Alternative sources of toxicology tests

Careful analyses of the community of insects encountered on a decomposing body, combined with knowledge of insect biology, ecology, and local environmental conditions, can often provide valuable forensic insights. These can include the estimation of time since death, movement of the remains after death, indication of antemortem injuries, and the presence of drugs or toxins.

Over the past two decades, there has been an apparent increase in the incidence of drug-related deaths reported within the United States and other countries. Decedents in such cases are, in many instances, not discovered for a substantial period of time (days or weeks). The resulting state of advanced decomposition and environmental recycling typically encountered in these situations often dictates the employment of various entomological methodologies. The entomological techniques most frequently utilized are based on comprehensive analyses of the insects and other arthropods associated with the remains, their development, and patterns of succession (Goff and Flynn 1991, Goff and Odom 1987, Lord et al. 1986).

The accuracy of entomological estimates in deaths involving narcotic intoxication has been subject to debate in recent years, as few available studies have explored the effects of drugs contained in decomposing tissues on fly colonization and ovipositional behavior, or on the rates of development of carrion-frequenting insects feeding on such food sources (Goff 1993). Additionally, relatively few studies have examined the effects of other tissue contaminants, such as toxins or environmental pollutants on these behaviors or the developmental patterns of the insects colonizing such tissues.

In recent years, interest has also focused on the potential use of carrion-frequenting insects as alternative toxicological specimens in situations where traditional toxicological sources, such as blood, urine, or solid tissues, are unavailable or not suitable for analysis. The use of anthropophagic fly larvae (maggots) as alternate toxicological specimens is well documented in the entomological and forensic science literature (Miller et al. 1994). Detection of various toxins and controlled substances in insects found on decomposing human remains has contributed to the assessment of both cause and manner of death (Lord 1990, Goff and Lord 1994, Nolte et al. 1992). With the development of hair extraction technologies, attention has recently focused on the analysis of chitinized insect remnants that are frequently encountered with mummified and skeletonized remains (Miller et al. 1994). In such cases, the standard toxicological specimens are often absent.

Studies of the use of carrion-feeding arthropods as alternative toxicological specimens, and of the impact that tissue toxins and contaminants have on the development of immature insects feeding on these substances, currently comprise the major avenues of exploration in the emerging field of entomotoxicology.

The potential value of larval and adult carrion-feeding insects, and their chitinous remnants, as alternative sources of toxicological information has been clearly demonstrated. As with other emerging technologies, however, great care must be taken in the interpretation and use of such data, particularly within the forensic arena. Given recent advances in analytical procedures, it has become more practical to use even decomposed tissues for analysis (Tracqui et al. 2004). The situation may still be encountered

where for various reasons there are no tissues remaining and the arthropods remain the only available material for analyses. In these instances, a qualitative analysis will be of value, but any attempt at quantitation must be viewed with skepticism. Much more research is required before the full potential of this discipline can be recognized.

Forensic toxicologists qualitatively and quantitatively identify drugs and poisons which may be relevant to cause and manner of death. In most cases, toxicological specimens are collected at autopsy. Alternatively, if a body is badly decomposed, bone, hair, and insect larvae and pupae are collected and analyzed.

The use of insects and insect remnants as toxicological specimens is well documented. Insect tissue or remnants (pupal cases, frass, etc.) can be used to identify drugs and toxins present in decomposing tissues. Literature to date has cited the use of arthropods as an alternative toxicological source since 1980.

Beyer was one of the first to use maggots to qualitatively assess drug presence in a suspected suicide case. A body of a 22-year old female was found skeletonized except for the skin. Larvae were collected and homogenized with the proteins precipitated out of solution. Gas chromatography was used to identify a phenobarbital concentration of 100 µg/g in larval tissue. The larvae were identified as Cochliomyia macellaria (Beyer et al. 1980).

Levine et al. (2000) described a case in which an unidentified male was found by a river and was decomposed and skeletonized. An empty bottle of

secobarbital was found near the body. Calf muscle and maggots were sent for toxicological analysis. No substances or drugs were detected in the calf muscle, but secobarbital was identified in the maggots by electron ionization gas chromatography/mass spectrometry.

