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ARAŞTIRMA MAKALESİ / **RESEARCH ARTICLE**

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PRELIMINARY OBSERVATIONS OF THE EFFECTS OF LORAZEPAM ON THE DEVELOPMENT OF *CALLIPHORA VICINA* AND *CALLIPHORA LOEWI* (DIPTERA: CALLIPHORIDAE) AND PMI ESTIMATION

ABSTRACT

Larvae of *Calliphora vicina* and *Calliphora loewi* were reared on minced cattle lung tissues treated with different Lorazepam concentrations based on levels of public common usage for therapeutical, narcotic and suicidal purposes to evaluate the reliability of insect larvae for toxicological investigations. The species are studied to determine time deviations caused by toxicological influences owing to their features like speed on heading the corpse, value on PMI estimation, briefness at developmental processes and easiness about rearing at laboratory conditions. Body length, weight, death ratio of larvae and pupa were compared within control groups. The results demonstrated that larvae feeding on drug containing tissues in slight dosages developed more rapidly and adult emergence was greater, but also higher concentrations created completely adverse results. The time required for pupation was significantly greater for colonies fed on tissues from lorazepam-dosed diets than for the control group. Results emphasize the importance of determining the contamination rates of lorazepam are essential to prevent deviation on PMI estimations.

Keywords: Forensic Entomology, Lorazepam, Calliphoridae, Larval growth rate, Postmortem interval, Benzodiazepine, Entomotoxicology.

CALLIPHORA VICINA VE *CALLIPHORA LOEWI* (DIPTERA: CALLIPHORIDAE) LARVALARININ GELİŞİM SÜRELERİ ÜZERİNDE LORAZEPAM'IN ETKİLERİNİN GÖZLENMESİ VE PMI TAHMİNİ

ÖZ

Halk arasında tedavi amaçlı, narkoz etkisi veya intihar amaçlı olarak kullanılan Lorazepam farklı konsantrasyonlarda kıyılmış koyun karaciğerinde yetiştirilen *Calliphora vicina* ve *Calliphora loewi* türlerinin besin ortamlarına eklenerek larvalar üzerindeki etkileri toksik araştırma amaçlı olarak incelenmiştir.Bu türler cesede ilk erişen örnekler oldukları için PMI hesaplamasında ve ölüm hakkında detaylı bilgi verebilmesi açısından ve labaratuvar ortamında rahatlıkla kültürlerinin kurulabilmesi yönünden çokça çalışılan türlerdir. Pupa ve larvaların vücut uzunlukları, ağırlıkları ve ölüm oranları kontrol gruplarıyla karşılaştırılmıştır. Sonuçlar göstermektedir ki düşük dozlarda Lorazepam etkisi larvalarda gelişimi artırmakta ve ergin evreye geçişi hızlandırmakta iken yüksek dozlara geçildikçe larvalarda ve pupalarda bozulmalar ve ölüm oranları hızla artmaktadır. Sonuç olarak PMI hesaplamasında ki sapmaların önüne geçebilmek için toksik etkinin miktarının belirlenmesinin önemi vurgulanmaktadır.

Anahtar Kelimeler: Adli entomoloji, Lorezepam, Calliphoridae, Larval gelişme oranı, Ölüm zamanı hesaplaması, Benzodiazepin, Entomotoksikoloji.

1. INTRODUCTION

An exclusive usage of forensically important necrophagous insects is to estimate the exact time of death (Smith, 1986). The time between death and discovery of a cadaver is known as the postmortem interval (PMI), (Goff, 1993). Researches about insect development and factors that affect the developmental processes have great importance on the branch of forensic science that insects and other arthropod cognates are used as evidence by forensic entomology. The principle depends on determination of larval growth rate and analysis of age differences for legal investigations to estimate PMI (Hall, 2001). In addition, the reliability of entomological evidence used in estimation of a postmortem interval can depend on many factors (Donovan et al., 2006; Adams and Hall, 2003; Kintz et al., 1990). In addition to forensic entomology, instead of commonly used methods like temperature, tissue type and environmental influence changes, by the help of the branch that examine drugs and heavy metals toxicity as a factor on vital processes of insects: entomotoxicology (Baselt 2004; Goodbrod and Goff, 1990; Archer, 2004; Grassberger and Reiter, 2002; Nabity et al., 2006). The principle aims to obtain variable PMI estimation by examinations about evaluation of factors produced by chemical components or elemental impacts that affect estimations of time period after death, especially on constitution of vital tissue feeding species development (Goff and Lord, 2001; Higley and Haskell, 2001; Monthei, 2009). Previous studies about drug or toxicant concentrations in decomposing tissues have been determined by analyses of Diptera larvae (Beyer et al., 1980; Goff et al., 1989). It is generally based on analyses of larvae, to identify presence of drugs and toxins in tissues as a reliable source of evidence in advanced decomposition state, where internal samples such as organs and urine are no longer available (Campobasso and Gherardi, 2004; Nolte et al., 1992). However if larvae are no longer present due to the longtime of body exposure, it is still possible to use insect puparia (Pien et al., 2004). On the other hand, it has been clearly determined that presence of certain toxins, widely used (and abused) illegal narcotics such as cocaine and benzodiazepines showed lethal effect, even in little dosages.

