HOW THE ALTITUDE AFFECTS THE CALLIPHORIDAE COMMUNITY DURING A CORPSE DECOMPOSITION

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Studying the sarcosaprophagous community is important for forensic practice since it can provide useful information in different aspects concerning death. Such community is affected by different environmental variables enhancing the need of studying it in different microclimatic environments, even if the different sites are close to each other. Seasonality is a factor to be considered because sarcosaprophagous community would vary through decomposition process, the community characteristics significantly varying across seasons

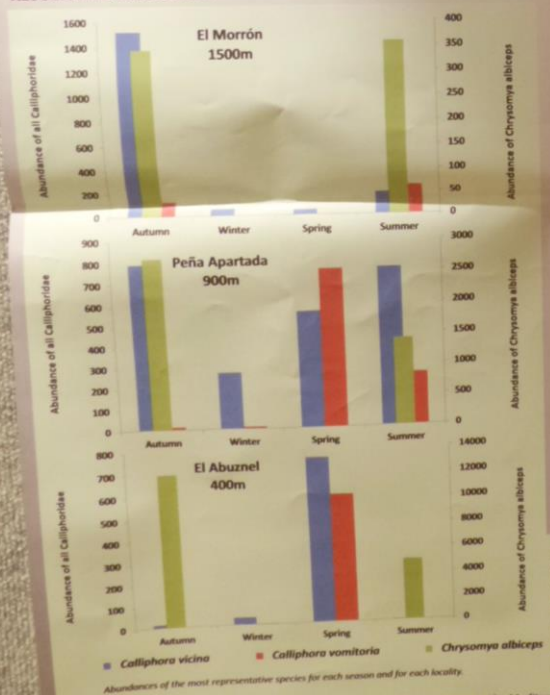
The most important group to be considered for forensic purposes is the necrophagous, mainly Diptera, some of which are known to be the first arthropods to access the corpse. Among them, Calliphoridae is the most important family, being the best indicators of postmortem interval and usually the first to reach the corpses. Samples were taken daily for the first two weeks from corpse exposition. The experiment was replicated at each altitude for the four seasons of the year, between autumn 2006 and summer 2007. The succession took different times depending on the altitude. While no significant differences in the faunal composition were found, some differences concerning the decomposition rates and the Calliphoridae community behaviour have been detected

METHODS

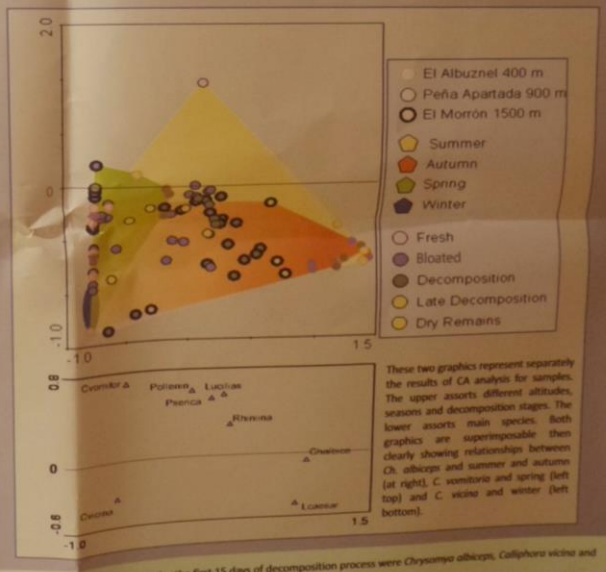
The Calliphoridae community was studied at three sites at different altitudes "El Abuznel" (400m), "Peña Apartada" (900m) and "El Morrón" (1500m) in the Regional Park of Sierra Espuña, one of the most important natural areas of the region located in the central area of the Murcia province.

Sampling was performed using a Schoenly trap (Schoenly *et al.*, 1991) of 60 cm high x 70 cm wide x 70 cm long as capture device, which is designed to collect both the arthropodian fauna accessing to a corpse acting as bait and that developing on it. The trap allows a continuous collection not depending on the collector. Thus, results obtained are comparable with others obtained with the same method (Schoenly *et al.*, 1991). A 5 Kg piglet (*Sus scrofa* L.) was selected as bait. Samples were taken daily for the first two weeks from corpse exposition. The experience was replicated at each altitude for the four seasons of the year. A PERMANOVA analysis, with Primer 6.0, was applied to check differences between the three altitudes. A Pairwaise test was then applied to show which are the differences in each decomposition stage. Finally a CA was performed to show how the different samples are arranged according decomposition stages, season and locality.

RESULTS AND DISCUSSION



Abundances of the most representative species for each season and for each locality.



These two graphics represent separately the results of CA analysis for samples. The upper assorts different altitudes, seasons and decomposition stages. The lower assorts main species. Both graphics are superimposable then clearly showing relationships between *Ch. albiceps* and summer and autumn (at right), *C. vomitoria* and spring (left top) and *C. vicina* and winter (left bottom).

The three most abundant species for the first 15 days of decomposition process were *Chrysomya albiceps*, *Calliphora vicina* and *Calliphora vomitoria*.

An altitudinal gradient can be observed; abundance of *Ch. albiceps* decreases with increasing altitude and the opposite for *C. vicina*, while *C. vomitoria* seems to have its upper altitudinal comfort at medium altitude. As seen above (CA results), seasonality also seems to affect the species abundance. Thus, *Ch. albiceps* mainly appears in summer and autumn, *C. vicina* is present all along the year but showing differential abundance depending the season and the particular locality and *C. vomitoria* seems to prefer spring, and summer if not too warm.

Comparing these results with those proceeding from an periurban site in the same region (Arnaldos et al., 2001) or close coastal island areas (Martínez Sánchez, 2003), it is worthy of note that the most representative species for these two environments, *Lucilia sericata*, was very scarce in all three localities of Sierra Espuña.

While *L. sericata* was related to spring in the periurban environment as well as in the Mediterranean coastal islands (Martínez Sánchez, 2003), it was *Ch. albiceps* that related to this season in the wild area except at the top mountain, where spring was too cold.

Nevertheless, if seasonally considered, some of our results match with some of the above as well as other proceeding of other areas from the Iberian Peninsula (Prado e Castro et al., 2012), especially in autumn and winter, due to *Ch. albiceps* in autumn and low diversity with special abundance of *C. vicina* in winter.

CONCLUSIONS

- Sarcosaprophagous Diptera community has been shown to be affected by altitude (species preferences and their own phenology on the one hand and environmental variables associated to altitude on the other hand) thus enhancing the need to study this community in different microclimatic environments even close to each other.
- There is a clear altitudinal and seasonal specific replacement in the studied wild mountainous localities the same as compared to other close but different environments.
- Our results show the importance of these taxa as seasonal, altitudinal and even environmental indicators or potential interest for forensic purposes.

NEW DATA ON LIFE CYCLE LENGTH OF *HYDROTAEA CAPENSIS* (DIPTERA: MUSCIDAE) BRED IN LABORATORY CONDITIONS

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Estimation of time from infestation, in cases of human or animal myiasis, or death, in criminal cases, on the basis of the life cycle development is one of the goals of Forensic Entomology. Some efforts have been made to detail developmental data of different Dipteran species, mainly those usually reported from forensic cases

Family Muscidae is an important group belonging to the sarcosaprophagous fauna due to its wide distribution and its synanthropic habits. Their females usually lay eggs on the natural openings of a corpse, wounds or clothes moisted by cadaveric fluids. *Hydrotaea capensis* (Wiedemann, 1818) is a cosmopolitan hemi or eusynanthropic species (Gregor et al., 2002) often found in the Mediterranean area. It can belong to the sarcosaprophagous fauna; some data point to its frequent presence in forensic cases (Lefevbre & Pasquerault, 2004). Despite such potential applied interest, its biology is still poorly understood. Lefevbre & Pasquerault (2004) tried to study its life cycle characteristics at different temperatures but did not achieve any conclusive data about the duration of each larval stage.

Hydrotaea capensis A. Male, B. Female

METHODS

The life cycle characteristics of this species have been studied using laboratory colonies bred from specimens collected from a forensic case in Murcia provice.

Flies were hold in an insectary at four thermal regimes, 18, 20, 25 and 30ºC with 50-60% RH and a photoperiod of 12:12 (light:dark) hours. Adults were fed with water and sugar *ad libitum*. Pig liver was supplied as laying substrate and larval food. The first larvae were collected within 2 h from larviposition. Five of the largest maggots were removed from the breeding cages every 12 h, with the exception of the first 12 hours in which samples were taken every 6 hours. Four replicates for each thermal regime were considered.

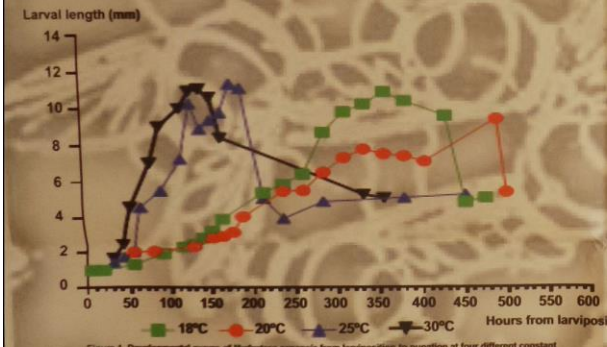

Figure 1. Developmental curves of *Hydrotaea capensis* from larviposition to pupation at four different constant temperatures

RESULTS

This contribution provides the first data about the duration of each preimaginal stage at four thermal regimes of *Hydrotaea capensis* (Fig. 1 and Tables 1,2,3). It also presents developmental data of southeastern Iberian populations of this species.

