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Insects as vectors of DNA in a forensic context

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Abstract

Insects have historically been used in criminal investigations to provide information in relation to postmortem intervals (PMIs) but the field of forensic entomology is expanding. It is now recognised that insects can act as vectors of human and mammalian DNA through the consumption of biological material, with extraneous DNA able to be extracted and genotyped from all stages of the life cycle, and insect faeces and regurgitant (artefacts). To date, DNA recovered from insects have been used to inform investigations into neglect and homicide, link body parts and to identify victims of crime. It may also potentially be used to identify assailants, confirm the food source of insects to determine their relevance in PMI calculations, determine if a crime has occurred, identify scenes of crimes, and link people to locations and other individuals. However, insects which have consumed biological material may also transfer DNA by travelling to new areas, or depositing it via their artefacts, either within crime scenes or laboratories, or at locations distant to the food source. This could contaminate forensic evidence, confound investigations, and/or falsely incriminate or exclude an individual. Therefore, it is important that there is increased awareness into both the utility of insects as vectors of forensically relevant DNA, and the potential for contamination.

Graphical/Visual Abstract and Caption

Human DNA can be recovered from insects which have fed on human biological material at a crime scene. This may provide useful information to investigators, but the insects can also transfer the DNA, potentially causing contamination events.

Introduction

When Sir Alec Jeffreys and his colleagues discovered a mechanism to isolate individualising regions of the human genome, they were arming crime investigators with a revolutionary biological tool (Jeffreys, Wilson, & Thein, 1985). In the years since, DNA analytical techniques have become increasingly sensitive and more powerful, with full DNA profiles able to be obtained from a single human cell (Ballantyne, van Oorschot, Mitchell, & Koukoulas, 2006; Dean et al., 2002; Findlay, Frazier, Taylor, & Urquhart, 1997; Hanson & Ballantyne, 2005; Park, Beaty, Boyce, Scott, & McIntosh, 2005; Spits et al., 2006). Adaptations of existing protocols have also proved beneficial in obtaining DNA profiles from previously insufficient amounts of DNA. These adaptations include the low copy number (LCN) and low template DNA (LTDNA) techniques, both of which involve increasing the

number of PCR cycles during the amplification step, performing replicate analysis and adopting more stringent interpretation guidelines when determining the final profile (Caragine et al., 2009; Gill, 2001; Murray et al., 2003).

These advances have enabled scientists to move beyond the traditional sources of genetic material, and they are now able to obtain reliable DNA profiles from a wide range of items (Wickenheiser, 2002). Human DNA has been recovered from a ~~myriad~~ wide variety of substrates that had previously been ineffectual sources, including surfaces as diverse as motor oil (van Oorschot, Gutowski, Robinson, Hedley, & Andrew, 1996), human skin (Graham & Rutty, 2008; Maguire, Ellaway, Bowyer, Graham, & Rutty, 2008), cheese (Sweet & Hildebrand, 1999), fingerprints (van Oorschot & Jones, 1997) and items touched only briefly (Kisilevsky & Wickenheiser, 1999; Phipps & Petricevic, 2007; Poy & van Oorschot, 2006; van Oorschot, Ballantyne, & Mitchell, 2010). This has highlighted the fact that potential sources of DNA may be invisible to the naked eye.

Due to the fact that DNA can now be obtained and genotyped from minute quantities of biological material, the deposition of DNA on a surface via indirect secondary or tertiary transfer from an initial source has become an area of considerable interest in forensic biology (Farmen, Jagho, Cortez, & Froyland, 2008; Goray, Eken, Mitchell, & van Oorschot, 2010; Goray, Mitchell, & van Oorschot, 2010, 2012; Goray, van Oorschot, & Mitchell, 2012; Ladd, Adamowicz, Scherczinger, & Lee, 1999; Phipps & Petricevic, 2007; Poy & van Oorschot, 2006; van Oorschot, Szkuta, Meakin, Kokshoorn, & Goray, 2019; Wickenheiser, 2002). While most research in this field focuses on person-facilitated DNA transfer, it has also been proven that DNA may be transferred through other vectors, such as insects.

Insects and their activity in relation to crime scenes (Benecke, 2001; Catts & Goff, 1992; Hall, 2000), incidences of neglect (Benecke, Josephi, & Zweihoff, 2004; Benecke & Lessig, 2001; Keh, 1985) or matters of public health (Hall, 2000) have long been used to assist in forensic investigations. The most common application of entomology with respect to crime scenes is the calculation of the minimum postmortem interval (PMI) which is an estimate of the shortest length of time that may have elapsed since a person has died (Ames & Turner, 2003; Catts & Goff, 1992; Ireland & Turner, 2006; Marchenko, 2001). However, blowfly colonisation may also occur in the wounds or orifices of living people, especially in instances of neglect (Benecke et al., 2004; Benecke & Lessig, 2001; Keh, 1985). For example, there have been reported instances of maggots found on the genitalia and rectal area of children after flies have been attracted to faeces festering in unchanged nappies (Benecke & Lessig, 2001). Determination of the age of the maggots can be used to estimate how long abuse has been occurring (Benecke & Lessig, 2001).

While the use of insects in a forensic context is dominated by the larval stages of blowflies, researchers are extending their scope to investigate the utility of other life stages in criminal and civil investigations. ~~Generally,~~The majority of insects will follow one of two life cycles – hemimetaboly or holometaboly (Gullan & Cranston, 2010). Hemimetabolous insects undergo incomplete metamorphosis where the morphological change is gradual from egg, to nymph, to adult, and does not include a pupal stage (Gullan & Cranston, 2010). The nymph stage of these insects is often similar to the adults but generally lacks wings and reproductive organs (Gullan & Cranston, 2010). Such insects include cockroaches, crickets, bed bugs, ticks, kissing bugs and lice. Holometabolous insects may oviposit (lay eggs from which maggots hatch) or larviposit (give birth to maggots). From the larval stage, the insect will undergo complete metamorphosis to adult, via a

pupal stage (Gullan & Cranston, 2010). The juvenile stage of such insects is very different morphologically and biochemically to the adult (Gullan & Cranston, 2010). Holometabolous insects include mosquitoes, flies, sandflies, wasps and beetles. The rate at which both lifecycles progress is dependent on temperature (Ames & Turner, 2003; G. Anderson, 2000; Dallwitz, 1984; Nicholson, 1934; Vogt & Walker, 1987), but are also affected by the availability of resources, time of day (Smith, 1983; Woolridge, Scrase, & Wall, 2007), and weather conditions (Digby, 1958; Mahat, Zafarina, & Jayaprakash, 2009). For example, if the temperature is too low or the environment too arid in the Canberra region of Australia, maggots will enter quiescence~~diapause~~ – a period of ~~suspended development~~dormancy – and will complete development when the temperature increases again (Dallwitz & Wardhaugh, 1984).

RETRIEVAL OF EXTRANEOUS DNA FROM INSECTS

Taking advantage of the advances in DNA analysis techniques, the discipline of forensic entomology has extended to include the investigation of insects as vectors of human and mammalian DNA. To date, human and mammalian DNA has been recovered from various life-stages of a number of insect species, including the adults of mosquitoes (Ansell et al., 2000; Chow-Shaffer, Sina, Hawley, De Benedictis, & Scott, 2000; Coulson, Curtis, Ready, Hill, & Smith, 1990; Curic, Hercog, Vrselja, & Wagner, 2014; Ibrahim, Alrakan, Alaifan, Al-Mekhlafi, & Kassab, 2015; Kent & Norris, 2005; Kreike & Kampfer, 1999; Mehus & Vaughan, 2013; Mukabana et al., 2002; Odongo & Irungu, 2002; Oshaghi et al., 2006; Rabelo et al., 2015; Reeves, Holderman, Gillett-Kaufman, Kawahara, & Kaufman, 2016; Sato, Furuya, Harada, & Suguri, 1992; Soremekun et al., 2004; Spitaleri, Romano, Di Luise, Ginestra, & Saravo, 2006; Vieira, Carvalho, & Silva, 2017), flies (Boakye, Tang, Truc, Merriweather, & Unnasch, 1999; Kester, Toothman, Brown, Street IV, & Cruz, 2010; Powers, van Oorschot, & Durdle, 2019), bed bugs (Raffaele, McCarthy, Raab, & Vaidyanathan, 2015; Schal et al., 2018; Szalanski et al., 2006), kissing bugs (Pizarro & Stevens, 2008), human body lice (Davey, Casey, Burgess, & Cable, 2007; Mumcuoglu, Gallili, Reshef, Brauner, & Grant, 2004), human head lice (Mumcuoglu et al., 2004), human pubic lice (Lord et al., 1998; Replogle, Lord, Budowle, Meinking, & Taplin, 1994), cockroaches (Kester et al., 2010), crickets (Kester et al., 2010), sandflies (Maleki-Ravasan et al., 2009) and ticks (Tobolewski, Kaliszewski, Colwell, & Oliver JR, 1992); blowfly maggots (Boakye et al., 1999; Chavez-Brones et al., 2013; Clery, 2001; Di Luise, Magni, Staiti, Spitaleri, & Romano, 2008; Kondakci, Bulbul, Shahzad, Polat, & Cakan, 2009; Li et al., 2011; Linville, Hayes, & Wells, 2004; Njau, Muge, Kinyanjui, Omwandho, & Mukwana, 2016; Powers et al., 2019; Wells et al., 2001; Zehner, Amendt, & Krettek, 2004); blowfly pupae (Carvalho, Dadour, Groth, & Harvey, 2005; Powers et al., 2019) and the empty puparium of blowflies (Marchetti, Arena, Boschi, & Vanin, 2013; Powers et al., 2019). Human DNA has also been recovered from the excreta of the adults of human pubic louse (Replogle et al., 1994), and the faecal and regurgitant artefacts of blowflies (Durdle, Mitchell, & van Oorschot, 2013; Durdle, Mitchell, & van Oorschot, 2011; Durdle, van Oorschot, & Mitchell, 2009; Kulstein, Amendt, & Zehner, 2015). However, the rates of amplification and genotyping success have been varied due to the fact that the food, and consequently the DNA contained within, breaks down due to digestive processes. There have also been demonstrated species-effect and this is likely due to the variable biophysical behaviour of flies, such as the rate at which different species empty their crops post-feeding (Campobasso, Linville, Wells, & Introna, 2005).

In several studies, researchers have focused on obtaining mitochondrial (mtDNA) to identify the species of origin (human or otherwise) of the contents of an insect gut. However, while identifying

an insect food source as human can be beneficial for an investigation, there is even more value in being able to identify a specific individual. To this end, studies have also investigated the capability of obtaining an individualising profile, primarily from nuclear DNA (nDNA). Even if a full DNA profile cannot be obtained, partial profiles can still be considered useful, particularly in an exclusionary capacity. It must also be noted that while human DNA is the focus on many forensic studies regarding the gut content of insects, forensically relevant DNA may also include that from other species – for example, DNA from illegally trafficked wildlife.