Lorazepam, a well-known benzodiazepine drug initially marketed under the brand names Ativan and Temesta, is indicated for the management of anxiety disorders or for the short-term relief of the symptoms of anxiety or anxiety associated with depressive symptoms. At the same time, lorazepam ranges widely on medical fields by application within different concentrations of other sedatives (Ungvari et al., 1993; Cock and Schapira, 2002). As it mentioned in case "Death of Michael Jackson" (Wikipedia 2010), it's been reported that benzodiazepines show serious effects on the brain, breathing and circulation when unrestrained usage occurs with other chemicals, especially it creates fatal cases when combined with alcohol (Aranko et al., 1985). Lorazepam binds to the gamma-aminobutyric acid (GABA) receptor effecting chloride movement through ion channels for the management of anxiety disorders (Di Lazzaro et al., 2005), the short-term relief of symptoms of anxiety or anxiety associated with depression, also effective for a common use on insomnia and panic attacks. In addition, like other relatives, it may lead to physical and psychological dependence risk that increases by long term use and dosage amounts (Perkins et al., 2009; Carvalho et al., 2001). Moreover, poisoning cases related to lorazepam could be seen on literature depending on wide usage and intensive accumulation metabolism caused by physical addiction and overdoses (Furnari et al., 2008; Bouchard et al., 2004). By the way, chemical poisonings associated with this class of drug are among the most and lorazepam can be easily determined from metabolic tissues such as urine, hair, oral fluid (Kintz et al., 2004; Cirimele et al., 1996).

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The accumulation amounts of lorazepam on human tissues are clearly studied in literature as dosage/accumulation rates and overdose limits on human metabolism by pharmacokinetics to determine where to search the drug accumulation on investigations (Greenblatt et al., 1978; Dussy et al., 2005; Allen et al., 1980). Otherwise on the subsequent stages of composition, these types of tissues are unhandy to obtain data for an investigation case (Knight, 1991). It is the main reason why we utilize attributes based on lorazepam in the scope of forensic entomology.

The main intention of our study was to gather information about Lorazepam influence on developmental processes by comparison at different concentrations to check the reliability of *Calliphora vicina* and *Calliphora loewi* on postmortem interval estimations. The effects of Lorazepam on the species are investigated to define morphological defections and the pattern of larvae development is created when exposed to different levels of the drug.

2. MATERIALS and METHODS

Adult samples were collected from pig (*Sus scrofa* L.) carrion, raised in the forests of Yarımca village (39º 53' N, 30º 37' E) (1171 m) in Central Anatolia in Turkey.

Adult *C. vicina* and *C. loewi* were maintained in rearing cages (50 cm X 50 cm X 50 cm) in an environmental chamber at 18-25 ºC and 60-70% humidity with cyclical artificial lighting 14 h daylight and 10 h darkness. Each cage was supplied with water-sugar mixture (5%) and fresh beef liver. When eggs were required, a dish containing approximately 30 g of sliced beef spleen was placed in a cage for oviposition and removed after 3 h. The egg mass was placed in a different dish and examined on each hour until hatching was initiated and process continued by the removal of individual larvae and separation into treatment groups.