In a similar study, Wilson et al. (1993) reared Calliphora vicina on human skeletal muscle from suicidal overdose victims of co-proxamol (propoxyphene and acetaminophen) and amitriptyline. Third instars were transferred to drug-free muscle or allowed to feed on drug-laden muscle for two more days prior to harvesting. The drug concentrations in the muscle food source were 0. 48 µg/g amitriptyline, 0. 38 µg/g nortriptyline, 0. 99 µg/g propoxyhene, and 14. 3 µg/g acetaminophen. The mean ratios of drug concentrations in larvae to the food source were 0. 5, amitriptyline; 0. 5, nortriptyline; and 0. 06 for propoxyphene. In all stages no drugs or metabolites were detected in puparia, pupal cases, or adults (Wilson et al, 1993).

Malathion, an organophosphate insecticide, was identified in larvae found on a decedent thought to be a suicide victim. Malathion was detected at a concentration of 2, 050 µg/g of larvae in specimens collected from the decomposing remains. Malathion exhibits low toxicity in mammals, yet a high toxicity to adult insects. In this case, the maggots were developing normally despite concentrations of malathion that were toxic to rats and adult species of blowfly (Gunatilake and Goff 1989). This case illustrates the importance of studying the effects many drugs and toxins have on insect species since drugs and toxins may affect insect development, mortality rates, and PMI.

The effects of various drugs and toxins to carrion-feeding insects have been investigated, but this area of study is still expanding. Tracqui et al. (2004) examined 29 necropsies in which various organic compounds (including benzodiazepines, barbiturates, antidepressants, phenothiazine, opiates, cannabinoids, meprobamate, digoxin, and nefopam) were detected in arthropod larvae sampled from human corpses. Larvae were collected from multiple sites on the cadaver, weighed, washed, and dried. The larvae were mechanically homogenized and then extracted using solid or liquid phase extraction procedures. Sample extracts were then analyzed by gas or liquid-chromatography. The results indicated that the concentrations of the drugs in insect tissues tended to be lower than those of cadaveric samples, and that concentrations varied between anatomic sites (i. e. within anatomic sites when larvae were grouped according to their site of sampling). Tracqui et al. (2004) also found only weak correlations between the concentrations of drugs in biofluids at the time of death and those in the larvae sampled from the cadaver at a later time.

Goff has conducted a number of entomotoxicology experiments with various drugs (Goff et al. 1989, Goff et al. 1991, Goff et al. 1992, Goff et al. 1993, Goff et al. 1994). When Goff did his studies he administered the drug to a living animal. He did this so that known and reproducible concentrations of drugs and metabolites in animal tissue could be used to approximate amounts normally encountered in human fatal overdoses.

Hédouin et al. (1999) established concentrations of morphine in an animal model before rearing larvae on tissues. Morphine, a metabolite of heroin, was injected intravenously into rabbits. The kinetics of morphine elimination

from blood after a single intravenous injection of morphine and the concentrations of morphine in tissues following a continuous perfusion were established. Morphine concentrations were determined using radioimmunoassay techniques. The rabbits that received a single injection received 2 mg/kg of morphine hydrochloride. Three rabbits received 2 mg/kg of body weight of morphine hydrochloride per hour for a period of 3 h using a continuous perfusion through a plastic catheter in the ear. Results from the continuous perfusion showed that the concentrations of morphine differed according to the organ analyzed, but were reproducible for organs between animals. This study permitted known and reproducible concentrations of morphine in the rabbit to be used as a substrate for rearing of larvae in entomological studies.

Goff used rabbits in his entomotoxicological studies of cocaine and heroin on Boettcherisca peregrina (Goff et al. 1989, Goff et al. 1991). The rabbits in the heroin study were given 6, 12, 18, and 24 mg of heroin by cardiac puncture. Boettcherisca peregrina were allowed to feed and develop on liver tissue containing heroin. From hours 18 to 96, larvae feeding on liver tissue containing heroin developed more rapidly than those feeding on the liver from the control. Time required for pupation was also greater for larvae that fed on tissue from heroin dosed rabbits than for the control larvae. The rates of development were sufficient to alter PMI estimates based on larval development by up to 29 hours (Goff et al. 1991).

In a similar study, three domestic rabbits received dosages of 35, 69, and 137 mg cocaine in 5 mL saline via cardiac puncture in the cocaine study. The dosages represent one-half the LD50, the normal LD50, and twice the LD50.