Necrophagous insects

Blowflies

Blowflies are the insects most commonly associated with crime scenes, and consequently several studies with a strong forensic focus have been conducted into the retrieval of human and mammalian DNA from blowflies (Table 1). In both the juvenile (maggot) and adult stages, food will be degraded by enzymatic, bacterial and physical activity, either prior to consumption as the insect breaks down solid tissue or dried food sources (Fraenkel, 1939; Graham-Smith, 1930; Hansen Bay, 1978a, 1978b; Hewitt, 1912; Kerlin & Hughes, 1992; Oschman & Berridge, 1970; Prince & Berridge, 1973; Waterhouse, 1957; Webber, 1957), or as it passes through the mid- and hindguts (Espinoza-Fuentes & Terra, 1987; Hobson, 1932). Consequently, the DNA present within the food source will also degrade to some extent (Lund & Dissing, 2004; Pääbo, 1989; van Oorschot et al., 1996). However, most food is initially stored in the crop – a pouch that extends from the foregut (Hobson, 1932) – where digestion does not actively occur (Chapman, 1985). It has been shown that the optimal point at which maggots should be sampled is that at which the crop is at its fullest - crops less than 1 mm in length have been found to be unreliable sources of DNA (Campobasso et al., 2005) due to the lack of food content. Crops will generally be this size in maggots less than 2 days old, and also in those in the pre-pupal stage during which maggots purge their gut contents (Campobasso et al., 2005). However, it is also possible that incomplete emptying of the crop occurs leading up to pupation so, while appearing empty visually, the crop may in fact still contain some food material, and maggots at this stage can still contain sufficient DNA to be amplified (Carvalho et al., 2005).

Furthermore, the DNA in a food source may be concentrated. Often, adult flies will ~~concentrate the liquid food by 'bubbling' where the fluid is exuded~~ repeatedly exude and reingest fluid ~~and reingested repeatedly~~, ~~and~~ with the fluid being suspended from the end of the proboscis in bubble form for several minutes after each extrusion (Dacks, Nickel, & Mitchell, 2003; Hendrichs, Fletcher, & Prokopy, 1993; Stoffolano, Acaron, & Conway, 2008; Thomas, 1991). This~~e~~ "bubbling" behaviour is thought to allow the evaporation of excess water (Hendrichs et al., 1993) ~~and~~or to concentrate the food (Hendrichs et al., 1993; Thomas, 1991), and has been observed to occur over several hours (Striman, Fujikawa, Barksdale, & Carter, 2011). The feeding apparatus of blowflies is most efficient when consuming liquid (Graham-Smith, 1914) but flies also readily consume dried biological materials (Durdle, Mitchell, & van Oorschot, 2016; Kulstein et al., 2015). One study has demonstrated a preference for dry blood or semen over the wet forms, depending on the age and gender of the fly (Durdle et al., 2016). The processing of dry food over wet would require an extra expenditure in energy, as would the "bubbling" of regurgitant, so it is evident concentrated food is of value to the flies. Dried food is likely to have a higher concentration of nutrients. For example,

free amino acids in semen increase in concentration for up to at least 60 min when left at room temperature (Jacobsson, 1950).

Conversely, flies also indulge in coprophagy (the consumption of faeces)(Durdle, van Oorschot, & Mitchell, 2013; Stevenson & Dindal, 1987), and emetophagy (the consumption of regurgitant) (Durdle, van Oorschot, et al., 2013; Striman et al., 2011). This means that a component of an adult fly's gut content may actually have been previously subjected to the digestive process, and the DNA within will be more extensively compromised.

Newly emerged flies will seek carbohydrates for nutrition and energy (Fraenkel, 1939; Hasset, Dethier, & Gans, 1950; Munro, 1951; Webber, 1957), and females will also seek proteins (Barton Browne & van Gerwen, 1992; Dethier, 1961; Readshaw & Van Gerwen, 1983; Roberts & Kitching, 1974; Williams, Barton Browne, & van Gerwen, 1979), predominantly for reproductive purposes (Thomsen & Lea, 1968). There are a number of forensically relevant food sources that flies have been shown to feed on to acquire vital nutrients. Research into the recovery of human DNA from blowflies has focused predominantly on maggots which have been retrieved from corpses, although additional food sources have included human liver, diabetic patients and human semen.

Blowflies are the first insects to inhabit a body after death (Benecke, 2001; Catts & Goff, 1992; Dadour, Cook, Fissioli, & Bailey, 2001; Keh, 1985; Marchenko, 2001). A corpse in the early stages of decomposition is a protein- and carbohydrate-rich source that both juvenile and adult flies exploit (Archer & Elgar, 2003a, 2003b; Arnaldos, Romera, Garcia, & Luna, 2001; Centeno, Maldonado, & Oliva, 2002; Clift & McDonald, 1976; Eberhardt & Elliot, 2008; Grassberger, Friedrich, & Reiter, 2003; Lang, Allen, & Horton, 2006; Marchenko, 2001; Payne, 1965; Sukontason et al., 2007; Velasquez, 2008; Webber, 1958; Wolff, Uribe, Ortiz, & Duque, 2001) due to the high quality protein that is available (Archer & Elgar, 2003a) and the availability of carbohydrates in the form of glucose in the putrefied tissue (Webber, 1957). Decomposition also produces tissue exudates such as inorganic salts that are necessary for egg maturation (Barton Browne, van Gerwen, & Bartell, 1980; Hobson, 1938; Williams et al., 1979).

While semen stains may not be as prevalent at crime scenes as blood and tissue, they are often found at scenes or on evidence involving sexual activity. Semen contains compounds of nutritional value for blowflies such as sugars (King & Mann, 1959; Nixon, 1964), carbohydrates (Webber, 1957), cholesterol (Eliasson, 1966) and essential amino acids (Webber, 1958), with on average, 225,000ng of DNA per mL (Lee & Ladd, 2001).

Table 1 Reported DNA recovery from flies after feeding on human and mammalian biological material

Species	Food source	Specimen	Amplification success	Reference
Cynomyopsis cadaverina (shiny blue bottle blowfly)	Human liver	Crop of third instar	mtDNA from 3 of 5 specimens	Wells et al., 2001
Calliphorid (blowflies), Sarcophagidae (flesh flies), Muscidae (house flies)	13 corpses – ranging from rigor mortis to advanced/highly mummified, with estimated PMIs of 1-16 weeks	Crop of third instar	mtDNA from 12 of 13 specimens; 7/13 full (10-loci) STR profiles, 2/13 partial STR profiles	Zehner et al., 2004
Calliphora.vicina (bluebottle blowfly)	Human tissue	Intact up to 2.5 days old, crop of third instar and post-feeding, killed immediately after collection	mtDNA from 2.5 days old to post-feeding maggots; nDNA from 2.5 days to late third instar maggots	Campobasso et al., 2005
Calliphora.vicina (bluebottle blowfly)	Human tissue	Intact up to 2.5 days old, crop of third instar and post-feeding, killed 24h after collection	mtDNA from third instar maggots; no nDNA detected	Campobasso et al., 2005
Calliphora.vicina (bluebottle blowfly)	Human tissue	Intact up to 2.5 days old, crop of third instar and post-feeding, killed 48h after collection	No mtDNA or nDNA detected	Campobasso et al., 2005
Calliphora.vicina (bluebottle blowfly)	Corpse in decay stage	Crop of "actively-feeding" larvae	Partial (6/10 loci) STR and (7/17 loci) Y-DNA profiles	Di Luise et al., 2008
Lucilia sericata (greenbottle blowfly)	Human tissue	Crop of third instar	3/8 full (16 loci) STR and 3/8 partial STR profiles. 8/8 (29 markers) SNP profile	Kondakci et al., 2009
Lucilia sericata (greenbottle blowfly)	Human semen (2, 3.5 or 6mL) on liver	Intact or crop of second instar; intact post-feeding and early and late pupae	1/20 full (4-loci) Y-STR profile from crop of second instar on 3.5mL semen/liver	Clery, 2001
Calliphora stygia (brown blowfly)	Human semen on liver	Adults fed semen - Crop of subsequent larvae, separated puparium and pupa	8/37 partial (1-7 alleles) STR profiles and 3/37 partial (8+ alleles) STR from all life stages	Powers et al., 2019
Calliphora stygia (brown blowfly)	Human semen on liver	Maggots fed semen - Crop of larvae, separated puparium and pupa	5/20 partial (1-7 alleles) STR profiles and 6/20 partial (8+ alleles) STR from all life stages	Powers et al., 2019
Calliphora augur (brown blowfly)	Human semen on liver	Adults fed semen - Crop of subsequent larvae, separated puparium and pupa	No STR profiles (21 loci) obtained	Powers et al., 2019
Calliphora augur (brown blowfly)	Human semen on liver	Maggots fed semen - Crop of larvae, separated puparium and pupa	2/16 partial (1-7 alleles) STR profiles from larvae	Powers et al., 2019
Calliphora dubia (lesser brown blowfly)	Sheep liver	Intact first, second, third instar, day 1, 2 and 3 post-feeding, and day 1, 2, and 3 pupa	87bp and 197bp nDNA fragments detected in all stages except day 3 pupa	Carvalho et al., 2005

Digestion will continue to occur in maggots once they have been removed from a food source, and DNA will continue to be degraded as a result (Table 2). It has also been shown that if a maggot leaves a body and continues to feed on a non-human source, less human DNA will be amplified than if the maggot had not continued to feed at all (Zehner et al., 2004). Predicting the time period during which STR profiles could be successfully obtained from flies that had fed on a corpse would be difficult, given the variability associated with DNA degradation, and the variable conditions to which a corpse may be exposed (Zehner et al., 2004). This highlights the fact that ideally an insect will be killed immediately after collection to best preserve any extraneous DNA in the gut.

Table 2 Degradation of DNA in maggots after removal from an original food source

Species	Food source	Days post-feeding	Maximum time for amplification success	Reference
Protophormia terraenovae (blue-assed fly)	3 corpses ranging from released rigor mortis to advanced decay	Crops from maggots starved for 0, 2, 4, 6, 8, 10 days	0-4 days – 30/30 full (16 loci) STR profiles; 6-10 days – no nDNA detected	(Njau et al., 2016)
		Crops from maggots fed on beef for 0, 2, 4, 6, 8, 10 days	0-2 days – 20/20 full (16 loci) STR profiles; 4-10 days – no nDNA detected	

Beetles

While blowflies are the early colonisers of corpses, other insects also appear in later waves of succession, including beetles, wasps, moths and ants; the stage of decomposition at which they exploit the body depends on their feeding habits and preferred diet (G. Anderson & VanLaerhoven, 1996; Bomemissza, 1956; Catts & Goff, 1992; Eberhardt & Elliot, 2008; Erzinclioglu, 2003; Payne, King, & Beinhart, 1968). Human mtDNA has been obtained from the maggots of *Omosita spp.*, collected after feeding on skeletalised human remains (DiZinno, Lord, Collins-Morton, Wilson, & Goff, 2002). While it is not clear from this study whether maggots were analysed individually, and how many samples were tested in total, the authors report that the profiles from all samples matched those from the bones on which the maggots were feeding, and a known blood sample from the donor of the remains.

