh of specific animal and hence those which occurs on the dead human body, also occurs on the flesh of any animal and hence for the study goat or other available flesh in the market was used. After one day of putrification, the liver and other meat was placed in open air for collection of flies. After some time the flies gathered on the rotten liver. The flies of calliphoridae family were collected by means of insect collecting net after identification they were released in insect rearing cages.

Treatment of Diazepam:

The flesh was finely chopped in the mixer and was mixed with the diazepam so as to make the concentration as 4 ppm, 8 ppm, 12 ppm and 16 ppm. The first instar maggots were released on the 50 gms each of the diazepam mixed flesh in separate culture chambers, one with only flesh was maintained as control. Fresh chopped meat was provided twice a day as food. Honey soaked in cotton was also provided as the source of sugar and glucose. Wet cloth piece was maintained on one side of the cage to maintain the humidity. The feed was changed on each day and the mortality was recorded.

The developing stages were collected on each day, were narcotized in menthol water and were stored in vials containing AGA solution (alcohol, glycerol and acetic acid). Narcotization inactivates the maggots at relaxed condition, and thus after preservation there is no contraction of the maggots. The vial was labeled as the stage collected, date and time. The stages were photographed and weighed on the electronic balance. Measurements of these stages were made by means of the microscope whose least count is 0.001. At the same time the temperature and humidity were recorded. Measurements of five maggots were done at each time and their average with the standard deviation. Difference among the mean values of control and treated were analyzed by Student's t-test. Difference were considered statistically significant when, $p < 0.05$. The data obtained is tabulated in the tables for different groups.

Results and Discussion

The flies belonging to the family calliphoridae of the order diptera found on the decaying flesh in Aurangabad region were *Lucilia cuprina*. *Lucilia cuprina* is considered one of the most important species of blow flies in forensic science. *Lucilia cuprina* can fly up to ten miles searching for food and can be found on anything ranging from carrion to decaying fruit. *Lucilia cuprina* is often used as a very helpful tool to aid medical and forensic professionals because it first occupies a corpse upon its death. Once it lands on a corpse it lays its eggs and continues its next generation. The eggs are followed by its larva, pupa and finally the adult. Forensic professionals may form a postmortem interval (PMI) by the life stage found on the corpse.

Medical doctors use maggots of *L. cuprina* for debridement therapy of patients who suffer from wounds that are healing slowly (Marsi and Nazni, 2005). The maggots cleanse the wound by eating the dead and infectious skin and preventing further infection and gangrene. Medico-legal entomology is the science of using insect life cycle data combined with other evidence of domestic crimes, such as homicide, suicide, movement of bodies, drugging, and torture (Starkeby, 2004). *Lucilia cuprina* (Wiedemann) and *Hemipyrellia ligurriens* (Wiedemann) are blow flies of forensic importance and their immature stages are found in human corpses, as previously reported in Thailand (Sukontason et al., 2007).

For the treatment of diazepam eggs of *Lucilia cuprina* were collected on first day. Then thirty eggs were placed separately on 0.0 ppm (Control), 4 ppm, 8 ppm, 12 ppm, 16 ppm diazepam containing chopped flesh. The observations were made each day with respect to the dose of concentrations and are given in the table 1.

The results showed that diazepam treated food cause the effect on growth of the larvae. As the quantity of diazepam increases in the food the larval development slows down and the pupal development was also delayed. The flies emerged first from control then from 4 ppm, while in higher concentrations, the pupation was delayed as per the dose of diazepam as given in table 1. The temperature variations and the humidity variations in the room conditions at the time of experiment are also mentioned in the table.