Boettcherisca peregrina were allowed to feed and develop on tissues containing cocaine. From hours 30 to 70, larvae developed more rapidly on tissue containing cocaine from rabbits injected with 69 mg and 137 mg of cocaine than on tissue from rabbits injected with 35 mg of cocaine or no cocaine. Total development times required for pupation and adult eclosion were also shortened. Differences between larvae developing on cocaine-dosed rabbit tissue compared to a control were sufficient to alter PMI estimates based on larval development in decomposing human tissues by up to 24 h (Goff et al. 1989). Goff's results indicate that an opiate (e. g., heroin) and a stimulant drug (e. g., cocaine) can both increase the rate of development in the Boettcherisca peregrina (Goff et al. 1989, Goff et al. 1991).

Bourel et al. (1999b) administered morphine chlorhydrate to three rabbits each at a different concentration. The three concentrations were 12. 5, 25. 0 and 50. 0 mg/h of morphine chlorhydrate via ear perfusion. A fourth rabbit was used as a control. Following administration of the drug, rabbits were sacrificed and 400 eggs of Lucilia sericata were placed in the eyes, nostrils, and mouth of each rabbit. Larvae were sampled daily to determine growth rate and weight. Puparia and emerging adults were also sampled. In this study, the larvae reared on the control and the rabbits that received 12. 5 and 25 mg/h of morphine developed at similar rates from hours 41 to 69, while larvae reared on the carcass given 50. 0 mg/h of morphine developed at a slower rate. From hour 91 to 165, the larvae from carcasses that received 12. 5 and 50. 0 mg/h developed at the same rate, which was slower than the control colony. Overall, the effects of morphine appear to be dose

dependent as the larvae feeding on the rabbit that received the greatest dosage were the slowest to develop. Based on results from this study, between hours 91 and 165 estimations of larval age based on total length can be significantly in error if the presence of morphine in tissues is not considered. The error can be as great as 24 h for Lucilia sericata larvae measuring from 8 to 14 mm total length.

In another case Bourel et al. (2001) used approximately 100 larvae of L. sericata reared on seven 250 g portions of minced beef combined with morphine hydrochloride solutions. After egg hatch, 10 specimens of second instar, third instar, post-feeding third instar and pupae were sampled and immediately frozen. After adults emerged, they were kept in a jar until they died and desiccated. Samples were homogenized, centrifuged, and the supernatant analyzed for morphine content using a specific radioimmunoassay. Concentrations of morphine were high in second and third instar larvae, almost proportional to concentrations in minced meat, but almost no morphine was detected in pupae. The results indicate that larvae excrete the drug during the post-feeding stage. A quantity of morphine is sequestered in the cuticle of pupae, but at minute concentrations. Morphine is sequestered in the cuticle during larval growth and in the formation of puparia (Bourel et al. 2001).

Elimination of drugs or toxins prior to metamorphosis has been shown in other studies. Sadler et al. (1995) was able to detect trimipramine, trazodone, and temazepam, in the larvae of Calliphora vicina, but was unable to detect the drugs in the pupae. The fact that drugs do not bioaccumulate throughout the life of the larvae suggests that elimination

mechanisms are present. Drug concentrations decreased when larvae were taken from drug laden meat and placed on drug free meat. The results of these studies indicate the importance of collecting larvae for toxicological analysis from those feeding actively on a corpse.

Introna et al. (1990) reared Calliphora vicina larvae on liver specimens from 40 cases in which cause of death had been determined to be opiate intoxication. Analysis of larvae and liver for opiates (morphine) was accomplished by radioimmunoassay. The concentration of opiates for all cases was found to range from 8 to 1, 208 µg/kg for larvae and 26 to 1, 769 µg/kg for the liver specimens. A significant difference was found between the opiate liver and larval concentrations.

Goff and Lord (1994) reviewed various studies in entomotoxicology and concluded that entomotoxicological testing was essential to accurate forensic entomology conclusions. Data indicating the presence of drugs allow for corrections to the data in cases when drugs affect insect development.

Future trends in forensic entomology

The precise estimation of PMI is the most important goal of forensic entomology by refining the techniques used.

Developmental and succession data, consideration of a greater number of geographical regions and a range of death scene scenarios are essential. Moreover there are several parameters which need further attention.

It is important to consider factors that might alter the time of oviposition, such as covering corpses with branches or tight wrapping with blankets,

carpets or plastic bags, and indoor placement, because these factors may delay initial oviposition (Higley and Haskell 2001).