It's reported that larval development of blow fly larvae in beef lung was faster and the death ratio was very low (Clark et al., 2005). Hence, in the present study we have used beef lungs as the main food component in both treatment and control groups. Diets were prepared as follows; 90 g beef lung, added 10 ml distilled water and range concentrations (0 µg/g, 0,25 µg/g, 0,50 µg/g, 0,75 µg/g, 1 µg/g, 1,5 µg/g and 2 µg/g) of lorazepam, homogenized and placed in sterile pots (diameter 10 cm, height 3 cm). Thirty newly hatched first-stage larvae were transferred to each pot within 30 min after hatching. Pots were located in larger glass aquaria containing a 5 cm deep layer of sawdust on the bottom, and covered with cotton mesh, held in place with an elastic band. The aquaria were kept in a growth cabin at 21-25 ºC and 60-70% humidity with cyclical artificial lighting 14 h daylight and 10 h darkness. In total there were 35 pots with 5 replicates of each concentration of lorazepam. Every 6h, alive and dead larvae were counted and death percentage determined. Body length and weight of all larvae were measured and each one was boiled and conserved in a 75:25 mixture of ethanol and acetic acid for morphological comparison. Adult flies were killed and sexed. Their size were recorded by measuring the inch-du cross-vein length of wings (Smith and Wall, 1997) using an eyepiece micrometer to compare development. Measurements were also taken from wild caught specimens to compare with the laboratory flies.

All data were statistically analyzed using Excel, GraphPad Prism and SPSS statistics software. Mean results were compared using one-way analysis of variance (ANOVA) and Duncan's multiple range tests.

3. RESULTS

2 µg/g concentrations of Lorazepam showed lethal effects on the larvae, all larvae died before the second instar stage. Nevertheless all *Calliphora loewi* larvae died in both control group and drug treated groups.

Table 1. Duration of mortal stage, larval mortaliy, puparial weights, durations and mortalities for colonies of *Calliphora vicina* reared on cattle lung tissues containing varying amounts of lorazepam at a constant temperature of 22 ºC.

Colony	Duration of Larval Stage (Hours)	Larval Mortality (%)	Pupal Weight (Mg)	Duration of Pupal Stage (Hours)	Total Mortality (%)	Total Development Time (Hours)
Control	174 a (SD = 9.7315)	0.0	11.72 a	342.8 a (SD = 12.3266)	3.6	516.8 a
0.25 µg/g	152 b (SD = 8.0625)	2.4	12.06 a	358.2 b (SD = 11.8324)	7.3	510.2 a
0.50 µg/g	156 b (SD = 10.8992)	3.1	12.15 a	362.4 b (SD = 10.5244)	12.4	518.4 a
0.75 µg/g	180 a (SD = 11.1523)	7.2	12.50 a	353.6 b (SD = 14.2245)	15.1	533.6 b
1.0 µg/g	198 c (SD = 14.3975)	11.6	12.55 a	354.2 b (SD = 12.3356)	19.7	552.2 c
1.5 µg/g	210 c (SD = 13.9989)	15.8	12.50 a	355.4 b (SD = 13.9775)	36.2	565.4 c

The concentration of lorazepam in the diet affected the length and weight: a step-wise change of larval development was observed with variable drug concentrations in the diet. In the first few hours of exposure, no difference of larval development was noticed, but from 12h on, it was observed that the presence of drug was significant for the fly development.

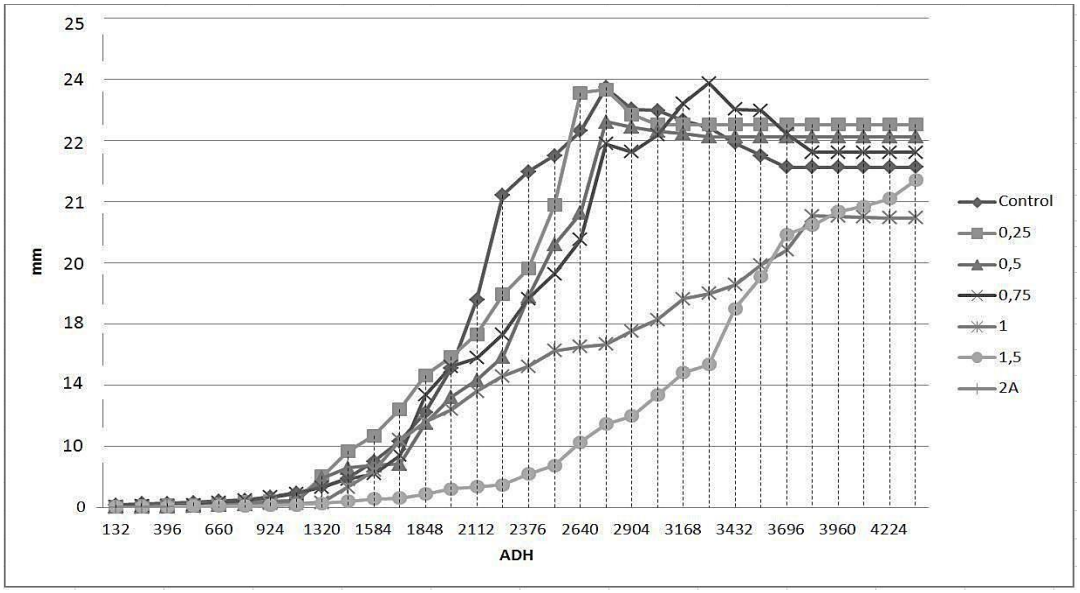

Figure 1. Growth rate of *Calliphora vicina* larvae fed on tissues containing lorazepam.