Haematophagous insects

Haematophagous insects are those which preferentially feed on blood. Human and mammalian DNA has been isolated from the digestive tracts of mosquitoes (Ansell et al., 2000; Chow-Shaffer et al., 2000; Curic et al., 2014; Ibrahim et al., 2015; Kent & Norris, 2005; Kreike & Kampfer, 1999; Mehus & Vaughan, 2013; Mukabana et al., 2002; Odongo & Irungu, 2002; Oshaghi et al., 2006; Rabelo et al., 2015; Reeves et al., 2016; Sato et al., 1992; Soremekun et al., 2004; Spitaleri et al., 2006; Vieira et al., 2017), sandflies (Maleki-Ravasan et al., 2009), tsetse flies (Boakye et al., 1999), bed bugs(Raffaele et al., 2015; Schal et al., 2018; Szalanski et al., 2006), and blackflies (Boakye et al., 1999) after the insects have consumed human and mammalian blood (Table 3). Several of these studies have also investigated the temporal effects on amplification and profiling success and reported decreasing amplification success the greater the time since feeding, regardless of whether nDNA or mtDNA was profiled (Chow-Shaffer et al., 2000; Curic et al., 2014; Ibrahim et al., 2015; Mukabana et al., 2002; Oshaghi et al., 2006).

In some instances it is only the adult female of the insect which consumes blood – this is true for mosquitoes and sandflies. Therefore it is the adult females that are the most important individual in a forensic sense as it is from them that human DNA may be retrieved. Mosquitoes and sandflies are holometabolous. The mosquito larval stage is aquatic, with eggs being predominantly laid on stagnant water. The natural habitat of the mosquito includes swamps and marshes, but they are also present in artificial environments such as receptacles that collect water in industrial and urban environments. Sandflies inhabit rural, peri-urban and urban environments, and in human dwellings prefer to occupy cool, dark and humid areas of the building.

Bed bugs and kissing bugs (a parasitic vector of Chagus disease) are hemimetabolous. Both the male and female will feed on blood, and the insects prefers dark, cool areas in a building. Human lice are also hemimetabolous and remain on the host's body throughout its lifecycle.

Studies have noted that the amount of human DNA that can be retrieved from an insect may be dependent on species. Mosquito species exhibit differences in the amount of blood consumed by a female in a single feeding event (Curic et al., 2014; Ibrahim et al., 2015), the length of time it takes to digest a meal (Christophers, 1960; Vinogradova, 2000), and also digestion behaviour. For example, *Anopheline* mosquitoes concentrate meals through pre-diuresis (Vaughan, Noden, & Beier, 1991), a form of evaporative cooling to reduce temperature during feeding (Lahondere & Lazzari, 2012). This would result in a greater concentration of human DNA in the gut content of this species (Mukabana et al., 2002).

Table 3 Reported DNA recovery from haematophagous insects after feeding on human and mammalian blood

Species	Hours post-feeding	Maximum time for amplification success	Reference
Mosquitoes			
Anopheles gambiae	0, 8, 16, 24, 32h	TC-11: 89%, 90%, 40%, 10%, 0%. VWA: 73%, 90%, 40%, 10%, 10% with increasing time points	Mukabana et al., 2002
Aedes aegypti	0, 8, 16, 24, 32, 40, 48, 56, 64, 72	0h – 10/10 full (1 VTNR and 3 STR); 8h – 10/10 full; 16h 6/10 full, 2/10 partial; 24h – 7/10 full, 3/10 partial; 32h – 1/10 full. 40-72h – all failed	Chow-Shaffer et al., 2000
Anopheles stephensi and *Culex quinquefasiatus*	1, 6, 12, 18, 24, 27, 33, 36	1-6h – 40/40 (1 marker); 12h – 19/20; 18-24h – 18/20; 30h – 17/20; 33 – 10/20; 36h – 20/20 failed	Oshaghi et al., 2006
Culicinae and Anophelinae	0, 8, 16, 24, 32, 40, 48, 56, 64, 72, 80, 88, 96, 104, 112, 120, 128, 136, 144, 152	*Culicinae* and *Anophelina*e species: 0-32h – 56/56 full (16 loci) STR profiles; *Culicinae* 40-48h – 74/74 full, 56h – 8/13 full; 64h – 3/14 full; 72h – 11/13 full, 80-88h full	Curic et al., 2014
Aedes aegypti maintained at 18°C	3, 6, 12, 24, 48, 72	3-48h – 50/50 full (16 loci) STR profiles; 72h – 10/10 failed	Ibrahim et al., 2015
Aedes aegypti maintained at 40°C	3, 6, 12, 24, 48, 72	3-6h – 20/20 full (16 loci) STR profiles; 12-24h 10/10 partial, 48-72h – 20/20 failed	Ibrahim et al., 2015
Culex pipiens maintained at 18°C	3, 6, 12, 24, 48, 72	3-48h – 50/50 full (16 loci) STR profiles; 72h – 10/10 partial	Ibrahim et al., 2015
Culex pipiens maintained at 1°C	3, 6, 12, 24, 48, 72	3-72h – 60/60 full (16 loci) STR profiles	Ibrahim et al., 2015
Bed bugs			
Cimex lectularius	0, 24h	30/35 – mtDNA (1 marker); 32/35 (1 loci) STR profile	Szalanski et al., 2006
Cimex lectularius	0, 12, 24, 48, 72, 96	0-72h – 45/45 full (5 loci) STR profiles; 96h – 9/9 failed	Raffaele et al., 2015
Cimex lectularius fed on male blood	0, 12, 24, 36, 48, 60, 72, 84, 96, 106	Full (16 loci) and partial STR profiles up to 84h	Schal et al., 2018
Cimex lectularius fed on female blood		Full (16 loci)* and partial STR profiles up to 96h; 3.5 × more DNA than male blood, 5.5 × more DNA than male/female blood	Schal et al., 2018
Cimex lectularius fed on 1:1 female:male blood		Full (16 loci) and partial STR profiles up to 72h	Schal et al., 2018
Human lice			
Pthirus pubis	Unspecified	8/8 mtDNA (1 marker)	Lord et al., 1998
Pthirus pubis	Unspecified	1/1 no STR (2 loci) profile obtained	Replogle et al., 1994
Pediculus humanus capitis	Unspecified	1/1 full (3 loci) profile (mixed from 2 individuals)	Mumcuoglu et al., 2004
Pediculus humanus capitis			Pilli et al., 2016
Pediculus humanus humanus	2, 4, 6, 8, 14, 17, 20, 24	2-20h – 13/14 full (3 loci) STR profile; 24h – 2/2 no STR profile obtained	Mumcuoglu et al., 2004
Pediculus humanus humanus	0, 2, 4, 6, 8, 12, 18, 24, 36, 48, 72h	0-6h – 8/8 full (2 marker) nDNA profile; 24h – max period of detection for 199bp marker; 12h – max period of detection for 283bp marker	Davey et al., 2007
Sandflies			
Phlebotomus duboscqi(Haouas et al., 2007)	4, 8, 12, 16, 20, 24, 37, 48h	4-24h – 6/6 full (1 marker) profile; 37-48h – 2/2 failed	*Haouas et al., 2007*

*Authors state that the female reference profile consisted of 29 human autosomal markers and one amelogenin marker for a total of 30 alleles in a full profile. However, Identifiler actually utilises 15 autosomal loci and the bi-allelic amelogenin, which would make a total of 32 markers for a full profile. Therefore, it is not clear if the reference profile was a partial profile, or if some "full profiles" were actually partial.

Extraneous DNA in field samples

There are several factors which impact the forensic utility of mosquitoes. Not all species consume human blood, as some species selectively feed on specific hosts such as birds, amphibians, reptiles and small mammals (Henderson & Senior, 1961). One study analysed several variables to determine which species should be targeted when collecting mosquitoes for the extraction of human DNA (Trajer, 2018). By assessing the willingness of 42 taxa to bite humans and calculating the probability that a human and an unfed female blood-seeking mosquito of a particular taxa would be in proximity, the authors identified *Aedes* mosquitoes as being the most suitable candidates for forensic analysis in Central Europe.

A number of studies have sampled haematophagous insects from the wild to determine the presence of human DNA, and in doing so, have given an indication of the likelihood of finding human or vertebrate DNA in a randomly sampled specimen. In one study, of 97 blood-fed sandflies females, human DNA was found in 5% of the females analysed, and in none of these instances was the human DNA mixed with the DNA from another mammal (Haouas et al., 2007). In another study, human DNA was also recovered from four mosquitoes collected from a hotel room shared by four people, and 6 mosquitoes from a bedroom used by one individual (Kreike & Kampfer, 1999). Of the hotel mosquitoes, three of the mosquitoes were found to have fed on only one individual each, but one of the mosquitoes had fed on two individuals. In a third study, of 24,250 mosquitoes collected from outdoor sites – farmland which contained a homestead, and a hardwood forest located in the same valley – 416 were able to be sequenced for vertebrate DNA and 391 were of sufficient quality that host origin could be determined (Mehus & Vaughan, 2013). Human mtDNA was found in 2 of the 147 amplified samples from the forest, and 6 of the 244 amplified samples from the farmland. A larger scale field study sampled 100 mosquitoes from 13 houses in a Thai village, with reference samples taken from 50 of the 56 residents of the village (Chow-Shaffer et al., 2000). The authors reported partial or full 4-loci profiles were obtained from 61% of the mosquitoes. Of the 20 full profiles obtained, it could be determined that 9 mosquitoes had fed on multiple individuals. Interestingly, 13 of the mosquitoes contained DNA from individuals who did not live in the house in which they were captured, or from individuals who had not been profiled.

After 81 kissing bugs (*Triatoma infestans)* at various stages of development were collected from domestic dwellings, or animal dwellings close to human habitats, vertebrate DNA could be retrieved from 69 samples (Pizarro & Stevens, 2008). Of these, human mtDNA was retrieved from 13, and for 8 of these, DNA from other vertebrates was found mixed with the human DNA. In another study, *Phlebotomus perfiliewi* and *Ph. (Adlerius)* sandfly species were collected from both indoor and outdoor sites, including human dwellings, animal dwellings, animal burrows, under bridges and river shores (Maleki-Ravasan et al., 2009). From a random sample of 200 collected insects, human mtDNA was found to be in 68 insects, either as human mtDNA alone, or with animal mtDNA.

Insect artefacts ~~(faeces and regurgitant)~~

In the context of bloodstain pattern analysis (BPA), insect stains are defined as "bloodstains resulting from insect activity" by the Scientific Working Group on Bloodstain Pattern Analysis (SWGSTAIN) (SWGSTAIN, 2009). These stains may include pre-existing bloodstains that are then altered by insects, or stains that are created through insects depositing faeces or regurgitant or transferring blood via external surfaces such as their tarsi (Rivers & Geiman, 2017). Faecal, regurgitant and insect-mediated transfer stains are also known as artefacts.