Seasonal influences, such as cold and rainy weather, may inhibit or even prevent fly activity and delay oviposition (Erzincliogl u 1996). However, Faucherre et al. (1999) observed flying as well as ovipositing Calliphora vicina under extreme conditions in the Swiss Alps, colonizing a corpse in a 10-m deep cave at a temperature of about 5_C.

The generally accepted assumption that activity of necrophagous flies ceases below an air temperature of 10_C (Williams 1984) or even 12_C (Smith 1986; Erzincliogl u 1996) may be questionable (see also Deonier 1940; Nuorteva 1965). However, the case described by Faucherre et al. (1999) occurred at an altitude of 1, 260 m and therefore a cold-adapted population of C. vicina may have been involved.

Blowflies usually show peaks of oviposition activity in the early afternoon (Nuorteva 1959a; Baumgartner and Greeenberg 1984, 1985; Greenberg 1990). These insects are not active at night and generally do not lay eggs during nighttime (Greenberg 1985). A postmortem interval estimation based on that assumption has to consider the possibility that a corpse which was found about noon and was infested by recently hatched maggots, could have been deposited there in the late evening of the previous day. Hence, fly eggs detected on a corpse during the night would lead to the conclusion that death occurred during the previous day or earlier (Nuorteva 1977). Greenberg (1990) presented the first experimental evidence of nocturnal oviposition by three forensically important blow flies, Calliphora vicina,

Phormia regina and Lucilia (Phaenicia) sericata. On the other hand, Tessmer et al. (1995) reported that blowflies fail to lay eggs at night both in urban (with lighting) and rural dark habitats.

However, Singh and Bharti (2001) supported the findings of Greenberg (1990). Hence nocturnal oviposition is a possibility and should be taken into consideration.

Diapause, the period during which growth and development of insects is suspended, is still a challenge for the forensic entomologist (see also Ames and Turner 2003).

Depending on the insect taxa, the major influences on larvae or pupae are photoperiod and temperature. Declining day length and/or decreasing temperatures indicate approaching winter and induce diapause, preventing development under unfavourable environmental conditions.

In many forensically important blowflies, diapause is under maternal control and exposure of females to short day lengths induces diapause in the offspring (Vinogradova 1991). Species with a large geographical range have to face changes in day length throughout the year.

The critical day length which induces diapause will be longer in populations from a northern range than in southern populations (McWatters and Saunders 1998).

The forensic entomologist working in a temperate region investigating a sample of dead maggots collected from a corpse during late September has to consider the possibility that these maggots had already entered diapause.

Besides day length, temperature may also influence the incidence of diapause (Vinogradova and Zinovjeva 1972).

Unlike photoperiod, temperature is not a noise-free signal, as it is subject to considerable variation both within and between years (McWatters and Saunders 1998). Increasing constant temperature is known to reduce the incidence of diapause in forensically important Dipteran species, such as Liopygia argyrostoma (Saunders 1975), Protophormia terraenovae (Vinogradova 1986) and Calliphora vicina (McWatters and Saunders 1998).

The duration of diapause is another important parameter.

McWatters and Saunders (1998) showed that in C.

vicina kept at temperatures of 15_C and 20_C, respectively, diapause was terminated in most larvae within 30 days. However, the diapause ended earlier in larvae whose parents had been kept at 20_C than those whose parents had been kept at 15_C. These observations should be a caveat for the forensic entomologist and points to the need for further studies on other species.

Competition may affect development and growth of the larvae. Smith and Wall (1997a, 1997b) presented data which indicate that the larvae of Lucilia sericata in carcasses experience significant levels of competition and that the intensity of this competition may be sufficient to reduce the numbers of adult L. sericata able to emerge successfully.

Reiter (1984), Smith (1986) and Erzinclioglu (1990) pointed to another factor which could complicate the estimation of the postmortem interval-precocious

egg development in flies. In some female flies, eggs may be retained in the oviduct, having been fertilized as they pass the spermathecal ducts in advance of the act of oviposition (Wells and King 2001). In cases where a suitable oviposition site is not available, the eggs may remain inside the fly until they have completed embryonic development. It has been reported for several species of

the tribe Calliphorini, including the forensically important Calliphora vicina, that the larva hatches from such eggs immediately following oviposition (Erzinclioglu 1990; Wells and King 2001). Precocious eggs are more likely to be found in bluebottles (Calliphora spp.) than in other lineages of carrion-feeding blowflies and the proportion of wild flies carrying an egg that is about to hatch can be quite high (Wells and King 2001).