	Thermal regime			
Stage	18°C	20°C	25°C	30°C
LI	96±0 h	39±6 h	54±12 h	18±0 h
LII	116±13,85 h	112±13,85 h	30±12 h	39±6 h
LIII	Failed pupation	204±13,85 h	129±35,83h	75±6 h
Pupae	—	—	165±6h	184±13,85 h

Table 1. Duration of preimaginal stages of *Hydrotaea capensis* under four temperature regimes, * mean ± S.D

	Thermal regime							
	18°C		20°C		25°C		30°C	
	hours	days	hours	days	hours	days	hours	days
Pupae	-	-	450	18,75	210	8,75	162	6,7
Adult	-	-	–	–	378	15,75	318	13,25

Table 2. Moment of appearance of first pupae and adult.

	Thermal regime	Duration of larval development	Duration of total development
Spanish population	18ºC	Failed pupation	–
	20ºC	14,79	–
	25ºC	8,87	15,75
	30ºC	5,5	13,16
French population (Lefebvre & Pasquerault, 2004)	17ºC	37,9	62,0
	24ºC	13,3	21,1
	30ºC	7	12,5

Table 3. Average of duration of larval and total development at 18°C, 20°C, 25°C and 30°C (in days).

At 18º C, in our study, larvae III failed to pupate, died and dried without this could be due to the breeding conditions. At 20ºC, although they reached pupae stage, didn´t complete their life cycle because they never reached the adult stage. At 25ºC and 30ºC the development took place normally. The more developmental temperature increased, the less duration of each of the larval stages decreased with exception LI duration at 25ºC (Table 1, 2). The fastest development occurred at 30ºC, followed by that at 25ºC. Both were quite similar, showing a rapid growth followed by a sharp reduction in size before the pupation started (Fig. 1), so fitting the usual pattern of other Diptera of forensic interest (Grassberger & Reiter, 2002). These results are consistent with those obtained for the total duration preimaginal and complete development (Table 2).

Comparing our data with only previous data (Lefebvre & Pasquerault, 2004), it is worthy to note that significant differences were found in this species for minimal duration of both larval and total development (until adult emergence) between our data and those from literature (Table 3). There are important differences in the development at lower temperatures; in our study at 18 and 20ºC this species never reached the adult stage and duration of larval development was significantly lower than obtained for French populations of this species at 17°C. However, when increasing temperature, values of duration of larval and total development for both populations approached, although they are always lower for the Spanish population. Thus, our results suggest a gradient of geographical variation among *H. capensis* populations.

All these results show that, as we remarked in other studies, it is absolutely essential a standardization of procedures for developmental studies and presentation of data in order to compare results from different populations. This would increase the utility of these studies for forensic practice. However, larger differences in developmental times do not necessarily have to be attributed to variation in experimental method. As Grassberger & Reiter (2002) remarked, geographic adaptations (intrinsic factors) could explain a difference in temperature-dependent development. The ideal experiment to clarify this issue would be carried out rearing specimens from different geographic regions under the same experimental conditions.

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THE IMPACT OF PARACETAMOL ON THE GROWTH RATE OF TWO SPECIES OF DIPTERA CALLIPHORIDAE (*Calliphora vicina* AND *Lucilia sericata*)

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Introduction

Forensic entomology mainly known to be used in the estimation of Post-Mortem intervals (PMIs). However, it is also used in different areas as the post-mortem transfer (Vanin et al., 2008) child neglect (Benecke and Lessig, 2001) and elders neglect (Benecke et al., 2004). Toxicological analysis is another area where insects can be used as samples for toxicological analysis in the absence of traditional sources (body fluids and internal organs normally taken for such purposes). Goff et al. (1994) stated that due to the fact that drugs and toxins in corpses and carcasses may be accumulated and stored in insects feeding on the contaminated tissues. These chemicals can be detected in the insects long time after the material they have been feeding on cannot be analysed anymore. Introna et al. (2001) stated that entomotoxicology which first appeared in the late 1970s, does not deal just with detection and determination of toxins and drugs in dead bodies, and claimed that it also investigates the impacts of drugs and toxins on arthropods development in order to assist the forensic PMI estimations. To our days it has been widely demonstrated that drugs and toxins had an impact on the insects growth rates which would certainly lead to wrong estimation of the PMI if not taken in consideration.

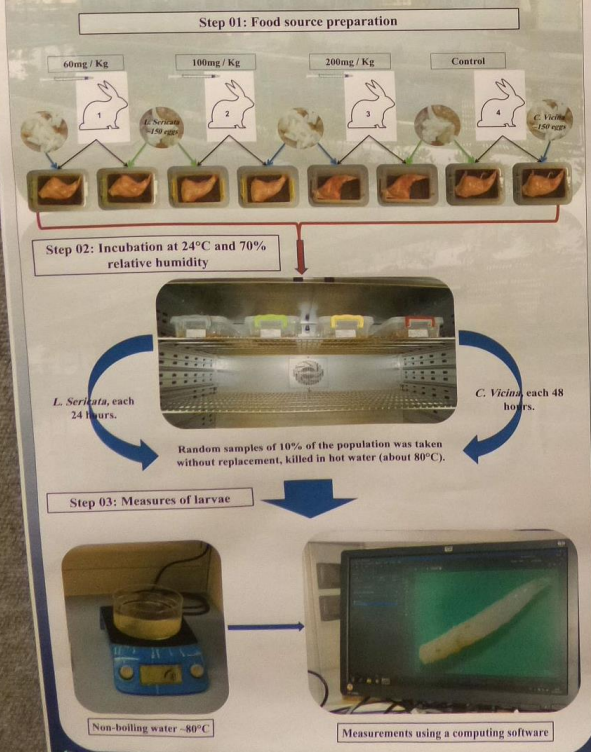
In this study we investigated the impact of paracetamol on the growth rate of *Lucilia sericata* (Meigen, 1826) (Diptera, Calliphoridae) and *Calliphora vicina* (Robineau-Desvoidy, 1830) (Diptera, Calliphoridae). In order to assess the impact of the drug on the growth rate of the target species, two parameters were assessed: length of the maggots in different periods (each 24 hours for *L. sericata* and 48 hours for *C. vicina*), and duration of the life cycle.

Method

Males and females of the two species were collected at the National Institute of Criminalistics and Criminology's campus in Bouchaoui, Algeria (36° 44' 0" N, 2° 55' 0" E).

The foodstuff was prepared; four rabbits (albinos) of the same sex (all males) and approximately of the same weight were injected through the marginal ear veins with different paracetamol doses (dose 01: 60 mg/kg, dose 02: 100 mg/kg, and dose 03: 200 mg/kg) every four hours during the day time (at 08:00, 12:00, and 16:00). A fourth rabbit was dedicated to serve as a control (00 mg/kg). Three days later, the rabbits were sacrificed 20 minutes after their last injection. Meanwhile, the insects were stimulated for oviposition.

A random sampling of 10% of the population was performed, larvae killed in hot water (about 80°C), and measured. The appearance of the first pupae (3 to 4) were considered as the end of the sampling processes and the appearance of the first adults (more than 3) was considered as the end of the life cycle.



Results

The results of the length measurement, for both species, showed that the higher the drug's dose in the substrate was, the bigger the maggots were compared to the controls. This was clearly observed during the first 72 hours of the maggots' life. During the estimated PMI calculation, it is observed that the blowflies' life cycle was not affected except for *L. sericata* larvae that have been feeding on paracetamol's dose 01 and 02, which extended the life cycle of the species for approximately 17 hours, that is clearly observed through the graphs (Fig. 3 and 4).

C. vicina

Tab. 01 and 02 Tables of the average size of larvae and pupae for the different species.

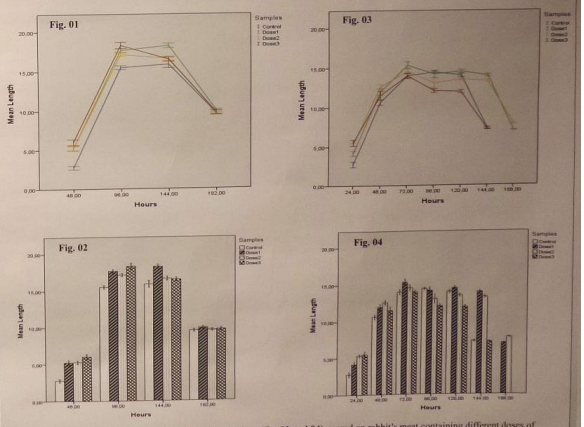
Tab. 01 Average size (mm)

Time (hours)	Control 00mg / Kg	Dose 1 60mg / Kg	Dose 2 100mg / Kg	Dose 3 200mg / Kg
48	2,76 ± 0,43	5,22 ± 0,58	5,25 ± 0,43	6,01 ± 0,64
96	15,49 ± 0,42	17,64 ± 0,41	17,11 ± 0,36	18,28 ± 0,70
144	15,87 ± 0,72	18,24 ± 0,45	16,61 ± 0,43	16,51 ± 0,41
192	9,58 ± 0,31	9,94 ± 0,28	9,62 ± 0,24	9,73 ± 0,33

L. sericata

Tab. 02 Average size (mm)

Time (hours)	Control 00mg / Kg	Dose 1 60mg / Kg	Dose 2 100mg / Kg	Dose 3 200mg / Kg
24	2,75 ± 0,64	4,16 ± 0,58	5,33 ± 0,38	5,52 ± 0,73
48	10,62 ± 0,57	11,97 ± 0,72	12,58 ± 0,55	11,50 ± 0,92
72	13,97 ± 0,55	15,32 ± 0,70	14,56 ± 0,60	13,99 ± 0,41
96	14,42 ± 0,25	14,18 ± 0,70	13,03 ± 0,93	12,05 ± 0,36
120	14,00 ± 0,32	14,49 ± 0,30	13,51 ± 0,27	11,92 ± 0,31
144	7,28 ± 0,18	13,97 ± 0,25	13,27 ± 0,24	7,18 ± 0,15



Figs. Length of *C. vicina* (**fig. 01 and 02**) and *L. sericata* (**fig. 03 and 04**) reared on rabbit's meat containing different doses of paracetamol compared to the control.

Tab. 03 and 04 Tables of p-Values by Scheffe's multiple comparison Post-hoc tests for the different species.