Pupariation was first observed in the 0.25 µg/g treated group at hour 144 (3168 ADH), followed by the 0.5 µg/g at hour 156 (3432 ADH), the control group at hour 174 (3828 ADH), 0.75 µg/g at hour 180 (3960 ADH), 1.0 µg/g at hour 198 hour (4356 ADH) and 1.5 µg/g treated group at hour 210 (4620 ADH).

The total duration of the larval stage was significantly different between colonies. Duration was the shortest for larvae from the 0.25 µg/g treated colony with a mean of 152 hour (144-160 h) and the longest duration from the 1.5 µg/g colony with a mean of 224 hour (210-238 h)(see Table 1). The larvae that developed on lungs treated with lorazepam weighed more than the control groups by forming a gradation of decreasing weight as the drug concentration increased: 0.25 µg/g to 1.5 µg/g, respectively.

Furthermore, larval and pupal death ratios in these pots were higher (7.3 %, 12.4 %, 15.1 %, 19.7% and 36.2% respectively) than control pots (3.6 %). Total durations for the immature stages (larvae and puparia) were greatest for the 1.5 µg/g colony and least for the 0.25 µg/g colony.

4. DISCUSSION

To evaluate whether lorazepam could alter the development of *C. vicina or C. loewi* and, therefore, bias the estimation of PMI, entomological methods based on the larval age determined from the larval weight, length or on the duration of insect development stage were used. The results showed significant differences in the mean larval weight and length growth curve of the larvae from treated groups as compared to the control group during the larval stage. If weight and length values achieved by the control group are considered as the ideal condition for pupation, the deviations on those values caused by lorazepam may induce deviations on PMI estimation in a time range from ± 6 up to 36h (-30h, +36h) (see Figure 1).

The presence of lorazepam appears to accelerate the growth rate for *C. vicina* during the larval stage in lesser concentrations, in contrast retarding on increasing portions. Moreover, *Calliphora loewi* adults generally prefer to avoid human activity and urban areas. Therefore under laboratory conditions, depending on the needs of natural habitats to continue vital processes, it's not been possible to rear larvae or adults even in control groups. Clearly, the drug or toxin contamination in tissue on which blow fly larvae feed does have an important effect on growth rate, and this needs to be taken into account more explicitly in the calibration of growth models when calculating post-mortem intervals.

5. CONCLUSIONS

In case of death investigations, all available materials must be considered for toxicological analysis, like suggested by *Levine et al*. "all reasonable steps must be undertaken to perform as comprehensive a drug screen must be undertaken" (Levine et al., 2000). Toxicological analyses are strongly disordered by using putrefactive tissues or fluids. The literature documented on the potential use of insects as alternative samples for detection of drugs and toxins generally have some limitations and include the bioaccumulation of drugs throughout the larval development and the potential correlation between drug concentrations in maggots and the tissues used as food source (Pounder, 1991). In skeletonized remains where no human soft tissues are present or decomposed to the point where drugs cannot be detected due to high levels of decomposition, insects can be more suitable specimen for analyses, with less decomposition interference (Kintz et al., 1990a; Nolte et al., 1992). During a comparative study, Kintz et al. obtained greater sensitivity using fly larvae, living material, instead of putrefied material. Thus drugs become hardly detectable over the time. Moreover, the stability of drugs in post-mortem tissues is strongly diminished (Schloegl et al., 2006).

In our study, the larvae of *C. vicina* were clearly reared on lung tissues spiked with lorazepam at different concentrations defined according to therapeutic, suicidal and common daily usage dosages of lorazepam. Based on consequences of the present study, between 12 and 42h, estimations of larval age based on total weight can be significantly in error, if presence of lorazepam in the tissues is not considered. In order to provide more accurate postmortem interval estimates, studies must be done in interpretation of arthropod development and succession patterns in cases where and how drugs or toxicants are a factor.

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