Parasitoid larvae feed exclusively on other arthropods, mainly insects, resulting in the death of the parasitoid’s host (Godfray 1994). The majority of parasitoids are either members of the order Hymenoptera or Diptera, representing an extremely diverse group and constituting about 8. 5% of all described insects (LaSalle and Gauld 1991; Godfray 1994). They also attack necrophagous taxa and therefore could appear on carrion. Fabritius and Klunker (1991) listed 83 parasitoid species, mainly wasps, which attack the larval and pupal stages of synanthropic Diptera in Europe. There are few reports on the use of parasitoids in forensic entomology (Smith 1986; Haskell et al. 1997; Amendt et al. 2000; Anderson and Cervenka 2002; Grassberger and Frank 2003b). The life-cycles of the common parasitoid species are known (e. g. Geden 1997) and, even if the adults have already emerged and left the host, the pupal exuviae of

the parasitic wasps can be identified for a long time afterwards (Geden et al. 1998; Carlson et al. 1999). The parasitoid developmental times can then be calculated and added to the time of development of the blowfly host.

Pupal parasitoids of blowflies may play an especially important role in the estimation of the postmortem period because their time of attack is often restricted to a small, well-defined window of time at the beginning of the pupal development of the host insect (Anderson and Cervenka 2002). An example of the practical application of these wasps involved a case where the early colonizers, individuals of the blowfly Protophormia terraenovae, had finished their development and already left the scene but adults of the parasitoid Nasonia vitripennis (Hymenoptera: Pteromalidae) were just about to emerge. These wasps need, at a constant temperature of 25_C, 350 accumulated degree days, equating to about 14 days, to reach adulthood (Whiting 1967; Grassberger and Frank 2003b). By contrast the host P. terraenovae needs about 9 days at this temperature to reach the stage appropriate for the parasitoid’s oviposition (Marchenko 2001; Grassberger and Reiter 2002a). It can therefore be assumed that the flies had access to the body for at least about 23 days before the corpse was found. The calculation of developmental times for the host and the parasitoid allowed the estimation of a greater minimum postmortem interval than the estimated development time of Protophormia terraenovae alone. This enabled the criminal investigators to disprove the testimony of a witness who claimed that he had seen the victim alive 20 days before the corpse was found. However, when thinking about the potential influence, especially of larval parasitoids, it is important to remember that this specialized group

might also create significant problems for forensic entomology. Holdaway and Evans (1930) described, for example, the change in developmental times for Lucilia sericata after the attack of its parasitoid Alysia manducator, which resulted in premature pupariation.

The role of freshwater and marine fauna in forensic investigations has received very little attention (Payne and King 1972; Nuorteva et al. 1974; Goff and Odom 1987; Haskell et al 1989; Catts and Goff 1992; Vance et al. 1995; Sorg et al. 1997; Davis and Goff 2000).

Knowledge about the role of aquatic arthropods during decomposition is still scanty (Keiper et al. 1997; Tomberlin and Adler 1998; Hobischak and Anderson 1999, 2002; Anderson 2001; Merrit and Wallace 2001; Anderson and Hobischak 2004). Compared with terrestrial habitats, decomposition in an aquatic environment is completely different. It occurs at a rate roughly half that of decomposition on land, mainly due to the prevention of insect activity and cooler temperatures (Knight 1991).

Merrit and Wallace (2001) have distinguished six decompositional stages ranging from submerged fresh, floating decay to sunken remains. Aquatic insects of forensic importance belong to the Ephemeroptera (mayflies), Trichoptera (caddis flies) and Diptera (true flies); the latter are mainly represented by Chironomidae (midges) and Simuliidae (black flies). However, these insects, unlike their terrestrial counterparts, are not obligatory saprophages, but instead use the submerged carrion both as a food source and a breeding site. The use of these insects for estimating the time of death is therefore more difficult and depends on the season and

on other conditions of the aquatic systems. No successional insect model exists which describes the different waves of colonization of a corpse in aquatic habitats (Merrit and Wallace 2001).

Finally, forensic entomology may help in investigations dealing with living, but ill, people by revealing neglect. The occurrence of maggots in the wounds or natural orifices of living persons may indicate such a neglect. Estimating the age of these maggots can reveal how long the neglect has been happening (Benecke 2003).