Tab. 03 : *Calliphora vicina*

p-Value (Significance level 5%)	Control	Dose 1	Dose 2
Dose 1	0.64		
Dose 2	0.34	0.04	
Dose 3	0.62	0	0.04

Tab. 04 : *Lucilia sericata*

p-Value (Significance level 5%)	Control	Dose 1	Dose 2
Dose 1	0.67		
Dose 2	0.62	0	
Dose 3	0.01	0.6	0.54

Conclusions

This study was carried out in order to investigate the impact of paracetamol on the growth rate of larvae that have been feeding on rabbit's meat which were injected by different doses of paracetamol (60 mg/kg, 100 mg/kg, 200 mg/kg). In this study it was demonstrated that the larvae fed on the food stuff containing known dosage of paracetamol have displayed a higher growth rate than the larvae fed on the control samples (00 mg/kg).

Paracetamol accelerated the larval growth rate of the both species. The higher the dose were, the bigger the larvae was found to be compared to the control samples during the three first days of development. However the life cycle length of both species was not affected except for *L. sericata* reared on the substrate containing dose 01 and dose 02; which extended the life cycle by approximately 17 hours. These results showed that paracetamol may affect the development of *Lucilia sericata* and *Calliphora vicina*, which is crucial to take into consideration to estimate the PMI correctly. Further work is strongly recommended in order to assess more parameters and doses.

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XIII EAFE MEETING Hungary 25-28 May 2016

Cases from Central Italy: indoors vs outdoors

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INTRODUCTION

In a previous work (Bugelli et al., 2015) the authors analyzed and compared indoor and outdoor cases from Central Italy in order to have a better understanding of the body colonization process and of the factors affecting it. Three new cases are here presented, the results confirm and strongly support the previous general results. In addition, the presence of *Megaselia scalaris* (Diptera: Phoridae) in indoor cases is confirmed.

CASE REPORT

Case 1

At the end of April, the body of a 49 years old man was found inside his car parked in a wood (fig.1). The body, in a supine position, was wearing a sweater, shirt, jeans and the shoes (fig.2). The victim was reported alive 6 days before the body discovery and the cause of death was indicated as a suicide (fig.4). Larvae belonging only to one species, *Lucilia sericata* (Diptera: Calliphoridae) were collected from the body (fig.3).

Taxon	Developmental stage
Calliphoridae	
Lucilia Sericata	eggs

Case 2

At the beginning of July, the body of a 77 years old man was found lying on the bed in a supine position wearing only underpants (Fig.5). The body was found in an advanced decay stage and no sign of injury was reported on the autopsy (Fig.6). The victim was reported alive few days before the body recovery. Larvae from two species were collected from the body and the crime scene: *Megaselia scalaris* (Diptera, Phoridae) and *Sarcophaga* sp. (Diptera: Sarcophagidae).

Taxon	Developmental stage
Phoridae	
Megaselia scalaris	L
Sarcophagidae	
Sarcophaga sp	L

Case 3

At the end of July, the body of an 84 years old woman was found lying on the floor in a supine position (Fig. 7). The body was in an advanced decay stage and no sign of injury was present (Figg.8-9). The victim was seen alive 6 days before the body discovery.
Three species, in larval stage, were collected from the body: *Chysomya albiceps*, *Lucilia sericata* (Diptera: Calliphoridae) and *Sarcophaga* sp. (Diptera: Sarcophagidae).

Taxon	Developmental stage
Calliphoridae	
Lucilia sericata	L
Chrysomia albiceps	L
Sarcophagidae	
Sarcophaga sp	L

Fig.1: crime scene. Car in the wood close to Pisa, Toscany.

Fig.2: body in the supine position in his car.

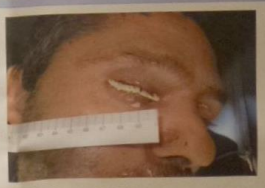

Fig.3: eggs mass

Fig.4: self-wound in his wrist

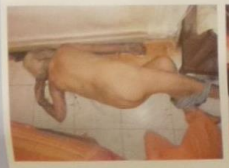

Fig.5: position of dead body in his home

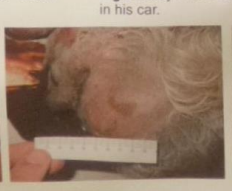

Fig.6: no sign of injuries.

Fig.7: crime scene in a field in Toscany

Fig.8: advanced decay stage

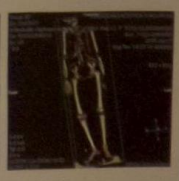

Fig.9: body CT-scan

HOW SHOULD LIVING ENTOMOLOGICAL SAMPLES BE STORED?

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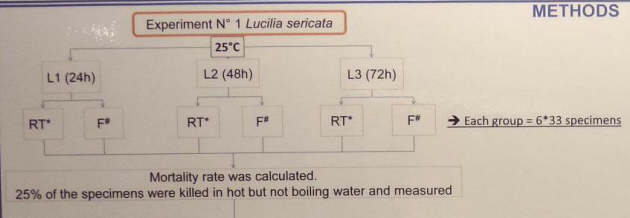
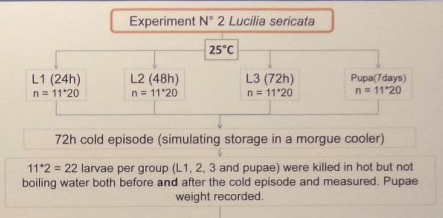
INTRODUCTION

Sampling, killing and transferring/storing of insect evidence is a very important task in forensic entomology because different methods can influence survival and growth rates of the living samples or bias the morphological examination of the dead specimens. The majority of "Best Practices" and "Guidelines" in forensic entomology recommend that fly larvae should kept under controlled or at least known conditions, most suitable at 2-6°C. They suggest in addition that larvae should be stored in vials with an air-permeable lid and that these vials should be equipped with coarse sawdust or paper for taking e.g. excretion liquids. Living samples should be then brought to an expert within 24 hours. While keeping this window of time seems to be a realistic approach, cooling the samples or catering them during storage seems to be a serious problem for some crime scene technicians or forensic pathologists. But neglecting these guidelines or best practice recommendations might lead to a weakening of the entomological evidence at court or even its exclusion. However, it is not always clear whether and which of the recommendations are based on experiences, opinions or scientific evidence. What happens if the cold chain is not maintained, and if there was no air-condition or no supply of coarse sawdust? We analysed this for different larval stages of the forensically relevant blow fly *Lucilia sericata*.

METHODS





H: hours after oviposition
T: 16h in falcons at room T°+ 8h styrofoam box at 8°C
F: 16h in falcons in the fridge + 8h styrofoam box at 8°C

The remaining specimens were transferred to the climate chamber (25°C) and reared to the adult stage. Every 24h checked for development and mortality rate.

The left mesothoracic tibia and the posterior cross vein dm-cu of the left wing were measured by the means of a dissecting microscope (Olympus SZ61) and computer software for visualization (AuxioVision, Zeiss).


Fig. 1: posterior cross vein dm-cu of left wing

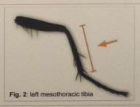


Fig. 2: left mesothoracic tibia

RESULTS and DISCUSSION

Larval length and Mortality Rate

Experiment N° 1

In this experiment, larvae in all three stages (L1-L3) didn't show a significant change in length in all groups. Significant differences with length of the control group. (fig.3-5). Mortality rate after "treatment" shows a significant difference between L1F & L2RT (p< 0.001), and between L2RT & L2F (p<0.05) (fig.6). No significant differences in mortality rate during development, in the first 144h for L1 and L2 (fig.7).

Lucilia sericata L1

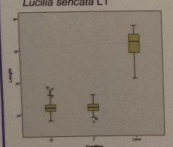


Fig.3: larval length L1 (24h old larvae) after 24h in a falcon without food and oxygene. 16h at Room Temperature (RT) and fridge (F) + 8h in a styrofoam box at 8-6°C.

Lucilia sericata L2

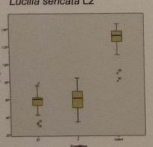


Fig.4: larval length L2 (48h old larvae) after 24h in a falcon without food and oxygene. 16h at Room Temperature (RT) and fridge (F) + 8h in a styrofoam box at 2-6°C

Lucilia sericata L3

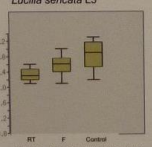


Fig.5: larval length L3 (72h old larvae) after 24h in a falcon without food and oxygene. 16h at Room Temperature (RT) and fridge (F) + 8h in a styrofoam box at 2-6°C.

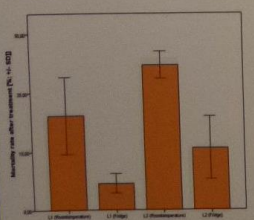


Fig.6: mortality rate L1-L2 after "treatment" (24h without food and oxygene).

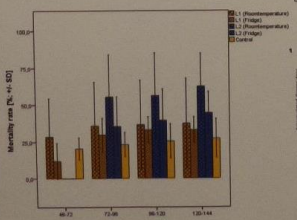


Fig.7: mortality rate L1-L2 during development (up to 144h).

Experiment N° 2

Group L1 (L_1) showed a significant growth during the 72h lasting cold episode ($p < 0,001$) (Fig.10). No significant change in L_2 –L_3 larval stage (Fig.10).
Pupae of the Control group showed a significant ($p < 0,05$) reduction of weight from 168 h after oviposition to 240 h after oviposition. (Fig. 11). No significant change in weight could be observed for pupae which were introduced in the cold episode (Fig.11).
Total mortality was higher in the groups L_1, L_2, L_3 and Pupae compared to the control group (7,42 ± 11,9 %), though a significant difference could be detected only for the groups L_2 (23,48 ± 12,3 %) and L_3 (21,97 ± 31,6 %) (Fig.12).