Blowflies excrete nitrogenous waste, mainly comprising uric acid, allantoin, amino acids, sugars, and ions (Wigglesworth, 1972). This excretion combines with other matter including partially digested and undigested food to forms faecal artefacts (Brown, Hawkes, Anderson Parker, & Byrd, 2001; Chapman, 1998). The process of regurgitating and bubbling by flies in order to digest or concentrate food sources may also leave regurgitant artefacts. The composition of both faecal and regurgitant artefacts is dependent on the diet of the fly, and what dietary constituents were consumed in excess (Wigglesworth, 1972).

In addition to faecal and regurgitant spots, artefacts may also be deposited by a fly walking through liquid and depositing it onto surfaces as it walks. Small round stains deposited by tarsi or pulvilli after flies have walked through blood, (Benecke & Barksdale, 2003; Bevel & Gardner, 2002; Brown et al., 2001; Rivers & McGregor, 2018) and asymmetrical linear stains created when flies have dragged their abdomen through wet blood (Rivers & McGregor, 2018), have been observed. However, others have noted the absence of tarsi tracks, despite the flies having been observed walking through blood (Fujikawa, Barksdale, & Carter, 2009; Striman et al., 2011; Zuha, Supriyani, & Omar, 2008) which may be due to the tarsi being unable to break the surface tension of the pools of blood (Striman et al., 2011).

While it is likely that DNA in any biological material consumed by flies would be broken down somewhat by digestive processes, the extent of digestion may vary before any food is excreted as waste (Chapman, 1998). Some food may be completely digested before the fly excretes any waste, but on other occasions the food may pass through the digestive system almost intact (Chapman, 1998).

Research has investigated the human DNA content of artefacts deposited after the flies have fed on human blood, semen and saliva (Durdle, Mitchell, et al., 2013; Durdle et al., 2011; Durdle et al., 2009), and putrefaction fluid from porcine trotters (Kulstein et al., 2015), and all food sources have proven to generate artefacts from which a DNA profile can be obtained.

The amount of human DNA that can be extracted from artefacts of flies which have fed on human blood, semen, saliva, depends on the type of biological material the flies have fed on (Durdle, Mitchell, et al., 2013). Human nDNA is more readily obtained from semen-based artefacts than blood-based artefacts deposited by *Lucilia cuprina* adults, and saliva-based artefacts have been proven to be poor sources of DNA. While full 10-loci STR profiles can be obtained from single artefacts in many instances, the likelihood of amplification success increases with increasing number of artefacts per sample. Artefacts derived from combined semen/saliva samples as per that generated by fellatio contain even higher amounts of human DNA than artefacts derived from semen alone. While this is counterintuitive, it may be possible that semen-derived artefacts introduce a PCR inhibitor that is diluted by the presence of saliva. Blood/saliva-derived artefacts do not contain more human DNA than artefacts derived from blood alone – it is possible there is more

saliva in these samples than in the semen/saliva samples, and any inhibition is more diluted as a result. Artefacts derived from putrefaction fluid or decomposing porcine blood have also proven to be good sources of extraneous DNA, with amplification success greater with artefacts derived from degraded blood than the putrefaction fluid (Kulstein et al., 2015).

Human DNA has proven to be robust in fly artefacts, with human DNA able to be obtained from swabs of 50 artefacts which had been left at 25°C, up to two years after the artefacts had been deposited (Durdle et al., 2011). Furthermore, in most instances, the artefacts also generated a full STR profile of 16 loci. The DNA does not degrade linearly over time, with the amount that could be obtained actually increasing over at least the first 400 days. This may indicate the presence of an inhibitor affecting the extraction process which eventually loses its potency over time, and also implies that it is not necessary to analyse artefacts immediately, and it may indeed be preferable to wait for up to two months before attempting to extract human DNA.

Human DNA has also been extracted from a sample containing approximately 10 pubic lice faecal pellets, each the size of a grain of rice (Replogle et al., 1994), with both STR markers able to be observed.

PRESERVATION AND ANALYSIS TECHNIQUES

The preservation and DNA analysis techniques used in the recovery of extraneous DNA from insects varies considerably amongst researchers, with few publications reporting on direct comparisons between techniques to determine optimal methods. Therefore, it is difficult to determine which protocol is preferable as the different combinations of preservation technique, storage time, storage medium, DNA extraction technique, target genes or regions, PCR cycles, quantitation methods and genotyping often vary between research studies, and in some instances, not all details are reported.

Presumptive and confirmatory tests

At the preliminary stage of an investigation, an initial screening analysis can be done using forensic tests for the biological fluid of interest, to determine if the insect's food source was blood, semen or saliva. This could prevent unnecessary genotyping of entomological evidence if the insects or artefacts prove to be derived from non-forensically relevant sources. Presumptive and confirmatory tests are used to screen for, and confirm the presence of, human biological material respectively, and can generally be performed both in the laboratory and at a crime scene (Virkler & Lednev, 2009). Presumptive tests are used to perform an initial screening of a stain to determine if a confirmatory test is necessary, as the latter is generally more expensive and labour intensive (Virkler & Lednev, 2009). Presumptive tests for blood, including Hemastix® (Benecke & Barksdale, 2003; Durdle, Mitchell, & van Oorschot, 2015; Fujikawa et al., 2009), Hemident™ (Durdle et al., 2015), Hemascein™ (Durdle et al., 2015), Sangur (Benecke & Barksdale, 2003), luminol (Benecke & Barksdale, 2003), phenolphthalein (Fujikawa et al., 2009), leucocrystal violet (Fujikawa et al., 2009) and fluorescein (Fujikawa et al., 2009) will test positive on artefacts or gut contents that are derived from blood. It must be noted, however, that HemaStix® commonly gives false positive results on fly artefacts derived from non-biological sources (Durdle et al., 2015).

The presumptive acid phosphatase test and confirmatory immunoassays ABAcard® p30 test and RSID™-Semen have been shown to return positive results for semen-derived stains in some instances

(Durdle et al., 2015). Phadebas® and SALIgAE® tests can be used to detect saliva-derived artefacts but the confirmatory RSID™-Saliva test cannot (Durdle et al., 2015). It is also possible to detect the prostate specific antigen in ~~flies from all stages of the lifecycle~~larvae and pupae after larvae have been reared on a food source upon which human semen has been deposited. (Clery, 2001).

While forensic tests are useful in testing whether an insect has fed on human biological material, there are some instances where it would be useful to actually distinguish between fly artefacts and unaltered bloodstains to determine if criminal activity has actually occurred. Crime scenes where blood has been shed often yield information about the sequence and occurrence of events through the analysis of bloodstain patterns (Barbaro, Cormaci, & Barbaro, 2006). Studies have been conducted into the morphology of fly artefacts derived from human blood (Durdle, van Oorschot, et al., 2013; Fujikawa et al., 2009; Rivers & McGregor, 2018; Striman et al., 2011), semen and saliva (Durdle, Mitchell, et al., 2013; Durdle et al., 2011; Durdle et al., 2009), chicken blood (Zuha et al., 2008) and putrefaction fluid from porcine trotters (Kulstein et al., 2015), and all food sources have proven to generate artefacts that bear a strong morphological similarity, in shape, size and colour, to unaltered bloodstains generated by a blood-letting event. Artefacts and bloodstains are even harder to distinguish when deposited on porous substances as porous surfaces cause both fly artefacts and blood to alter shape (Durdle, van Oorschot, et al., 2013; Kulstein et al., 2015). However, it is impossible to compile a definitive and finite collection of fly artefact morphologies as fly artefacts may also vary greatly in colour, shape and size due to the different processes involved in their formation (Benecke & Barksdale, 2003; Durdle, van Oorschot, et al., 2013; Fujikawa et al., 2009; Fujikawa, Barksdale, Higley, & Carter, 2011; Rivers & McGregor, 2018; Striman et al., 2011; Zuha et al., 2008), meaning that it can be very difficult to distinguish between artefacts and unaltered bloodstains by visual assessment only.

Complicating the issue further, modern bloodspatter and entomology texts (Brown et al., 2001; Byrd & Castner, 2001; Englert & Passero, 2010; Wonder, 2001) make some attempt to describe the defining features of artefacts, but these descriptions are incomplete and often contradictory and, as such, can be misleading. For example, one book only describes faecal artefacts as comma-shaped, noting that the tails of such spots are formed when the fly's abdomen moves as it walks and excretes simultaneously (Brown et al., 2001). Another states that confounding fly artefacts are the result of regurgitation only, and describes faecal artefacts as beige and uniform in shape (Wonder, 2001). However, the morphology of faecal and regurgitated artefacts, and the mechanisms behind their deposition, have been shown to be much more varied than this (Benecke & Barksdale, 2003; Durdle, van Oorschot, et al., 2013; Fujikawa et al., 2009; Rivers & McGregor, 2018).

Therefore, if fly artefacts are misidentified as genuine bloodstains, the reconstruction of events can be compromised. However, commonly used forensic tests have been shown to be ineffective in distinguishing between blowfly artefacts and unaltered human biological material (Durdle et al., 2015; Fujikawa et al., 2009; Fujikawa et al., 2011). It has been shown that confirmatory immunoassay tests Hematrace® and Hexagon OBTI are only able to make the distinction on relatively fresh artefacts artefacts but often fail to make the distinction on older artefacts (Durdle et al., 2015). This age effect also applies to semen- and saliva-based fly artefacts, with the presumptive acid phosphatase test and confirmatory immunoassays ABAcard® p30 test and the RSID™-Semen returning negative results for fresh semen-derived artefacts but positive results for older artefacts, and the Phadebas® and SALIgAE® tests being positive for both fresh and older saliva-derived

artefacts (Durdle et al., 2015). The confirmatory RSID™-Saliva test was the only one which could distinguish between saliva-based artefacts of any age, and unaltered saliva (Durdle et al., 2015). It has also been observed that faecal artefacts with tails fluoresce at 465nm when viewed through an orange filter, whereas human blood does not fluoresce at this wavelength (Fujikawa et al., 2011). However, many other biological and non-biological material may also fluoresce a this wavelength, so it is unlikely that this would be a rigorous test to distinguish between artefacts and the unaltered food source (Rivers & Geiman, 2017).

Currently, one research group is investigating enzymes with the aim of devising an immunological assay that will enable the distinction of fly artefacts from human bloodstains (Rivers et al., 2018; Rivers, Acca, Fink, Brogan, & Schoeffield, 2014; D. B. Rivers et al., 2019). They have identified the presence of a pepsin-like enzyme in the crop of the adult fly (Rivers et al., 2014). An antisera has been developed to this enzyme and tested on the artefacts of 31 species from 11 different families (Rivers et al., 2018; D. B. Rivers et al., 2019). The specificity of the test has proven to be high, with no binding with canine, porcine, rat or feline blood (Rivers et al., 2018). The authors report that the anti-serum is able to detect the synthetic peptide created for the development of the assay to a dilution of 1:500,000. While the occasional false negative has occurred, the test shows real promise (Rivers et al., 2018; D. B. Rivers et al., 2019). Strong positive reactions have been obtained from artefacts stored for 3, 5 and 7 years at 25°C. However, the group is yet to test whether chemical alteration of the artefacts through application of cleaning products or extended exposure to environmental conditions such as UV light, might impact on the efficacy of the test, and whether the antisera would be effective on fly artefacts deposited after feeding on human biological material other than blood, or artefacts deposited in the field (Rivers et al., 2018; D. B. Rivers et al., 2019).