Table 1. Effect of diazepam on the Morphometric parameters of life cycle stages of *Lucilia cuprina.*

PMI Days	Stages	Conc of Diazepam	Length (mm)	Width (mm)	Weight (mg)	Temperature ^{0}C			Humidity %		
						Max	Min.	Recorded	Max	Min.	Recorded
1	Ist Instar	Control	4.0±0.039	1.0±0.02	04±0.21	34.7	30	31.9	37	14	28
	IstInstar	4 ppm	3.9^{NS}±0.037	1.0 ^{NS} ±0.01	04 ^{NS} ±0.23						
	IstInstar	8 ppm	3.8 ^{NS} ±0.038	0.9 ^{NS} ±0.008	03 ^{NS} ±0.18						
	Ist Instar	12 ppm	3.6*±0.035	0.7*±0.008	02*±0.15						
	Ist Instar	16 ppm	3.4*±0.031	0.6*±0.008	02*±0.14						
2	IInd Instar	Control	7.6±0.28	1.7±0.014	15±0.71	36.6	30.1	33.6	35	13	25
	IInd Instar	4 ppm	7.5 ^{NS} ±0.27	1.6 ^{NS} ±0.012	14 ^{NS} ±0.52						
	IInd Instar	8 ppm	7.4 ^{NS} ±0.24	1.5 ^{NS} ±0.013	13*±0.41						
	IInd Instar	12 ppm	7.2*±0.22	1.2*±0.012	12*±0.38						
	IInd Instar	16 ppm	7.1*±0.21	1.1*±0.011	12*±0.36						
3	IIIrd Instar	Control	10±0.33	2.4±0.33	40±1.8	33	28	30.5	41	17	32
	IIIrd Instar	4 ppm	9.9 ^{NS} ±0.31	2.3 ^{NS} ±0.32	39 ^{NS} ±1.7						
	IIIrd Instar	8 ppm	9.7*±0.30	2.2 ^{NS} ±0.31	38*±1.6						
	IIIrd Instar	12 ppm	9.5*±0.26	2.0*±0.28	38*±1.7						
	IIIrd Instar	16 ppm	9.2*±0.24	1.8*±0.24	37*±1.5						
4	Pre-pupa	Control	9.0±0.19	2.8±0.37	42±1.9	33.3	27	30.8	40	16	31
	Pre-pupa	4 ppm	8.7*±0.22	2.7 ^{NS} ±0.39	41 ^{NS} ±1.8						
	IIIrd Instar	8 ppm	9.7*±0.31	2.6 ^{NS} ±0.37	43 ^{NS} ±2.1						
	IIIrd Instar	12 ppm	9.6*±0.32	2.5*±0.35	42 ^{NS} ±2.0						
	IIIrd Instar	16 ppm	9.3*±0.32	2.4*±0.32	40 ^{NS} ±1.8						
5	Pre-pupa	Control	8.7±0.34	2.6±0.032	38±1.8	35.3	28	31.6	39	15	29
	Pre-pupa	4 ppm	8.6 ^{NS} ±0.32	2.5 ^{NS} ±0.031	37 ^{NS} ±1.7						
	Pre-pupa	8 ppm	8.5 ^{NS} ±0.31	2.6 ^{NS} ±0.032	36*±1.6						
	IIIrd Instar	12 ppm	9.8*±0.39	2.6 ^{NS} ±0.031	43*±2.2						
	IIIrd Instar	16 ppm	9.5*±0.35	2.5 ^{NS} ±0.030	41*±2.0						
6	Pupa	Control	8.0±0.3	2.9±0.036	39±1.8	36	29	32.3	38	14	29
	Pre-pupa	4 ppm	8.5*±0.31	2.8 ^{NS} ±0.033	38 ^{NS} ±1.7						
	Pre-pupa	8 ppm	8.4*±0.32	2.7 ^{NS} ±0.035	37*±1.6						
	Pre-pupa	12 ppm	8.3*±0.31	2.7 ^{NS} ±0.034	36*±1.5						
	Pre-pupa	16 ppm	8.2 ^{NS} ±0.30	2.5*±0.032	36*±1.6						
7	Pupa	Control	7.5±0.23	3.0±0.35	36±1.8	36.5	29.3	32.6	36	14	27
	Pupa	4 ppm	7.1*±0.20	2.9 ^{NS} ±0.32	35 ^{NS} ±1.6						
	Pupa	8 ppm	7.0*±0.21	2.8 ^{NS} ±0.32	34*±1.5						
	Pupa	12 ppm	8.2*±0.34	2.8 ^{NS} ±0.31	38*±1.9						
	Pre-pupa	16 ppm	8.1*±0.30	2.6*±0.31	37 ^{NS} ±1.7						
8	Adult	Control	8.2±0.33	4.2±0.38	46±2.3	37.3	30	33.2	35	13	26
	Adult	4 ppm	8.1 ^{NS} ±0.31	4.0 ^{NS} ±0.37	44*±2.1						
	Pupa	8 ppm	7.6*±0.28	3.0*±0.34	33*±1.2						
	Pupa	12 ppm	7.4*±0.24	3.0*±0.32	37*±1.6						
	Pupa	16 ppm	7.2*±0.22	2.8*±0.30	36*±1.6						
9	Adult	8 ppm	7.6*±0.28	4.0 ^{NS} ±0.038	42*±2.1	36	28	30.7	42	18	30
	Pupa	12 ppm	7.4* ±0.25	3.0*±0.034	36*±1.7						
	Pupa	16 ppm	7.2*±0.23	2.7*±0.030	35*±1.5						
10	Adult	12 ppm	7.2*±0.21	3.5*±0.032	41 ^{NS} ±1.8	37	28	30.9	41	17	30
	NoAdult	16 ppm	Dead pupa	-	-						