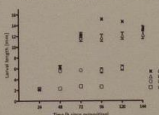


Fig.10: Influence of the 72 lasting cold episode (–8 °C) on larval length of *Lucilia sericata*. L_1: cold episode starting 24 h after oviposition, L_2: cold episode starting 48 h after oviposition, L_3: cold episode starting 72 h after oviposition

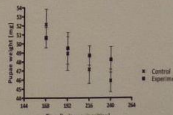


Fig.11: Influence of the 72 h lasting cold episode (–8 °C) during pupal development (from 168 h to 240 h after oviposition) on pupae weight of *Lucilia sericata*

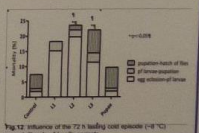


Fig.12: Influence of the 72 h lasting cold episode (–8 °C) on mortality rate of *Lucilia sericata*

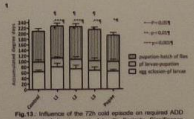


Fig.13: Influence of the 72h cold episode on required ADD per period of development of *Lucilia Sericata*. Significance differences of total required ADD compare to control.

Accumulated degree days (ADD)

Regarding total development from egg eclosion to hatching of the adult, the groups L_1 (226 ± 17,4 ADD), L_2 (225 ± 13,1 ADD) and L_3 (215 ± 11,9 ADD) required significant more accumulated degree days compared to the Control group (207 ± 9,7 ADD) (Fig.13). The group Pupae needed the lowest amount of accumulated degree days (193 ± 12,2 ADD), a statistically significant difference compared to the control group could be shown (Fig.13).

Adult Measurements

Experiment N° 1

The data show no significant differences in the mean tibial length of all L3 groups with control. (Fig.8). No significant differences between RT and F in dm-cu length. (Fig.9).

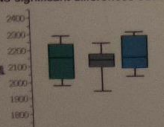


Fig.8: influence of the "treatment" to the length of the mesothoracic tibia in L3.

Fig.9: influence of the "treatment" to the length of the dm-cu of left wing in L3.

Experiment N° 2

The data shows that the mean tibial length of the males in the control group was significantly higher than that of male individuals from the other groups ($p < 0.001$). L3 females show a significantly shorter mean tibial length than the females in the control group ($p < 0.001$). Overall, the mean tibial of the control group with 2.39 mm was significantly greater than that other groups (Fig.14).
The average length of the posterior cross vein dm-cu of left wing of group L3 was significantly below than all other groups (Fig.15). The group L3 shows a significant difference ($p < 0.001$) with the control group. The individuals from the groups L1, L2 and pupae disclosed in any evaluation a significant difference to the control group (Fig.15).


Fig.14: influence of the cold episode (–8 °C) to the length of the mesothoracic tibia in L1, L2, L3 and pupae. * significance compared to control

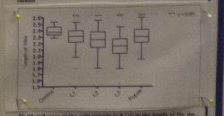


Fig.15: influence of the cold episode (–8 °C) to the length of the dm-cu of left wing in L1, L2, L3 and pupae. * significance compared to control

CONCLUSIONS

- Our experiment suggests that the storing living samples in a fridge, i.e. at temperatures of about 6C, is a practice to prefer over the storage of the samples at room temperature as mortality will increase and might lead to a loss of specimens.
- Storage time of living samples should not exceed 24 hours, as a longer period may modify the time of development of the specimens.
- Increased mortality and modified time of development might hamper or even bias an appropriate evaluation of entomological evidence.
- Future studies might include other species as well and test the sampling and storage of larger larval masses.

DNA Barcoding in Forensic Entomology –

A NGS approach for fast and reliable identification of species compositions on dead bodies.

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Background Information

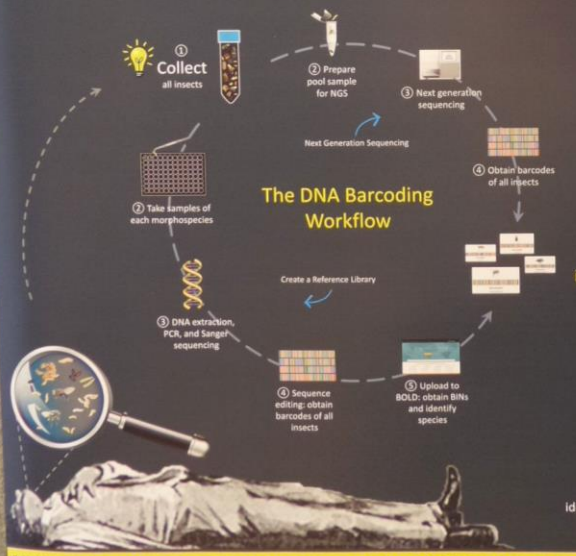
Forensic entomology aims to use insects to estimate the post mortem interval of a carcass. A major problem however is the rapid, exact, and reliable identification of the insect species. Eggs and early larval stages of many insects share similar features, making it difficult to distinguish between species based on morphology. Often, collected larvae need to be reared under constant conditions to the imago, which offers better differential features. This time consuming method is not beneficial if fast species identification is crucial. A promising alternative is species identification using molecular markers. The Bavarian State Collection of Zoology (SNSB-ZSM) uses DNA barcoding to obtain genetic fingerprints of all animals, fungi, and plants found in Germany. Approximately 17,000 animal species were already successfully barcoded and more than 100,000 barcode sequences were provided for the international Barcode of Life Database (BOLD). In this study, we created a DNA barcode reference library for arthropod species playing an important role in forensic entomology and we applied NGS techniques in order to obtain simultaneous species identifications of bulk samples.

Procedure

We investigated insects, which were extracted from 30 human bodies and stored in 50mL Falcon tubes, provided by the local morgue. For reference library construction, we sorted out visually distinct morphospecies and different developmental stages of all sample containers; tissue samples were removed from each specimen and transferred into 96 well plates for subsequent DNA extraction. Sequence data and trace files were then uploaded to BOLD. In a second step, we tested the potential of bulk extractions and subsequent species identification using NGS and the application of the barcode reference data in one single analysis step.

Results

The analysis of the NGS data comparison with the BOLD database revealed 31 different BINs of insect species, of which only 13 could be identified by references of the forensic insect library which we created on BOLD. The whole procedure took a total of 30 working hours from DNA extraction to species identification for the whole bulk sample, which clearly indicates that NGS is especially promising, as it saves time and costs in comparison to conventional methods. This study illustrates that DNA Barcoding might be a promising addition for forensic entomologists, because it facilitates accurate and fast species identification of all developmental stages even when specialized taxonomists are lacking.



A **major** problem in forensic entomology is the **accurate** identification of larvae based on their **morphology** alone.

?

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Which species were found based on the habitat?

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CLASSIFICATION OF LARVAL SILPHINAE (COLEOPTERA: SILPHIDAE) ACCORDING TO INSTAR

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INTRODUCTION

Larvae of beetles may be classified according to instar based on measurements of quantitative morphological features. For this purpose statistical classifiers of the species level were created and a very high accuracy of classification was achieved. Here we test the concept that subfamily level classifier may be similarly successful in the classification task. For this purpose, larvae of four closely related species of carrion beetles i.e. *Necrodes littoralis*, *Oiceoptoma thoracicum*, *Thanatophilus sinuatus* and *Thanatophilus rugosus* (Silphidae: Silphinae) were studied.

MATERIAL & METHODS

LARVAE

- *T. sinuatus* and *T. rugosus* - reared in laboratory condition.
- *O. thoracicum* - collected on pig carcasses during 2011 field studies.
- *N. littoralis* - measurement data from our previous study.

MEASUREMENTS

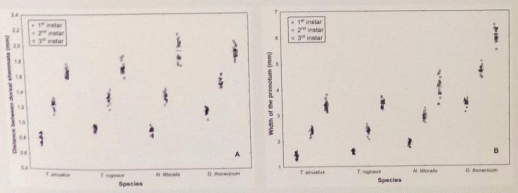
Second instar larva of *T. sinuatus*:
A: distance between dorsal stemmata,
B: width of the pronotum,
C: width of the mesonotum.

CLASSIFIERS

- **Subfamily level classifier** was created using 60 training larvae (20 per instar) of each species. Classifier was tested with training larvae and 30 test larvae (10 per instar) of each species.
- **Genus level classifier for *Thanatophilus* larvae** was created using 60 training larvae (20 per instar) of *T. sinuatus* and *T. rugosus* larvae. Classifier was tested with training larvae and 30 test larvae of each species.

RESULTS

- Distance between dorsal stemmata and width of the pronotum were used to create classifier (Table 1). The width of the mesonotum was excluded due to its almost perfect correlation with the width of the pronotum.



Measurements of distance between dorsal stemmata (A) and width of the pronotum (B) in larval stages of *T. sinuatus*, *T. rugosus*, *N. littoralis* and *O. thoracicum* (training larvae); symbols: raw data, —: mean.

Table 1. Classification functions in the subfamily level classifier for larval Silphinae.

Feature	Larval instar		
	I	II	III
Stemmata	110.1322	155.9285	204.461
Pronotum	-9.5795	-13.4486	-17.456
Constant	-43.4476	-86.3388	-148.4

- Validation with test larvae gave perfect results in the case of *N. littoralis* and *O. thoracicum*. Few misclassifications were however observed in the case of *T. sinuatus* and *T. rugosus* (Tables 2, 3).

Table 2. The classification matrix for test larvae of *T. sinuatus*.

	1st instar	2nd instar	3rd instar	Percentage correct
1st instar	10	0	0	100
2nd instar	0	10	0	100
3rd instar	0	3	7	70
Total	10	13	7	90

MATERIAL & METHODS

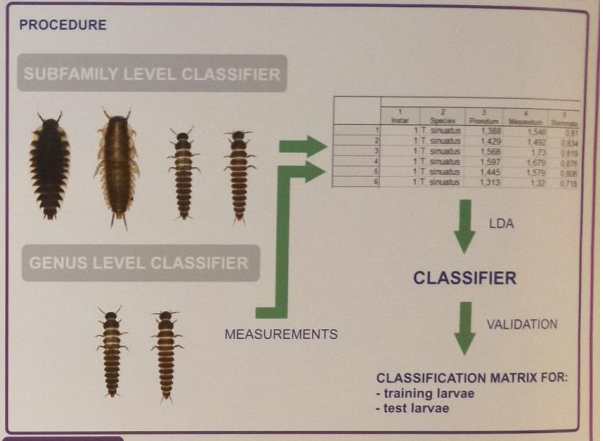
DATA ANALYSES

- Cluster analysis
- Linear discriminant analysis (LDA) - The analysis generates classification functions which may be used to classify larvae according to instar with the following formulas:

$$S_i = c + w_1 * f_1 + w_2 * f_2 + \ldots + w_i * f_i;$$

where S_i is the classification value for an instar x, c is the constant, w represents the weight for a measured feature and f is the measurement of a feature.