It has been argued that artefacts can easily be distinguished by experienced criminalists from genuine bloodspatter due to the inconsistent directionality and colour variation within a pattern (Ristenbatt III, 2019; Shaler, 2012), and therefore a confirmatory test for fly artefacts is unnecessary (Ristenbatt III, 2019). However, this argument does not consider the usefulness of such a test for identifying isolated artefacts that are not part of a pattern (Durdle, van Oorschot, et al., 2013), those which are deposited on porous substrates which alter artefact and bloodstain morphology (Durdle, van Oorschot, et al., 2013), or those which may be derived from semen or saliva which, predominantly being colourless, are even more difficult to distinguish from the source fluid. Given that full human DNA profiles can be derived from even single artefacts and can thus falsely place a person at a crime scene, (Durdle, Mitchell, et al., 2013), it is important that the provenance of the DNA can be confirmed, rather than assumed based on a visual assessment, in much the same way that confirmation of blood is required in order to conclude that a DNA profile has been obtained from that source (D. B Rivers et al., 2019). Furthermore, not all crime scene personnel attending a scene may be experienced in bloodstain pattern analysis, particularly in jurisdictions which are not well resourced.

Preservation

Entomological evidence is often preserved after collection at a crime scene for later examination by a specialist. Historically, the focus has been on preserving the specimens in such a way that the morphological features of the insects important in identifying species and stages of development remain intact. However, with the recognition that forensically relevant DNA may be obtained from

entomological evidence, the preservation technique used must not comprise the integrity of the gut contents. Two studies have directly compared preservation techniques, and made recommendations based on the findings (Table 4). One study determined storage at -70°C in no fluid was optimal, as DNA recovery was high and the morphological features of the specimen were retained (Linville et al., 2004). However, storage in ethanol made dissecting out the crop more difficult as it became fragile (Linville et al., 2004).

Table 4 Studies comparing the impact of various preservation methods on DNA amplification and genotyping.

Species	Time period	Preservation method	Amplification success	Reference
Calliphora vicina maggots	2 weeks, 8 weeks, 6 months	-70°C in no fluid	6/6 mtDNA (1 marker) at 2 and 8 weeks, 3/6 at 6 months; 5/6 STR profiles (8 loci)* at 2 and 8 weeks, 1/6 at 6 months	Linville et al., 2004
		4°C in no fluid	6/6 mtDNA (1 marker) at each time point; 5-6/6 STR profiles (8 loci)* obtained up to 6 months	
		24°C in no fluid	6/6 mtDNA (1 marker) at 2 and 8 weeks, 4/6 at 6 months; 6/6 STR profiles (8 loci)* at 2 weeks, 4/6 at 8 weeks	
		70% ethanol at 24°C	6/6 mtDNA (1 marker) obtained up to 6 months, 2-3/6 STR profiles (8 loci)* obtained up to 6 months	
		95% ethanol at 24°C	6/6 mtDNA (1 marker) obtained up to 6 months, 2-3/6 STR profiles (8 loci)* obtained up to 6 months	
		70% ethanol at 4°C	6/6 mtDNA (1 marker) obtained up to 6 months, 3-4/6 STR profiles (8 loci)* obtained up to 6 months	
		Kahle's solution (30mL 95% ethanol, 12mL formaldehyde, 4mL glacial acetic acid and 60mL water) 24°C	6/6 mtDNA (1 marker) obtained at 2 weeks; 4/6 STR profiles (8 loci)* obtained at two weeks. No DNA detected after 2 weeks.	
		Formaldehyde at 24°C	1/6 mtDNA (1 marker) at 2 and 8 weeks; No STR profiles (8 loci)* generated	
Aedes aegypti mosquitoes	7, 30, 90, 180 days	-20°C	7d – 3/4 mtDNA (1 marker); 30d – 2/4; 90d – 0/4; 180d – 1/4	Reeves et al., 2016
		Desiccation with silica beads at 30°C at 80% humidity	7d – 1/4 mtDNA (1 marker); 30d – 1/4; 90d – 1/4; 180d – 0/4	
		95% ethanol room at 30°C at 80% humidity	7d – 3/4 mtDNA (1 marker); 30d – 3/4; 90d – 3/4; 180d – 2/4	
		FTA card at 30°C at 80% humidity	7d – 4/4 mtDNA (1 marker); 30d – 4/4; 90d – 3/4; 180d – 4/4	

* Completeness of profile not specified

However, it must be noted that researchers have still been able to recover extraneous DNA from entomological evidence preserved using methods that are not necessarily the 'optimal' method. For example, while one comparative study determined desiccation to be a poor preservation method (Reeves et al., 2016), another study demonstrated DNA from mosquitoes which had been killed 30h post feeding was able to be amplified after the mosquitoes were stored as desiccated specimens for 2-7 months at room temperature. Both studies used only the abdomen of the mosquito for analysis, and used 35 cycles during PCR but the DNA extraction techniques and target genes differed. Furthermore, the increasing sensitivity of DNA analysis techniques, the development of kits specifically for degraded DNA and samples containing potential PCR inhibitors, and the increasing availability of next generation sequencing and SNP analysis should overcome a lot of the issues associated with sub-optimal preservation.

Sample preparation

It is important to remove potentially contaminating DNA from the exterior surfaces of samples before DNA analysis, particularly for specimens to be used in criminal investigations (Campobasso et al., 2005; Carvalho et al., 2005). However, decontamination measures must not compromise or degrade the forensically-relevant DNA that may be contained within the specimen. Washing and overnight soaking of maggots in 20% bleach has been found to be the optimal decontamination technique as it breaks down the DNA present on the outside of the insect, but has no impact on the amount of DNA that can be recovered from the gut (Linville & Wells, 2002). However, other techniques have also shown to be effective decontaminants without compromising extraneous DNA recovery, including washing and overnight soaking in distilled water, or treatment with DNase solution (Linville & Wells, 2002). These techniques do not necessarily destroy the contaminating DNA but do sufficiently remove it from the outer surface of the maggot. Overnight soaking may not be necessary, as repeated washing and vortexing of samples in Milli-Q water has also proved to be an effective decontamination technique (Carvalho et al., 2005).

In order to attract the maximum extraneous DNA from the gut of insects, it is important that the gut contents are readily available. Some researchers have achieved this by cutting an incision into the abdomen of the insect to allow it to leak into the sample (Lord et al., 1998; Pilli et al., 2016). Other researchers homogenised or crushed the bed bugs and lice prior to DNA extraction (Mumcuoglu et al., 2004; Raffaele et al., 2015; Replogle et al., 1994; Schal et al., 2018). Intact mosquitoes, or mosquito abdomens (used to maximise DNA concentration), are routinely squashed onto filter paper or tissue (Ansell et al., 2000; Chow-Shaffer et al., 2000; Kreike & Kampfer, 1999), or directly into tubes (Ibrahim et al., 2015; Mehus & Vaughan, 2013; Oshaghi et al., 2006). Analysis of intact insects can lead to complete amplification failure (Mumcuoglu et al., 2004), or in PCR products being visualised as non-specific bands on gels (Chow-Shaffer et al., 2000).

Most researchers use only the crop of the maggot when extracting DNA from the gut. This contains the least digested gut contents, and also maintains the integrity of the external features of the maggot (Campobasso et al., 2005; Linville et al., 2004); . However, the fragility of the crop may depend on the preservation technique used, making it difficult to dissect out intact in some circumstances (Linville et al., 2004). Furthermore, the crop is only useful prior to the maggot entering the post-feeding stage, at which point contents of the crop pass into the mid and hind gut and are eventually excreted (Campobasso et al., 2005). Therefore it is important that maggots selected for analysis are those who have fed for several days but have not entered the post-feeding stage, where possible (Campobasso et al., 2005; Carvalho et al., 2005). As the crop is visible when a maggot is feeding, it should be easy to discern which maggots are suitable for analysis. Ideally, post-feeding maggots will not be analysed intact, as they have a high concentration of lipids (Campobasso et al., 2005) which are known inhibitors of the polymerase chain reaction (PCR). It is also not just the retrieval of DNA that is affected by the use of whole insects. One study that investigated the detection of PSA in entomological evidence noted that PSA could not be detected in whole maggots that had been sonicated, but was able to be detected in crops that had been dissected out of the maggots (Clery, 2001).

Two studies noted that more amplification success was obtained from bloodmeals that were still visibly red as this generally indicated less digestion of the blood meal (Chow-Shaffer et al., 2000; Raffaele et al., 2015). However, it has also been noted that there was no link established between bloodmeal size and success of amplification (Mukabana et al., 2002).

DNA extraction

A number of DNA extraction methods have been utilised to obtain extraneous DNA from insects (Table 5). While Chelex has been used in a number of studies, it is with varying levels of success. In studies which have made direct comparisons between Chelex and other selected methods – phenol/chloroform (organic) (Mumcuoglu et al., 2004), Prepfiler (Marchetti et al., 2013), DNA IQ (salting out method) (Di Luise et al., 2008), and Qiagen™ DNA MicroKit (silica columns) (Di Luise et al., 2008) – Chelex has always proven to be the least effective method, possibly due to less purification steps than other methods (Di Luise et al., 2008; Mumcuoglu et al., 2004). The efficacy of Chelex has been improved by the addition of bovine serum albumin (Replogle et al., 1994).

Table 5 Extraction methods which have been used to successfully amplify extraneous DNA from entomological evidence.