Where, *p<0.05 (Significant t test), NS- Not significant

The blowfly eggs of many genera has forensic importance and have been studied in many parts of the world (Kitching, 1976; Greenberg and Szyska, 1984; Erzinclioglu, 1989; Liu and Greenberg, 1989; Greenberg and Singh, 1995; Greenberg and Kunich, 2002). Watanabe et al., (2002) reported identifying species found in association with a corpse is one of the first steps a forensic entomologist performs in seeking to estimate the post-mortem interval (PMI). Liu and Greenberg (1989) have been developed keys and diagnostic descriptions for egg, larval and pupal stages for several flies of forensic importance.

The most important use for entomological data is the estimation of the postmortem interval (PMI) (Hall, 2001). The Postmortem Interval (PMI) provides an important information in homicide investigations and untimely deaths (Byrd and Castner, 2001). Developmental data for blow flies provide the most accurate information for estimating the PMI (Greenberg, 1991).

Important work of entomotoxicology is the investigation of the effects of toxins and drugs on arthropod development (Goff and Lord, 1994). Goff et al., (1991) observed that use of drugs prior to death can result in an inaccurate estimation of PMI based on insect development. Bourel et al., (1999) reported that morphine can cause an underestimation of the PMI in *Lucilia sericata* by 24 hours. Ethanol caused significant variations in maggot length for third instars feeding on treated meat compared to an untreated control in field conditions (Tabor et al., 2005). Monthei (2009) observed effects of different concentrations of ethanol on maggot development of the black blowfly, *Phormia regina* in which he reported that in ethanol treated tissue, development of maggot take more time than its control.

Studies show that use of different drugs and toxins can affect maggot development rates, resulting in inaccurate estimations of postmortem intervals (PMI) based on insect development (Goff et al., 1992, Bourel et al., 1999). Goff et al., (1993) showed the effects of amitriptyline on larvae of *P. ruficornis* (Fabricius) reared on tissues of rabbits that were administered by different doses of the drug. Arnaldos et al., (2005) also observed in laboratory experiments investigating the effects of heroin on time taken to complete individual larval stages in *Sarcophaga tibialis* was considerably longer, in contrast to those larvae which were not fed heroin.

Entomology-toxicology is a recognized method of estimation of postmortem interval, but relatively little research has carried out in the use of larvae in forensic entomology-toxicology in India. The most commonly used sedative drug, diazepam were used in the present study and their impact on different stages of life cycle of the *Lucilia cuprina* and morphometric parameter helps in crime investigations. This drug can affect the duration of the life cycle stages and hence in such condition, it is essential to have the standard data related to such fact. Hence effect of dizepam on the duration of the life cycle stages and the impact on their morphometric measurement was done.

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