PROCEDURE



RESULTS

Table 3. The classification matrix for test larvae of *T. rugosus*.

	1st instar	2nd instar	3rd instar	Percentage correct
1st instar	10	0	0	100
2nd instar	0	10	0	100
3rd instar	0	1	9	90
Total	10	11	9	96.6667

- Because classifiers created for forensic purposes should be more accurate, it was decided to generate genus level classifier for *Thanatophilus* larvae (Table 4). Its validation with training and test larvae was 100% correct.

Table 4. Classification functions in the genus level classifier for larval *Thanatophilus*.

Feature	Larval instar		
	I	II	III
Stemmata	105.5746	137.978	148.949
Pronotum	45.3618	80.206	131.842
Constant	-82.2481	-185.553	-351.543

CONCLUSIONS

- Larval instar classifiers of the subfamily and genus level were tested in the case of forensically important species of carrion beetles (Silphidae: Silphinae).
- Measurements of distance between dorsal stemmata and width of the pronotum were found to be useful for instar determination.
- Subfamily level classifier classified correctly all larvae of *N. littoralis* and *O. thoracicum*, as well as the fully sclerotized larvae of *Thanatophilus*.
- For the *Thanatophilus* larvae just after ecdysis it is suggested to use the genus level classifier.

Frątczak K., Matuszewski S. 2016: Classification of forensically-relevant larvae according to instar in a closely related species of carrion beetles (Coleoptera: Silphidae: Silphinae). Forensic Science, Medicine, and Pathology, 12(2): 193-197.

XIII. International Meeting of the European Association for Forensic Entomology, HUNGARY, 25 - 28. May 2016

ULTRAMORPHOLOGY OF PREIMAGINAL STAGES OF *HYDROTAEA CAPENSIS* (DIPTERA: MUSCIDAE)

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1. Introduction

Despite its interest for forensic purposes, family Muscidae has not been deeply studied concerning its preimaginal stages although, recently, some papers (i.e. Grzywacz et al., 2013, 2014) have paid attention to the comparative study of such stages of several species. Concerning *Hydrotaea capensis* (Wiedemann, 1818), a species of forensic interest (i.e. Lefebvre & Pasquerault 2004, Arnaldos et al. 2014) few data are available on its immature stages. This contribution provides the first images of all preimaginal stage of *H. capensis*, as well as those of the cephalopharyngeal skeleton of the three larval stages.

2. Material and Methods

A laboratory colony of *H. capensis* was founded with larvae and puparia from a forensic case in Murcia. Once the adults emerged it was confirmed that they belonged to *H. capensis*. Specimens of all preimaginal stages were obtained and studied on SEM using the methodology adopted in Paños-Nicolás *et al.* (2015). Larvae were also studied by light microscopy to observe their internal structures.

3. Results

Eggs

Larva I

Larva II

Larva III

Light microscopy anatomy

Pseudocephalon

Thorax

Abdomen & anal division

Puparium

1-9 Eggs morphology. **10, 11, 16, 17, 21 & 22** Cephalopharyngeal skeleton outlines. **12** Mouthhooks. **13 & 23** Pseudocephalon. **14, 19 & 25** Posterior spiracles. **15 & 26** Anal protuberance. **18** Anterior spiracle. **20 & 22** Intermediate sclerite. **27, 32 & 36** Pseudocephalon. **28,35 & 37** Detail of antenna. **29, 34 & 39** Maxillary palpus. **30, 33 & 40** Detail of labial lobes and labial organ. **31, 36 & 38** Ventral organ. **41 & 55** Lateral morphology of first thoracic segment. **42, 49 & 53** Spines of first thoracic segment. **43, 51 & 54** Thoracic spines. **44, 48 & 52** Dorsal morphology of first toracic segment. **45 & 56** Anterior spiracle. **46 & 57** Detail of Keilin's organ. **50** Keilin's organ and lateral hole in third toracic segment. **58, 64 & 71** General view of abdominals segments. **59, 65 & 72** General morphology of anal division. **60, 69, 74 & 77** Posterior spiracles. **61, 68 & 75** Detail of creeping welt. **62** Anal protuberance. **63 & 70** Detail of anal papillae. **66 & 73** Morphology of spiracular field. **67 & 76** Sensilla of posterior papillae. **78-82** General morphology and details of puparium. **83** Detail of creeping welt. **84** Detail of respiratory horn. **85** Detail of posterior spiracle.

Abbreviations: *a (1-7)*- abdominal segments; *ads*- additional sensillae; *an*- antenna; *ans* antennal sensillae; *ao*- anal opening; *ap*- anterior pole; *ap(1-3)* anal pad; *ap-m*- middle anal pad; *apl*- anal plate; *as*- anterior spiracle; *asb*- anterior spiracle band; *asp*- anterior respiratory papillae; *b*- button; *br*- basal ring; *bsc*- basal sclerite; *bp*- basal pore; *c*- chorion; *cs*- chorionic sculpture; *cw*- creeping welt; *d*- denticulate layer; *db*- dorsal bridge; *dbv*- antennal groove; *ds*- dental sclerite; *eh*- hatching line; *ep*- hatching plead; *es*- stomal sclerite; *fl*- segment folding; *h*- hole; *ip*- intersegmentary groove; *ir*- intermediate sclerite; *ke*- Keilin's organ; *l*- labium; *las*- antennal lateral sensilla; *ll*- labial lobe; *lo*- labial organ; *lp*- lateral pads; *l*- labial sclerite; *ls-a*- anterior labial sclerite; *ls-p*- posterior labial sclerite; *mc*- micropyle; *mcp*- micropylar plate; *mh*- mouthhook; *mo*- mouth opening; *mp*- maxillary palpus; *mv*- muscular vesicle; *o*- orifice; *ob*- oral bar; *or*- oral groove; *p (1-6)*- posterior papillae; *pa*- anal protuberance; *ph*- parastomal bar; *prf*- peritreme; *ps*- posterior spiracle; *psh*- peristigmatic buff; *pts*- pit sensilla; *rh*- respiratory horn; *rs*- respiratory slit; *s*- sensilla; *sb*- basiconica sensilla; *sb(1-3)*- basiconica sensillae; *sc(1-3)*- trichoidea sensillae; *s(1-3)*- thoracic segments; *tm*- tegumentary fold; *vb*- ventral bridge; *vc*- ventral cornu; *vo*- ventral organ; *vp*- vertical plate; *wr*- forehead *wr*. *29 & 34*. *pit sensilla in maxillary palpus; 31, 36 & 38 sensillar elements in ventral organ.*

4. Conclusions

-The micromorphology of all preimaginal stages of *Hydrotaea capensis* and the morphological correlation among its three larval instars, as well as a comparison with other *Hydrotaea* species are provided.

-The best morphological features of taxonomic value have been found in the pseudocephalon, anal division and cephalopharyngeal skeleton.

- Combining light and electronic scanning microscopy techniques allows a more accurate, detailed and complete description of preimaginal morphology.

- It would be desirable to establish a set of standart features for further descriptions of preimaginal morphology in order to perform future complete identification keys.

High Resolution Melting (HRM) Analysis: Application on forensically relevant fly species (Diptera: Brachycera)

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Kindly supported by FORENSISCHES FORUM

GOETHE UNIVERSITÄT FRANKFURT AM MAIN

Rechtsmedizin Frankfurt am Main

Introduction

Estimating the minimum postmortem interval (PMI_{min}) by age determination of necrophagous insects, constitutes one of the crucial challenges in forensic entomology. Since the development from egg to adult stage varies between different species, reliable identification is essential. But also succession studies for example demand for fast, cost effective and accurate species determination. Therefore, the currently used methods, e.g. the use of morphological keys, which requires sufficient expertise, or the cost and labour intensive sequencing of DNA-fragments, could be complemented by High Resolution Melting (HRM) Analysis.

HRM combines a real-time PCR with an immediately following stepwise increase of temperature, causing DNA-denaturation. The analysis provides a specific melting temperature of a certain DNA-fragment. Therefore, a dsDNA-intercalating fluorescent dye is incorporated in the growing double strand during the preceding PCR and is released when the dsDNA melts into single strands. A simultaneous fluorescence measurement enables the real-time monitoring of this process. The DNA-melting temperature depends on the length and the composition of the analyzed fragment and therefore varies between genetically diverging species.

It has been demonstrated that this technique is useful for the detection of mutations or nucleic variants in medi-clinical researches like tumor genetics for instance (*Dirican et al.*, 2014). Moreover, Malewski et al. (2010) demonstrated the applicability of this method to forensic entomology, by analyzing *Calliphoridae* species.

To test if this method may overcome the difficulty of reliable species determination and thereby help to ensure the most accurate calculation of the minimum postmortem interval (PMI_{min}), HRM was additionally applied to 6 other forensically relevant fly families.

Materials & Methods

Fig.1: Overview of the analyzed fly (Brachycera) families

Specimen: In total, 118 individuals from 33 different species of 7 forensically relevant fly families (see Fig.1) were analyzed. The samples derived from the institutes own breed or were trapped.

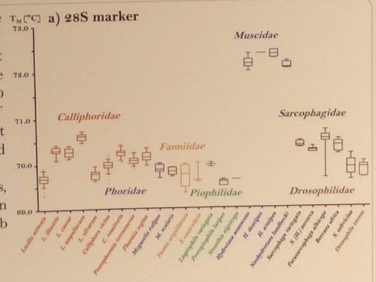
Extraction & Identification: DNA was extracted from adult or larval specimens using phenol-chloroform. The species were verified by sequencing the common COI Barcoding-fragment (*Folmer et al.*, 1994).