Extraction method	Basis	Species	References
DNA IQ™ System (Promega)	Salting out	Maggots, pupae	Powers et al., 2019
		Fly artefacts	Durdle, Mitchell, et al., 2013; Durdle et al., 2011; Durdle et al., 2009
Chelex® 100 (Bio-Rad)	Resin	Human body and head lice	Mumcuoglu et al., 2004
		Pubic lice	Replogle et al., 1994
		Mosquitoes	Curic et al., 2014
		Maggots	Chavez-Brones et al., 2013; Di Luise et al., 2008
Insta-gene™ Matrix (Bio-Rad)	Chelex – resin	Mosquitoes	Ansell et al., 2000
Organic	Phenol/chloroform	Pubic lice	Lord et al., 1998
		Bed bugs	Schal et al., 2018
		Mosquitoes	Chow-Shaffer et al., 2000; Ibrahim et al., 2015; Mehus & Vaughan, 2013; Mukabana et al., 2002
		Maggots	Carvalho et al., 2005; Chavez-Brones et al., 2013; Zehner et al., 2004
		Fly artefacts	Kulstein et al., 2015
Guanidine/ethanol protocol (Tkach & Pawlowski, 1999)		Mosquitoes	Mehus & Vaughan, 2013
QIAmp DNA Mini Kit (QIAGEN)	Silica membrane	Sandflies	Haouas et al., 2007
		Body lice	Pilli et al., 2016
HotSHOT protocol (Truett et al., 2000)	Sodium hydroxide and Tris	Mosquitoes	Vieira et al., 2017
		Maggots	Wells et al., 2001
DNeasy kit (QIAGEN)	Silicon beads	Kissing bug	Pizarro & Stevens, 2008
		Maggots	Linville et al., 2004
Isopropanol precipitation at room temperature		Bed bugs	Raffaele et al., 2015
UltraClean™ BloodSpin™ purification kit (Mo Bio)	Silica spin column	House flies, German cockroaches, cave crickets	Kester et al., 2010
Prepfiler® (Applied Biosystems)	Salting out	Blowfly puparia	Marchetti et al., 2013

DNA profiling

Amplification and genotyping success will be somewhat dependent on the DNA analysis techniques used, and the type of DNA targeted. To date, both mtDNA and nDNA have been recovered from insects, and each has their advantages. The use of mtDNA and gel electrophoresis is often a faster and cheaper method, with a greater likelihood of success, than STR or next generation sequencing (NGS) analysis. mtDNA analysis may provide sufficient identification to resolve a case, or can be used as a screening test to determine the source of the food in the insect's gut. However, If

individualisation of the DNA is required beyond that which a simple nDNA analysis can provide, mtDNA, STR or SNP sequencing can subsequently be performed.

Mitochondrial DNA

mtDNA is contained within mitochondria organelles, of which there can be hundred or thousands within a cell (Wilson, Stoneking, Hollad, Dizinno, & Budowle, 1993). It is inherited through the maternal line and can be used to link a person to a maternal relative. mtDNA can be particularly useful in instances where tissue is heavily degraded due to its high copy number (Wilson, DiZinno, Polanskey, Replogle, & Budowle, 1995). Many researchers use mtDNA to determine the presence of extraneous DNA in insects, particularly as it is assumed digestive and feeding processes are likely to have degraded any DNA consumed. For human mtDNA, the markers most commonly targeted are Hypervariable Regions 1 (HV1) and 2 (HV2) (S. Anderson et al., 1981; Li et al., 2011; Linville et al., 2004; Lord et al., 1998; Lutz, Weisser, Heizmann, & Pollak, 1996; Sullivan, Hopgood, & Gill, 1992; Vieira et al., 2017; Wells et al., 2001; Zehner et al., 2004). The protein coding genes cytochrome *b* (Kent & Norris, 2005; Kocher, Irwin, & Wilson, 1991; Mehus & Vaughan, 2013; Oshaghi et al., 2006; Zehner, Zimmermann, & Mebs, 1998) and cytochrome oxidase subunit 1 (Mehus & Vaughan, 2013; Reeves et al., 2016) have also been used to detect human and mammalian mtDNA. However, it is not the case that mtDNA will have higher amplification success than STR markers in all instances. For example, in one study, the researchers were able to amplify the HV1 marker for 30/35 bed bugs, but could amplify the STR marker for 32/35 bed bugs (Szalanski et al., 2006).

Human nDNA

A number of nDNA markers have been successfully amplified using gel electrophoresis. For autosomal loci, these include HUMTHO1 (Mukabana et al., 2002; Raffaele et al., 2015; Replogle et al., 1994), D1S80 (Chow-Shaffer et al., 2000; Kreike & Kampfer, 1999; Replogle et al., 1994), TPOX (Raffaele et al., 2015), FIBRA (Raffaele et al., 2015), CSF1PO (Raffaele et al., 2015), ALU (Pizarro & Stevens, 2008; Raffaele et al., 2015), VWA (Mukabana et al., 2002; Raffaele et al., 2015), PNOC gene (Haouas et al., 2007), D12S77 (Ansell et al., 2000), D18S61 (Ansell et al., 2000), D15S127 (Ansell et al., 2000), D7S493 (Ansell et al., 2000), D21S268 (Ansell et al., 2000), HLA-DQα (Kreike & Kampfer, 1999), and for Y-DNA, these include DYS19 (Clery, 2001), DYS389I (Clery, 2001), DYS389II (Clery, 2001), DYS390 (Clery, 2001). A number of commercial Short Tandem Repeats (STR) profiling kits using capillary electrophoresis have also been used (Table 6).

Table 6 Commercial STR profiling kits which have been used to successfully genotype extraneous DNA recovered from entomological evidence.

Commercial kit	Number of loci	Species	Reported profile	Reference
AmpFℓSTR® Profiler Plus® (Applied Biosystems)	10*	Houseflies, German cockroaches and cave crickets	At least one allele	Kester et al., 2010
AmpFℓSTR™ Identifiler™ (Applied Biosystems)	16*	Bed bugs	Partial and full	Schal et al., 2018
		Mosquitoes	Partial and full	Ibrahim et al., 2015
		Maggots	Full and partial	Chavez-Brones et al., 2013
AmpFℓSTR™ MiniFiler™ (Applied Biosystems)	8 miniSTR plus amelogenin	Mosquitoes		Curic et al., 2014
AmpFℓSTR NGM Select (Applied Biosystems)	16*	Body lice	Partial/degraded	Pilli et al., 2016
		Fly puparia	Full	Marchetti et al., 2013

Ion AmpliSeq™ Identify Panel (Life Technologies)	9 markers	Body lice	Full	Pilli et al., 2016
AmpFlSTR® Yfiler™	16 loci	Maggots	Full	Di Luise et al., 2008
STR III Multiplex (Promega)	3 loci	Head and body lice	Full	Mumcuoglu et al., 2004
SNaPshot® (Applied Biosystems)	29 SNPs	Maggots	Full	Kondakci et al., 2009
PowerPlex® 21 System (Promega)	21 loci*	Maggots, pupae	Partial	Powers et al., 2019
AmpFlSTR® Profiler Plus® (Applied Biosystems)	10 loci*	Fly artefacts	Full and partial	Durdle, Mitchell, et al., 2013; Durdle et al., 2011; Durdle et al., 2009

* including amelogenin

Cockroaches, crickets and house flies

DNA exists on surfaces due to transference from primary and/or secondary sources, and may accumulate over time to quantifiable levels (Poy & van Oorschot, 2006; Raymond, van Oorschot, Gunn, Walsh, & Roux, 2009; van Oorschot et al., 2010; Wickenheiser, 2002). This background DNA can even be present in environments where DNA anti-contamination measures are regularly undertaken, such as forensic laboratories (Ballantyne, Poy, & van Oorschot, 2013; Poy & van Oorschot, 2006) and morgues (Schwark, Poetsch, Preusse-Prange, Kamphausen, & von Wurmb-Schwark, 2012). A study investigating the potential recovery of this environmental human DNA from insects sampled from house flies (*Musca domestica*), cockroaches (*Blattella germanica*) and cave crickets (*Ceuthophilus spp*) (Kester et al., 2010). The insects were allowed to forage on indoor surfaces in rooms with varying levels of dust and human traffic. The authors report that the mean amount of human DNA recovered from individual houseflies was 0.028ng, 0.027ng from cockroaches, and 0.011ng from crickets. They also stated that there was no correlation found between the amount of DNA that could be retrieved and the levels of dust or human traffic. However, it is not clear whether the levels of dust and human traffic were assessed contemporaneously as the results pertaining to the successful retrieval of human DNA from the dust samples themselves were actually those from a paper published two years previously. Therefore, it is possible the determination of dust levels may also be taken from that publication and are not an accurate reflection of the level of dust the insects actually foraged on.

IMPLICATIONS FOR FORENSIC SCIENCE

The ability to extract human and mammalian DNA from insects and their excreta has wide-ranging implications for forensic science. In a sense, the insects are assisting in preserving the DNA from environmental degradation and may harbour evidence that criminals do not know exist, and therefore cannot dispose of.

Identification of crime scene

Analysis of DNA extracted from the gut content or artefacts of insects collected from a suspected crime scene where a body is no longer present may prove informative in guiding an investigation. If DNA from the relevant species (eg human or protected wildlife) is obtained from entomological evidence, indicating insects at a particular location were feeding on a body there prior to the body's relocation, a forensic investigation can be triggered (Carvalho et al., 2005; Li et al., 2011; Mukabana et al., 2002; Wells et al., 2001; Zehner et al., 2004). The recovery of extraneous DNA from

necrophagous insects may also be useful in determining if a reportedly missing person is actually deceased (Kulstein et al., 2015), as it is unlikely such insects will feed on the tissue of a living person, except in instances of neglect.

The recovery of human DNA from human head lice (*Pediculus humanus capitis*) was used to assist in the investigation of a case of potential neglect of an elderly person prior to death (Pilli et al., 2016). The suspect claimed she or a carer occupied a particular room at the victim's residence – insects were collected from this room and the bloodmeals of 10 were extracted and pooled for DNA analysis. SNP analysis was successful and the likelihood ratio was calculated as being "5.49×10^{37} more likely the evidence came from the victim rather than from some unknown person belonging to the European population". This supported the theory that the victim had been neglected by the suspect, and a crime had indeed been committed.

Identification of a victim

DNA retrieved from a body in an advanced stage of decomposition, or which has undergone considerable trauma, may be too degraded to generate a DNA profile of sufficient quality to identify the individual. However, there may be sufficient high-quality DNA preserved in entomological evidence that would allow a DNA profile to be obtained, and compared to reference samples collected from items regularly used by the individual, family members or DNA databases (Chavez-Brones et al., 2013; Marchetti et al., 2013).

The first reported use of human DNA extracted from blowfly maggot guts in a forensic investigation was published in 2011 (Li et al., 2011). DNA profiles were sought in order to definitively link a skull to a badly decomposed decapitated body found 500m away. Third instar *Aldrichina grahami* maggots were collected from both the skull and the body, and full 16-loci STR profiles were obtained from their crops. While STR and mtDNA profiles were able to be obtained directly from the human remains, negating the need for the maggot gut evidence, this case gives a clear indication of the quality of samples that can be recovered from entomological evidence collected from badly decomposed corpses.

A 2013 case report described how DNA genotyping of maggot gut contents was also used in a criminal investigation to identify a burnt human body (Chavez-Brones et al., 2013). Due to the state of the corpse, only a small portion of the liver was available for DNA analysis and attempts to obtain a genetic profile were unsuccessful. Three blowfly and flesh fly maggots (*Calliphoridae* and *Sarcophagidae*) that had been retrieved from the body and preserved were subjected to DNA analysis, and a partial profile was able to be obtained, with 12 out of 15 loci, plus amelogenin, able to be genotyped. While a reference profile of the suspected victim was not available, the profile was able to be matched to her father with a probability of paternity calculated to be 99.685%.