Analysis: For the analysis, one nuclear (*28S rRNA* gene (28S)) and two mitochondrial (*cytochrome-c-oxidase subunit 1* gene (S9, COI2)) markers were designed. The HRM samples were prepared using ABI MeltDoctor Mastermix and analyzed on a StepOnePlus RT-Cycler. Subsequent data evaluation was performed using StepOne (v2.3) and HRM Software and included the evaluation of Amplification and Difference Plots, as well as the Aligned and Derivative Melt Curves. Finally, a statistical analysis was carried out using GraphPad Prism and ANOVA.

Results

When comparing the results obtained using the three different markers and analyzing several specimens of each species, it becomes obvious, that only slight differences occur between the up to 7 fly families (see Fig. 2a, b, c). The melting temperatures of about 30 species spread over a temperature range of approximately 3°C. Though, there is a significant difference between the nuclear (69,5°C to 72,5°C) and the mitochondrial (71,5°C to 74,5°C) markers.

In contrast to the high similarity between the species, a conspicuously high variance within one species can be observed (e.g. see Fig. 2a *Fannia aequilineata*, 2b *Protophormia terraenovae* or 2c *Liopiophila variegata*).



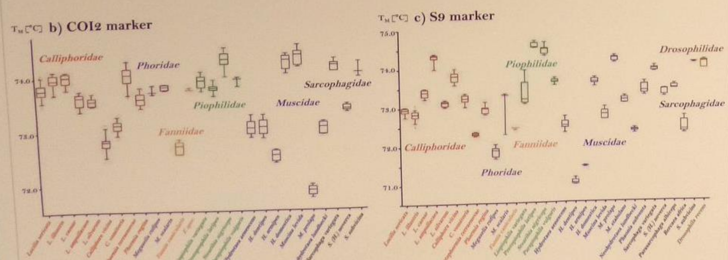


Fig.2: Boxplots showing the melting temperatures obtained from 1 - 30 specimens per species, using either the nuclear marker ((a) 28S) or a mitochondrial marker ((b) COI2 and (c) S9)

Discussion

Several aspects, potentially influencing the measurement results, were taken into consideration.

DNA-Amount: In order to exclude an influence of the DNA quantity on the melting temperature, a series of experiments including the insertion of increasing DNA-concentrations was performed. It revealed that the measurement results within one species remained stable (see Fig. 3). Hence, it can be concluded that the melting temperature does not depend on the DNA-concentration.

GC-Content & Sequence Variation: Furthermore, the base composition of all analyzed fragments was compared. The count and position of all sequence variations and the resulting GC-content for each analyzed specimen were recorded and compared. However, no correspondance between the sequence analysis and the melting temperatures could be found.

Difference Plot: Resulting graphs could not be used to distinguish between species (Data not shown).

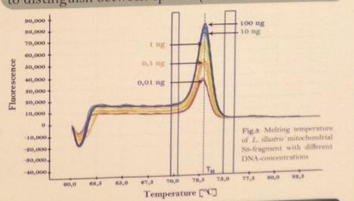


Fig.3: Melting temperature of *L. illustris'* mitochondrial S9-fragment with different DNA-concentrations

Conclusion

High Resolution Melting (HRM) Analysis has been applied on the identification and differentiation of forensically relevant fly species. The evalutation of the results revealed a low interspecific variance on the one hand, but a high intraspecific variance on the other hand. The melting temperatures as obtained could not be explained by different DNA quantities, species specific sequence variations or the related change of the GC-content, though.

The statistical analysis via ANOVA additionally predicted a failure in a sound species identification of 20 – 25%.

Thus, the resolution, as well as the reproducibility of this method seem to be insufficient for the scientific issue required here. It can be concluded, that HRM is not a reasonably accurate method to distinguish between individuals with a close relationship as in this research, especially with regard to forensic issues. Also, the current study does not confirm the allegations by Malewski et al., declaring HRM as a sufficient method for species determination.

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Identification of carrion visiting Muscidae (Diptera) by means of wing measurements

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Introduction

- The presence of Muscidae on decomposed bodies has been recorded in numerous case reports and carrion succession experiments. Dead animal and human bodies attract adult houseflies, both common elements of carrion insect assemblages and rare visitors. Thus application of identification keys with a broad taxon coverage for the geographic region of interest is recommended instead of those exclusively orientated for identification of "forensically important" species.
- The identification of adult Muscidae may be considered difficult, particularly by non-experts. Thus many studies have identified adult muscids only to the genus or family level.
- The aim of our study was to investigate the possibility of semiautomated muscid identification by means of wing measurements as an alternative for classic morphology and DNA-based approaches.

Material and methods

- We have used 583 specimens representing 11 genera and 2 species of Muscidae visiting carrion and cadavers.
- Both wings have been detached from the body and their image were obtained using USB camera with resolution of nine megapixels.
- On the wing images 15 landmarks have been determined in IdentiFly software (Fig. 1). Coordinates of the landmarks were analysed using methods of geometric morphometrics.
- Identification of the species was based on Canonical Variate Analysis. Identification error was assessed using leave-one-out cross-validation.

Table. 1. Identification error of animal and human bodies visiting muscid genera assessed using leave-one-out cross-validation. Rows represent a given genus and columns predicted genus. Correct identifications are in the diagonal and incorrect are marked in red.

	Hydrotaea	*Graphomya*	*Thricops*	*Muscina*	*Stomoxys*	*Mydaea*	*Eudasyphora*	*Phaonia*	*Helina*	*Musca*	*Azelia*	**Total**	**Correctly classified (%)**
Hydrotaea	318	-	-	-	-	-	-	-	-	-	-	318	100
Graphomya	-	15	-	-	-	-	-	-	-	-	-	15	100
Thricops	-	-	28	-	-	-	-	-	-	-	-	28	100
Muscina	-	-	-	78	-	-	-	-	-	-	-	78	100
Stomoxys	-	-	-	-	13	-	-	-	-	-	-	13	100
Mydaea	-	-	-	-	-	14	-	-	-	-	-	14	100
Eudasyphora	-	-	-	-	-	-	28	-	-	-	-	28	100
Phaonia	-	-	-	-	-	1	-	33	-	-	-	34	97,06
Helina	-	-	-	-	-	-	-	1	23	-	-	24	95,83
Musca	-	-	-	-	-	-	-	-	-	17	-	17	100
Azelia	-	-	-	-	-	-	-	-	-	-	13	13	100
Total	318	15	28	78	13	15	28	34	23	17	13	583	99,66

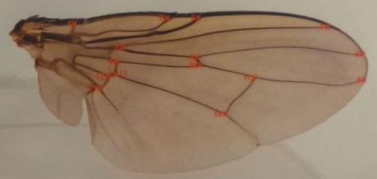

Figure 1. Muscidae wing with the vein junctions used in the analysis numbered.

Table. 2. Identification error of animal and human bodies visiting muscid species assessed using leave-one-out cross-validation. Rows represent a given genus and columns predicted genus. Correct identifications are in the diagonal and incorrect are marked in red.

	H. meteorica	*H. cyrtoneurina*	*H. aenescens*	*H. dentipes*	*H. armipes*	*H. similis*	*G. maculata*	*H. ignava*	*H. pilipes*	*T. simplex*	*M. pascuorum*	*S. calcitrans*	*M. levida*	*M. stabulans*	*M. prolapsa*	*M. lateritia*	*E. cyanicolor*	*P. subventa*	*H. depuncta*	*M. domestica*	*A. aterrima*	**Total**	**Correctly classified (%)**
Hydrotaea meteorica	17	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	18	94,44
Hydrotaea cyrtoneurina	-	29	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	30	96,67
Hydrotaea aenescens	-	-	55	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	55	100
Hydrotaea dentipes	-	1	-	42	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	44	95,45
Hydrotaea armipes	-	-	-	-	29	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	31	93,55
Hydrotaea similis	-	-	-	-	-	52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	52	100
Graphomya maculata	-	-	-	-	-	-	15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15	100
Hydrotaea ignava	-	-	-	-	-	-	-	51	-	-	-	-	-	-	-	-	-	-	-	-	-	51	100
Hydrotaea pilipes	-	-	-	-	-	1	-	-	36	-	-	-	-	-	-	-	-	-	-	-	-	37	97,30
Thricops simplex	-	-	-	-	-	-	-	-	-	28	-	-	-	-	-	-	-	-	-	-	-	28	100
Muscina pascuorum	-	-	-	-	-	-	-	-	-	-	30	-	-	-	-	-	-	-	-	-	-	30	100
Stomoxys calcitrans	-	-	-	-	-	-	-	-	-	-	-	13	-	-	-	-	-	-	-	-	-	13	100
Muscina levida	-	-	-	-	-	-	-	-	-	-	-	-	16	-	-	-	-	-	-	-	-	16	100
Muscina stabulans	-	-	-	-	-	-	-	-	-	-	-	-	-	9	-	-	-	-	-	-	-	9	100
Muscina prolapsa	-	-	-	-	-	-	-	-	-	-	-	-	-	1	22	-	-	-	-	-	-	23	95,65
Mydaea lateritia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14	-	-	-	-	-	14	100
Eudasyphora cyanicolor	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	28	-	-	-	-	28	100
Phaonia subventa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	33	1	-	-	34	97,06
Helina depuncta	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	23	-	-	24	95,83
Musca domestica	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	17	-	17	100
Azelia aterrima	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13	13	100
Total	17	30	55	43	30	53	15	51	39	28	30	13	16	10	22	14	28	34	24	17	13	583	98,28

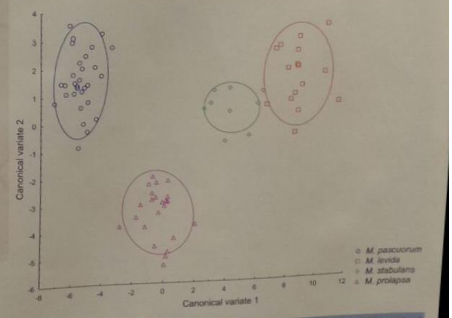

Figure 2. Differences in wing venation between representatives of *Muscina* visiting animal and human bodies obtained by Canonical Variate Analysis. First two canonical variates (CV1–CV2) are presented. The ellipses indicate 95% confidence limits.