Human DNA was also successfully obtained from blowfly pupal casings collected as evidence from two cases (Marchetti et al., 2013). *Lucilia sericata* maggots from multiple developmental stages were collected from bodies in two separate cases and allowed to pupate, with puparia being preserved after seven days. In the first case, approximately 0.1-0.2ng/µL of nDNA was obtained from the puparium extracted, and a full 15-loci plus amelogenin STR profile was obtained. In the second case, 8-10ng of good quality nDNA were obtained from the puparia and full profiles obtained.

Victim identification may also be achieved through the recovery of a dead person's DNA profile from insect artefacts present at a location from which a body has been moved, and at which spilled blood or decomposition products are absent (Durdle, Mitchell, et al., 2013; Durdle et al., 2011; Durdle et al., 2009). In order to be able to target artefacts as a potential source of DNA, it is important to know where they are likely to occur. Currently, the prevailing view conveyed in forensic texts is that artefacts will primarily be found in sun-warmed areas on ceilings and walls due to phototaxis (positive response to light stimuli) (Bevel & Gardner, 2002; Brown et al., 2001; Byrd & Castner, 2001; Rivers & Geiman, 2017). However, some studies contradict this assumption (Durdle, Verdon, Mitchell, & van Oorschot, 2018; Meyer, 1978). While flies are known to be phototaxic (Cornwell, 1955), whether flies move toward or away from the light is dependent on the age of the fly (Meyer, 1978). Two-day-old *Calliphora erythrocephala* blowflies have a tendency to move toward a light source, and are more active in terms of flight, but 3-day-old flies of the same species are more likely to move away from light, and spend more time on the ground and on food sources (Meyer, 1978). It has been observed that the location of artefacts are neither limited to ceilings and walls, nor will they only be concentrated around food or light sources (Durdle et al., 2018). Under some conditions, artefacts may be found in low areas in large number (Durdle et al., 2018).

Identification of assailant

It may be possible to identify an assailant from human DNA recovered from insects or artefacts (Kreike & Kampfer, 1999; Marchetti et al., 2013; Replogle et al., 1994). It is not uncommon for intruders to injure themselves and, subsequently, shed their own blood at a crime scene (Osterburg & Ward, 1992). Consequently, insects may feed on the perpetrator's DNA and retain it in the gut, or deposit it in artefacts. A perpetrator may also deposit their DNA through vomiting or spitting. However, these are less likely to generate DNA profiles due to the low concentration of DNA in vomit (Vandewoestyne, Van Hoofstat, Franseen, Van Nieuwerburgh, & Deforce, 2013), and the demonstrated lack of attraction of flies to saliva (Durdle et al., 2016). Semen, on the other hand, has been shown to be attractive to adult flies, being the preferred, or second-most preferred, food source out of semen, blood, saliva, canned tuna, honey and pet food, depending on the age of the fly (Durdle et al., 2016). This makes it a potentially valuable source of DNA in sexual assault cases. Human sperm present on a corpse will be destroyed as the body decomposes (Di Maio & Di Maio, 1993; Moyer, Rimdusit, & Mishell, 1970). In instances where a sexual assault has occurred prior to the death of a victim, valuable evidence may be lost if the body starts to putrefy before the body is found. However, given the ability of scientists to generate profiles from the juvenile, pupal and adults stages of blowflies, it appears that insects may protect sperm from degradation by consuming it, providing investigators with the capacity to identify the donor (Clery, 2001; Powers et al., 2019). While the donor may be the perpetrator of a sexual assault, sperm may also be present in or on a body due to consensual sexual activity and, if the body is not able to be identified, identification of the sperm donor may provide investigators with an alternative lead.

While blowflies have been shown to have a high affinity to semen as a food source, a high mortality rate in adult *Lucilia cuprina* blowflies feeding on semen has been reported, and flies have been observed to appear to suffer paralysis in some legs after consuming semen (Durdle et al., 2015). It is possible the detrimental impact of semen on flies may be due to the high DNA content of semen (Butler, 2005), as guanylic acid, a component of DNA, is known to be toxic to many organisms, including fly maggots (Burnet & Sang, 1963) and chick embryos (Karnofsky & Lacon, 1961). This

means that deceased flies in the vicinity of a deceased victim of suspected sexual assault may contain the DNA of the assailant.

However, there is an indication from laboratory studies that semen on tissue is not an ideal laying environment for blowflies. A cohort of *Calliphora augur* flies that had fed on semen as maggots, showed wing and abdomen deformities in 10% of cases (Powers et al., 2019). Furthermore, low rates of egg hatching after maggots had fed on semen were also observed, with 80% of *C. augur* eggs and 50% of *Calliphora stygia* eggs failing to hatch, and *C. augur*, a facultatively larviporous species, laying both eggs and maggots, indicating that human tissue bearing semen may not be an ideal laying environment. This raises the question of whether flies attracted to body orifices bearing semen would actually ovi- or larviposit there, and consequently, how likely it would be that semen-based entomological evidence from maggots feeding on semen could be located.

Linking person to person, or person to location

Several studies have identified DNA from multiple individuals (both human and mammalian) in haemophagous insects (Ansell et al., 2000; Chow-Shaffer et al., 2000; Kent & Norris, 2005; Kreike & Kampfer, 1999; Mukabana et al., 2002). This provides a unique way to potentially link an offender and a victim if an insect has consumed blood from both people in temporal proximity (Trajer, 2018).

As pubic lice are transferred by skin-to-skin contact (Campobasso et al., 2005), and head and body lice transferred by close physical contact (Mumcuoglu et al., 2004), the finding of another human's DNA in the gut of lice recovered from a victim, or in the vicinity of the victim, would provide a very strong connection between those individuals (Campobasso et al., 2005; Mumcuoglu et al., 2004). Alternatively, insects such as mosquitoes and sandflies may contain bloodmeals from multiple individuals in the gut at any given time, and given these insects don't travel far between feeds, a link may be established between those individuals (Haouas et al., 2007; Trajer, 2018). There are a number of lice for which humans are hosts. Pubic crab lice (*Pthirus pubis*) live predominantly in pubic hair, and occasionally may also be found in other coarse hairs such as eyelashes (Turgut, Kurt, Catak, & Demir, 2009; Wu, Zhang, & Sun, 2017). The lice rarely leave the body of the host and transfer between individuals is facilitated by body-to-body contact, often during sexual activity (Mumcuoglu et al., 2004).

It is also possible for DNA obtained from an insect to provide a link between a person and a location (Szalanski et al., 2006), particularly those insects that are not obligate feeders on one type of host. For example, if an insect that has be transferred to a crime scene by an offender contains DNA from a dog, this may be an indication there was a pet at the location where the offender lived. Similarly, a dumped body may contain insects with DNA from other animals, providing an investigative lead. The extracted DNA may even be able to link the insect to a specific animal, if sufficient DNA is available for sequencing.

Bed bugs are insects which feed on blood at all life stages (Schal et al., 2018), and have a propensity to aggregate close to sleeping or resting areas (Booth et al., 2012). In lower-income housing, bed bugs have been observed at a population density of hundreds per square metre (Raffaele et al., 2015). Furthermore, they are wingless and unlikely to walk long distance so any movement of a bed bug will likely be due to translocation via human-mediated transport (Schal et al., 2018). However, they do not stay on the host after feeding (Szalanski et al., 2006). Given this, they may be an

excellent source of DNA at a location where a hostage or a kidnap victim was held, prior to relocation, as they will be easy to find at a suspected scene *en masse*, with a high probability that any recovered insects will have consumed a bloodmeal, regardless of developmental stage. Compared to some other insects, the size of a blood meal consumed by a bed bug is relatively large (Daba, Daba, Shehata, & El Sawaf, 2004; Ogunrinade, 1980; Sierras & Schal, 2017). It must be noted that while bed bugs could be useful in linking a person to a location, their propensity to feed on only one host per meal means there is only a small chance of the gut contents of a bed bug linking a person to another person (Raffaele et al., 2015; Schal et al., 2018).

The first reported use of human DNA extracted from a mosquito bloodmeal stain in a criminal investigation was published in 2006, and the evidence provided a strong link between a location and a victim (Spitaleri et al., 2006). In this instance, a murder victim was found partially hidden in bushes near a beach but inquiries established the murder may have occurred in a suspect's house some distance from the location of the body. The only potential biological evidence available was a dried bloodmeal stain from a squashed mosquito found on an interior wall of the home. An almost complete 15-loci STR profile was able to be obtained, and this matched that of the victim. It was noted that the mosquito was *Culex pipiens*, and that this species is not known to traverse distances as great as that between the location of the victim and the suspect's house under normal conditions, minimising the possibility that the presence of the victim's DNA in the suspect's house was purely coincidental.

Insects may also be used to link a person to a location where a violent crime has not occurred, and human blood, semen or a body is not, or has not, been present. For example, people may be linked to a location where criminals have consorted pre- or post-commission of a crime, or a victim of a kidnapping was held (Oliveira-Costa, 2011). This is possible due to the capacity for human DNA to be retrieved after insects have fed on environmental DNA, likely predominantly comprising shed epithelial cells (Kester et al., 2010). Analysing the gut content of insects may allow for a more efficient manner of testing for DNA in a location than swabbing of areas that investigators believe offenders may have touched (Kester et al., 2010). Furthermore, due to the possible presence of DNA from more than one individual in environmental DNA, it may be possible to link a person to another person. However, it must be noted that for environmental DNA to be useful in a forensic investigation, the investigator would need to know when surfaces had last been cleaned, as the insects may have consumed cellular material from people who had been present in the vicinity well before any criminal activity had taken place.

Transfer of DNA via insect vectors

Given that insects can be mobile vectors of extraneous DNA, it is possible that they may transfer human DNA from one location to another. Insects vary in terms of their ability to traverse great distances under their own power. While insects such as bed bugs and lice will remain close to a host (Campobasso et al., 2005; Mumcuoglu et al., 2004; Schal et al., 2018), and others such as certain mosquito species disperse only short distances (Costero et al., 1998), some can travel several kilometres. For example, *Lucilia* blowflies have the capacity to fly 3.2 km before exhaustion (Hocking, 1953) and other forensically relevant blowflies, such as *Phormia regina*, travel much further than *Lucilia* species (Cragg & Hobart, 1955). The dispersal rate of *Aedes* mosquitoes is 0.15 to 1.5km per day but this can be increased tenfold in strong winds (Trajer, 2018). Furthermore, it is also possible

that insects may be mechanically transported via assisted travel (e.g. in a car or aeroplane) or wind-assisted dispersal, meaning they may transfer DNA a considerable distance from its original source.

Given the time it takes for an insect to digest a meal depends on a number of factors such as ambient temperature, concentration and type of food, and level of hunger (Gelperin, 1966), there may be ample time for an insect to transfer DNA to a location distant from where it consumed biological material. The rate of digestion in a mosquito is dependent on the environment – in tropical species, digestion will take 2-3 days, but will take longer in moderate climate species, and it can take up to 80 hours before a fly excretes a meal (Bowdan & Dethier, 1986; Kerkut & Glibert, 1985). It is therefore feasible that an insect may leave one building after feeding on human biological fluids and enter another building, possible depositing an artefact, being killed or collected in the second location.