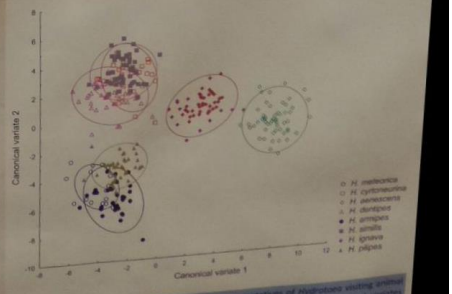

Figure 3. Differences in wing venation between representatives of *Hydrotaea* visiting animal and human bodies obtained by Canonical Variate Analysis. First two canonical variates (CV1–CV2) are presented. The ellipses indicate 95% confidence limits.

Results and conclusions

- Most genera were identified without error (Table 1). Misidentification between genera occurred only in two cases. A single specimen of *Phaonia* was identified as *Mydaea* and a single specimen of *Helina* as *Phaonia* (Table 1).
- Identification of species within *Muscina* (Figure 2) was high, with only a single specimen of *M. prolapsa* misidentified as *M. stabulans* (Table 2). Correct identification rate of species within *Hydrotaea* (Figure 3) was lower and ranged from 93 to 100% (Table 2).
- Our results revealed relatively high success in both genus and species identification of carrion visiting Muscidae. Automated identification by means of wings measurements can be used by non-experts and does not require sophisticated equipment. The method can be an alternative to more difficult and more time-consuming identification based on taxonomic keys.

The present work was supported financially by the Ministry of Science and Higher Education, Poland (IUVENTUS PLUS grant no. 0146/IP1/2015/73).

Duration of the post feeding and intra-puparial stage of the blow fly *Calliphora vicina* (Diptera: Calliphoridae) as a function of the pupariation substrate

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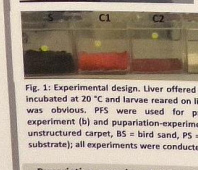
Kindly supported by FORENSISCHES FORUM

Introduction

Growth rates of blow flies are major tools used to estimate the PMI_{min} in forensic entomology casework and considerable effort has gone into generating developmental data for a number of temperature regimes and species of forensic importance (Ames & Turner 2003; Donovan et al. 2006; Grassberger & Reiter 2002). Under laboratory conditions several types of pupariation substrate are offered to post-feeding larvae e.g. sawdust or sand. However, real case scenarios are highly variable regarding their possible pupariation substrates and this might influence the time spent in dispersal and finally the pupariation process itself (Mai & Amendt 2012).

Hence, the present study is concerned with the hypotheses, that different kinds of substrates affect the duration of the post-feeding and intrapuparial stages of the forensically relevant blow fly *Calliphora vicina*, and that post-feeding larvae might show a specific preference for a substrate. Moreover we examine whether interactions between post-feeding larvae maintained at different larval densities might influence the duration of the pupariation process by a natural disturbance factor.

Material & Methods



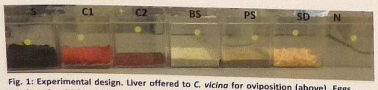


Fig. 1: Experimental design. Liver offered to *C. vicina* for oviposition (above). Eggs incubated at 20 °C and larvae reared on liver at 24°C until post-feeding stage (PFS) was obvious. PFS were used for preference-experiment (a), disturbance-experiment (b) and pupariation-experiment (c: S = soil, C1 = woolen carpet, C2 = unstructured carpet, BS = bird sand, PS = playground sand, SD = sawdust, N = no substrate); all experiments were conducted at 12°C

Pupariation-experiment: 10 larvae were placed on each substrate: soil, woollen carpet, unstructured carpet, bird sand, playground sand, sawdust and "no substrate" (Fig. 1). Substrate-boxes were incubated at 12°C. Pupariation-timings were noted by checking every 3 hours during daytime. Once all individuals had pupariated, temperature was increased to 20°C. Adult eclosion timings were checked twice per daytime and ADHs were calculated. Each substrate was replicated 5 times, the entirety of the experiment was replicated 3 times.

Preference-experiment: 10 larvae were placed in choice-boxes (5 per side): bird sand vs. playground sand, sawdust vs. soil, sawdust vs. playground sand, woollen carpet vs. playground sand, soil vs. woollen carpet and soil vs. playground sand. After 2 weeks at 12°C , puparia per side were recorded.

Disturbance-experiment: 1, 2 or 5 Larvae were placed in tubes (7ml). 1 larva per tube was replicated 20 times, 2 larvae per tube 15 times and 5 larvae per tube 10 times. Tubes were incubated at 12°C. Pupariation- and adult eclosion timings of the first and last individual per tube were noted. The experiment was replicated 4 times

Results

Results showed a significantly longer duration of the post-feeding stage in the soil, the woollen carpet and on "no substrate" in two of three experimental runs ($F(6) = 4,67$, $p = 0,01$; $F(6) = 2,47$, $p = 0,04$). The pupariation-timings of all experimental runs showed similar results (Fig.2). Timings of adult eclosion showed no significant differences in the duration of the intrapuparial stage. Plotting the means of all accumulated degree hours (ADH) taken until the adult stage (Fig. 3), shows an overlapping of all values by their standard deviation. This validate, that there is not a significant difference.

When post-feeding larvae were given a choice for their pupariation substrate, soil was preferred the most, whereas sand was avoided (e.g. $T(9) = 6,74$, $p = 4,25\ E^{-05}$; $T(9) = 2,82$, $p = 0,01$)(Tab. 1).

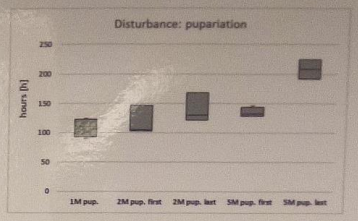


Fig. 4: Pupariation timings for different number of larvae. Boxplots based on mean values of all experimental runs. Boxes show the median and the quartiles and bars show the minimum and maximum values. Tubes containing 1 larva pupariated earlier than tubes containing 2 or 5 larvae. Overlapping of extreme values indicates no significant difference.

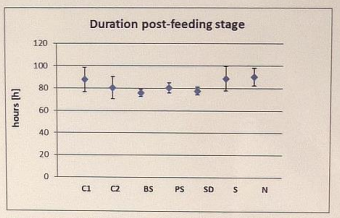


Fig. 2: Pupariation timings on different kinds of substrates. Plotted are the mean values of all three experimental runs. Bars show the standard deviation. The longest duration of the post-feeding stage could be observed in the soil, the woollen carpet (C1) and on "no substrate".

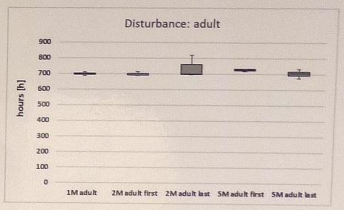


Fig. 5: Adult eclosion timings for different number of larvae. Boxplots based on mean values of all experimental runs. Boxes show the median and the quartiles and bars show the minimum and maximum values. Overlapping of extreme values indicates no significant difference.

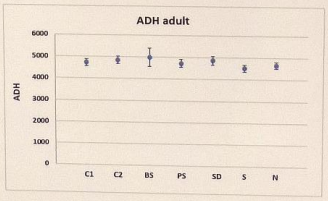


Fig. 3: ADHs taken to reach the adult stage on different kinds of substrates. Plotted are the mean values of all three experimental runs. Bars show the standard deviation.

Tab. 1: Offered choices and observed pupariation-preferences. Soil was preferred the most, whereas sand was avoided.

Choice	Preference
Carpet 1 vs. Playground sand	Carpet 1
Carpet 1 vs. Soil	Soil
Playground sand vs. Bird sand	No preference
Sawdust vs. Playground sand	Sawdust
Soil vs. Playground sand	Soil
Soil vs. sawdust	Soil

The disturbance-experiment showed a significantly longer duration of the post-feeding stage in case of 5 larvae per tube (e.g. $F(4) = 28,74$, $p = 4,12\ E^{-08}$; $F(4) = 26,03$, $p = 1,3\ E^{-12}$). No significant differences could be observed between 1 and 2 larvae per tube (Fig. 4).

Adult eclosion timings were significantly different for 5 larvae per tube in two of four experimental runs ($F(4) = 5,34$ $p = 0,001$; $F(4) = 2,75$, $p = 0,03$), whereas two experimental runs showed no significant differences between the groups. Plotting the mean values of all runs (Fig. 5) indicates a slightly longer duration of the intrapuparial stage only when there were 5 larvae per tube.

Discussion & Conclusion

Results showed that different kinds of substrates can significantly influence the duration of the post-feeding stage of the blow fly *C. vicina*, but not the duration of the intrapuparial stage. Longest post-feeding stage could be observed in the soil, the woollen carpet and on "no substrate". Holmes et al. (2013) performed a similar experiment: they placed post-feeding larvae of the black soldier fly *Hermetia illucens* (Diptera: Stratiomyidae) on five different substrates (wooden shavings, potting soil, topsoil, sand and nothing) and studied the developmental timings of pupariation and adult eclosion. Results showed a longer duration of the post-feeding stage on the topsoil and without any pupariation substrate. The duration of the intrapuparial stage took less time on no substrate, whereas no differences could be observed between the other treatments. Their results equate to the results of this study. Hence, using the same substrate for all developmental studies avoids miscalculations of the PMI_{min} in forensic entomology casework.

The preference-experiment showed that post-feeding larvae try to avoid pupariating on sandy substrates. This might indicate that sand is too dry and abrasive to the skin of post-feeding larvae and that they might prefer a moistened and less harsh substrate. Further studies with a higher amount of replicates should be carried out to confirm the results of this study.