Even the ability for insects to travel short distances can have forensic implications, and in some instances, this transfer of DNA can be advantageous. For example, fly artefacts can be targeted at crime scenes where, for example, the perpetrator has cleaned the areas in which they have been present, being unaware that insects may have transferred their DNA to areas within the scene where they haven't been (Durdle, Mitchell, et al., 2013).

The capacity for insects to transfer DNA can also lead to contamination events, and may confound criminal investigations and/or falsely incriminate or exclude a person from involvement in a crime. It has been demonstrated that flies may move around a building due to both phototaxis, and to search for food, particularly in climatic conditions during which they are most active (Durdle et al., 2018). Consequently, they may deposit artefacts *en masse* in an area away from the food source. Given it can be difficult to distinguish fly artefacts and bloodspatter patterns, due to their similar morphology (Benecke & Barksdale, 2003; Durdle, van Oorschot, et al., 2013; Fujikawa et al., 2009; Rivers & McGregor, 2018; Striman et al., 2011; Zuha et al., 2008), and the current lack of a confirmatory test to definitively identify fly artefacts, this may cause problems for investigators who confuse a mass of fly artefacts for impact spatter or other unaltered bloodspatter patterns. The misidentification of artefacts as genuine bloodspatter may contradict the version of events according to the victim, suspect or witnesses. For example, a person may deny having been in a certain area of the crime scene. If a fly artefact found in that area is mistaken for a genuine spot of biological material, and the DNA profile subsequently derived from it is found to be consistent with the DNA profile of the person in question, investigators may not believe their account of the incident. In reality, an insect may have fed off the person's bodily fluids and subsequently regurgitated or excreted in an area the individual had not in fact visited.

It is important to note that the deposition of artefacts may continue for some time after the fluid has become available at the scene because flies will continue to be attracted to it, regardless of whether the fluid is wet or dry (Durdle et al., 2016; Kulstein et al., 2015). Deposition of artefacts may also occur after the food source has been removed, either through cleaning up by an offender or complete consumption by flies. While flies of different ages and sexes may vary in their feeding patterns and attraction in regard to semen or blood (Durdle et al., 2016), flies at a crime scene will be of both sexes and of varied ages and it can be expected that there would be no specific periods where all the flies present will, or will not, be feeding on the biological fluid. Due to this behaviour, it

is necessary to immediately disperse any flies present at a crime scene, to ensure they don't continue to transfer DNA after discovery of the scene.

Additionally, investigators may sample a single drop of blood that does not appear to be part of a pattern originating from a victim in the hope that it either contains DNA from the offender or provides information to assist with the reconstruction of events (Durdle, van Oorschot, et al., 2013). Given that full human DNA profiles may be derived from single fly artefacts (Durdle, Mitchell, et al., 2013; Durdle et al., 2011; Durdle et al., 2009), confusing a fly artefact with a blood spot could prove problematic, especially if the artefact was brought into the scene from an unrelated biological source.

If an insect were to travel a considerable distance, albeit assisted, there is the possibility that an innocent person may have their DNA transferred to a crime scene, implicating them in activity in which they were not actually involved. Given full human DNA profiles can be obtained from fly artefacts up to 2 years after deposition (Durdle, van Oorschot, et al., 2013), the contaminating artefact may actually have been deposited years earlier, and may contain DNA of a former resident or other person. While aged mosquito stains have not been tested, it is quite probable that these too could cause contamination years after the stain has been formed.

The finding of human DNA in insects and their artefacts is not only forensically significant at crime scenes, but also in forensic laboratories. For artefacts to constitute a risk, DNA would need to be transferred via the insect to an evidentiary item in an amount sufficient to interfere with the interpretation of the DNA profile derived from the evidence – that is, in an amount that is above the level of detection by the genotyping method used and sufficient to constitute a detectable minor component. The level of DNA transferred would depend on the amount of DNA present in the artefact, the number of artefacts transferred, the physical nature of the artefacts e.g. wet or dry and the manner of transfer (Goray, Eken, et al., 2010). If, for example, a fly has left artefacts on an examination bench and the examiner inadvertently touches these, and then the evidentiary item, there is potential for any profile collected from this item to be compromised if there was sufficient DNA in the initially deposited fly artefacts. Alternatively, it is feasible that an insect in a forensic laboratory could feed on blood or semen on evidentiary items left unattended and exposed in one area for an extended period of time and later excrete or regurgitate on surfaces or evidence (from the same or other cases) in other areas within the laboratory. Some semen- and semen/saliva-based artefacts contain amounts of DNA that could prove problematic even when more complex levels of transfer occur (Durdle et al., 2009). Furthermore, single or low numbers of artefacts could contribute to the accumulation of background DNA on items and structures. The human DNA contained in the artefacts could then be passed on, along with other extraneous DNA, to an evidentiary item through secondary or tertiary transfer. Additionally, since it has been shown that DNA can be extracted from insects which have consumed environmental DNA (Kester et al., 2010), it is not impossible that they could transfer sufficient quantities of this DNA to constitute a contamination risk by depositing artefacts on exposed evidentiary items.

Artefacts, and any extraneous DNA within, may be further transferred, subsequent to deposition. Certain aspects of fly artefact morphology make the possibility of this occurring more likely. For example, pools of fly regurgitant can remain moist for several days after deposition and dried artefacts on non-porous surfaces can be easily detached and may be fractured under light pressure

(Durdle, van Oorschot, et al., 2013). Therefore, secondary or tertiary transfer of the DNA in the artefact upon contact with another surface may occur.

While there has been no studies to date specifically investigating the DNA content of blood or other bodily fluids transferred via contact with the external surfaces of insects such as their tarsi or abdomens, it must be noted that this is still a viable mechanism by which DNA may be transferred. DNA in these artefacts will not have been subjected to digestive degradation as the bodily fluid will be transferred in its original form. This form of insect-mediated DNA transfer is most likely to result in artefacts in close proximity to the body fluid source. However, it is possible an insect may acquire the body fluid on its outer surface and then fly to a different location and deposit the fluid on a surface distant to the source. For this to occur, there would need to be sufficient fluid on the insect that it would remain wet until it reached the secondary location, or that the insect has acquired sufficient fluid that this flakes after drying. Alternatively, the insect may pick up a flake of already dried bodily fluid and transfer this to a secondary location. -

Confirming relevance of entomological evidence

Insects can provide very useful information in regards to the PMI of a corpse, but to ensure that the estimated PMI is valid, the investigator must be confident that the entomological evidence used in the calculations is derived from the body of interest. However, it has been shown that the presence of blood or a body at a crime scene does not mean blowflies will preferentially feed on these, but may feed on other biological material if it is present and openly available (Durdle et al., 2016). Indeed, other human food sources may be preferable, and consequently, investigators must consider whether any DNA obtained from an insect or its artefacts is actually related to the commission of the crime or whether it originated from an innocuous source. Furthermore, insects at a crime scene may not feed at all. Variables which influence the likelihood of a fly feeding include the length of time since the fly's last meal (Waterhouse, 1957), fullness of its crop (Bowdan & Dethier, 1986; Simpson, Barton Browne, & van Gerwen, 1989), how much water it has consumed (Dethier, Solomon, & Turner, 1965), food taste and concentration (Bowdan & Dethier, 1986; Hasset et al., 1950; Simpson et al., 1989), type of food (Dethier & Bowdan, 1992; Hasset et al., 1950), gender (Dethier & Chadwick, 1948) and environmental factors such as temperature and humidity (Dethier & Chadwick, 1948). One variable that does not influence the response of flies to food is nutritional value (Dethier, Evans, & Rhoades, 1956; Hasset et al., 1950). Therefore, it is necessary to detect forensically-relevant DNA in the insects' guts so the insects can be confidently used to assist in the determination of PMI or other investigative analyses (Carvalho et al., 2005; Kondakci et al., 2009; Li et al., 2011; Wells et al., 2001; Zehner et al., 2004). However, if amplification of human DNA fails, which may occur for a variety of reasons, species-specific analysis can be performed to determine if the maggot has actually been feeding on an alternative animal by targeting markers such as the cytochrome b gene (Barallon, 1998; Bataille, Crainic, Leterreux, Durigon, & de Mazancourt, 1999; Kent & Norris, 2005; Zehner et al., 2004; Zehner et al., 1998), or utilising other species-specific primers (Carvalho et al., 2005).

It has been shown that if an insect moves to a second food source after feeding on a human food source, it may be difficult to obtain a human STR profile (Njau et al., 2016). However, this may also be informative in terms of determining how long ago the maggot was feeding on the human body – if it can still be detected, the feeding may have been a very recent event.

Another factor to consider when determining the relevance of entomological evidence in relation to a specific crime scene, is whether the evidence could be present due to coincidental circumstances, or whether the context means the likelihood of coincidence is sufficiently low as to make it improbable. For example, consideration must be given to whether an insect has the capacity to travel the distance between the biological source on which it fed, and where it, or its artefacts, was collected. If this distance is outside its usual dispersal range, the probability that any DNA transfer was adventitious is much less likely than if the distance was within that which the insect could reasonably travel (Spitaleri et al., 2006).

Conclusion

The ability to retrieve human and mammalian DNA from insects has wide-reaching forensic implications in terms of both contamination and as a potential source of DNA to be targeted. The utility of insects as vectors of DNA has been demonstrated through their occasional use in forensic investigations but increasing the awareness of this growing branch of forensic entomology would be of benefit to investigators. To fully understand the extent to which insect-facilitated transfer may affect forensic investigations, research must be conducted on the effect or influence of a number of variables. The demonstrated variability in amplification and genotyping success highlights the need to isolate which components in insects and/or their artefacts are affecting the extraction and amplification efficiency, and how these effects can be mitigated. It would also be extremely useful for more comparative studies to ascertain the optimal sampling, preservation, extraction, amplification and genotyping methods, with the ultimate aim of developing a recommended protocol that maximises DNA recovery but also maintains necessary morphological features so as not interfere with other entomological analyses, such as species identification.

Further research into the morphology of insect artefacts, particularly those deposited by insects yet to be tested, would be informative, but it is not necessary to continue to test all species of a given insect. It has already been established that it would be impossible to catalogue all possible morphologies of artefacts and the development of a test to conclusively identify artefacts, in particular, would be of more benefit. To this end, the immunoassay currently under development (Rivers et al., 2018; D. B. Rivers et al., 2019) is a strong start in this direction.

The level of forensically relevant information that can be gained from insects, and conversely, the risk of contamination from insect-facilitated DNA transfer, will increase as DNA detection techniques continue to become more sensitive and DNA recovery from insects and their artefacts more achievable. To this end, it is important that crime investigators and scientists are made more aware of insects and their relevance as vectors of biological material and DNA in a forensic context.

References