The disturbance-experiment showed a significantly longer duration of the post-feeding stage in the case of 5 larvae per tube. This indicates that there is a natural disturbance factor on the pupariation process by wandering post-feeding larvae. Thus, the PMI_{min} could be underestimated by approximately 20 hours in the case of a large larval mass and limited dispersal space around the corpse.

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ALTERNATIVE USES FOR BLOWFLY LARVAE IN THE DETECTION OF SEMEN FOR POST MORTEM SEXUAL ASSAULT CASES

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Aims

- To determine the effectiveness of current semen collection protocols at post-mortem sexual assault scenes.
- To explore the potential role for forensic entomology in future post-mortem sexual assault investigations.

Objectives

- To determine the degree in which insect activity impacts successful semen recovery at post- mortem sexual assault scenes, using DNA profiling techniques (Fig. 1) to identify the presence and persistence of semen throughout decomposition.
- To explore the effectiveness of semen recovery from the guts of feeding larvae, comparing the presence and persistence of the Homeobox gene (Fig. 1) with current swabbing techniques.

Background

- There have been significant developments in research exploring semen persistence within living victims following a sexual assault (Jones, 2015), leading to the development of semen collection protocols used universally for both living and post mortem victims. However there is little literature on the impact decomposition and subsequent insect activity have on the presence and persistence of semen following death (Dziak, Parker, Collins, & Johnston, 2011).
- With the increased levels of sexual assaults being observed (National Crime Survey, 2015), tied with the pressure of budgeting within policing, it is crucial that new processes are explored. Having a range of techniques available enables cases to be addressed quickly, as well as improving the quality of results.
- Research has shown that blowfly larvae will feed on biological material present at post mortem scenes (Durdle, Mitchell & van Oorschot, 2015), retaining genetic material from its food source within the gut (Clery 2001).
- This study will explore the necessity for further research into the use of forensic entomology as a tool in post-mortem sexual assault cases, examining the impact insect activity has on semen persistence at a crime scene. Furthermore, this study will compare the potential for larvae gut content analysis as an alternative tool for offender identification.

Methodology

Study 1 – Does insect activity impact successful semen recovery?

- *Controlled colony size*
- *Species variation*
- *Fluctuating temperatures*

Liver doused in boar semen will be exposed to the above conditions. Daily dry swabs will be taken until pupation is observed. Swabs will undergo DNA profiling (Fig.1) for the identification of the Homeobox gene on the Y chromosome, verifying the presence of male specific DNA. Comparison graphs will be generated to demonstrate the variation in DNA concentration. Once established, STR profiling will be carried out on high impact scenarios to further determine the effects on profile deterioration.

Study 2 – Can insect crop content analysis be used as an alternative to current collection protocols?

- ***Percentage***

3 liver samples will be doused in boar semen, with 100%, 50% and 25% coverage. Larvae will be sampled from random locations on the liver daily, until pupation is observed. The percentage of larvae containing the Homeobox gene within the gut will be determined (Fig. 1).

- ***Quantity***

Liver will be doused in 6ml, 3ml and 0.5ml of boar semen respectively. Percentage of guts containing the Homeobox gene will be determined and compared to control swabs following current swabbing protocols.

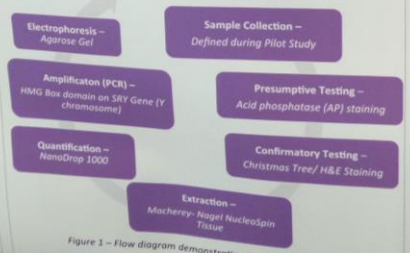

Figure 1 – Flow diagram demonstrating DNA profiling process.

Results

- Initial experiments are being conducted to optimise DNA recovery from boar semen using the Macherey-Nagel NucleoSpin tissue extraction kit.

Table 1 – Table showing NanoDrop 1000 results for 2 extraction types

Protocol	Conc.	260/280	ng/uL
1	200uL	0.97	5.4
1	100uL	1.14	3.1
1	50uL	0.91	2.0
2	200uL	2.64	12.8
2	100uL	2.81	7.5
2	50uL	3.96	7.2

Protocol 1:

- Blocked spin columns
- High protein contamination
- Lower than expected DNA concentrations
- Expected increase in DNA concentration between volumes

Protocol 2:

- Additional pre-spin wash steps with modified wash buffer
- Increased concentrations of DNA recovery
- Reduced protein contamination

Where Next?

- Increased proK concentration
- Additional wash steps
- Longer incubation stages
- Pilot Study

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STAY FROSTY!
THE USE OF ICE-SPRAY FOR SAMPLING AND KILLING BLOW FLY LARVAE

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Rechtsmedizin Frankfurt am Main

Introduction

In forensic entomology, the correct sampling and storing of fly larvae as evidence is of great importance. Best practice paper often recommend the killing of the fly larvae by means of hot water and the subsequent storing in > 70% ethanol for guarantee an accurate and correct measurement of the larval length.

Hot or even boiling water is rare at a crime scene. Ice-spray for medical or industrial use is a simple to get and easy to store equipment as a serious alternative at the crime scene. We examined the killing of larvae (L_2 & L_3) of the two forensically important blow fly species, *Calliphora vicina* and *Lucilia sericata*, by using a medical or an industrial ice-spray before storing the specimens in 96% ethanol.

Material and Methods

- Larvae of both taxa were reared on pork ground meat at 25°C until the larval stages L_2 (~48h) and L_3 (~62h) were reached.
- Groups of 10 larvae of each, *Calliphora vicina* and *Lucilia sericata*, were treated as follows:
  - a) poured and killed by hot water **(HW)**
  - b) killed in 96% ethanol **(ETH)**
  - c) sprayed with a medical ice-spray for about 4 seconds **(MI 4s)**
  - d) sprayed with an industrial ice-spray for about 4 seconds **(II 4s)**
  - e) sprayed with a medical ice-spray for about 8 seconds **(MI 8s)**
  - f) sprayed with an industrial ice-spray for about 8 seconds **(II 8s)**

All specimens were transferred (or kept: see treatment b) and stored in 96% ethanol

- Right after killing (Day 0) and every 24 hrs until day 6, the larvae were measured by means of a geometric micrometer. Only the ETH larvae were measured on day 1 for the first time.

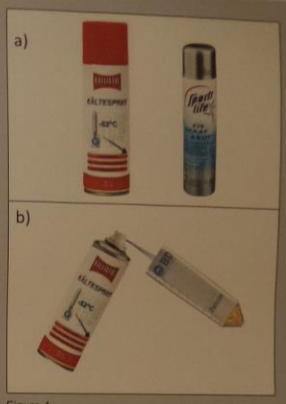

Figure 1
a) left: Industrial ice-spray – „Ballistol– Kältespray"
→ -52°C;
right: Medical ice-spray – „Sports life – Eis Spray Akut"
→ -45°C
b) Illustration of how to apply e.g. the industrial ice-spray on larvae in a falcon vial

Results & Discussion

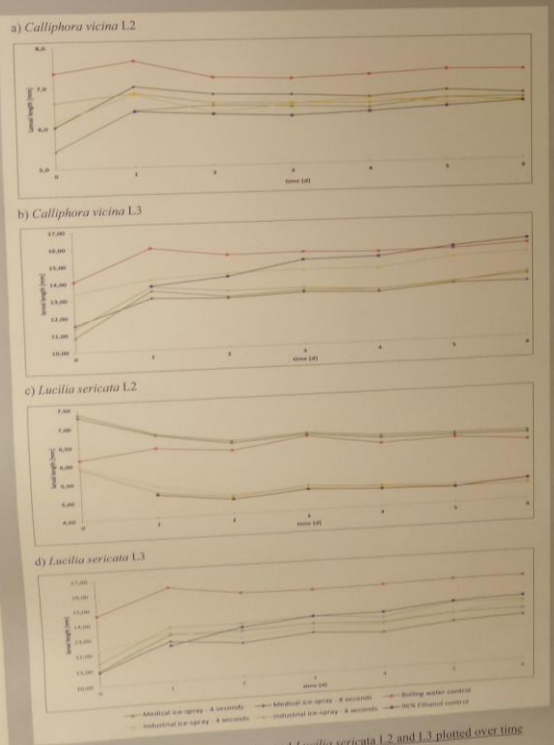

Figure 2: Larval length of *Calliphora vicina* and *Lucilia sericata* L2 and L3 plotted over time

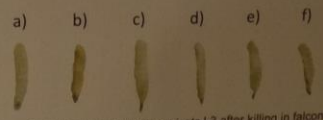

Figure 3: Colouring of *Lucilia sericata* L3 after killing in falcon tubes with a) HW b) ETH c) MI 4s d) II 4s e) MI 8s f) II 8s

Almost all larvae treated with an ice-spray shrunk at day (0) immediately after killing. At day (1) and later on there was a increase in size. The water in the larvae forms ice crystals which damages the tissue. When defrosted, the water may leave the cells and the larvae expand and increase in size again .

Especially due to the fact that the ice-spraying results very often in a fast and sometimes long lasting shrinkage of specimens, killing in hot water and storing in ethanol shows the most reliable results. This is valid especially for *Lucilia sericata*, which shows much more unusal performance than *Calliphora vicina*.
However, when comparing the influence of killing and storing the larvae in ethanol vs. killing them with ice-spray before storing in ethanol, the latter might be a promising alternative.

Outlook

- More specimens and longer storing times have to be analysed before a final evaluation can be made
- Future examinations may vary the application time of the ice-spray and the distance between the larvae and the spray can
- Another research topic will be the application of ice-spray for a short period (approximately 2 seconds) to stun the blow fly larvae temporarely for developement studies and measurements

A molecular, morphological and physiological comparison of English and German *Calliphora vicina* (Diptera: Calliphoridae)

Institute of Legal Medicine Frankfurt am Main

NATURAL HISTORY MUSEUM

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Royal Golden Jubilee Ph.D. Program (PHD/0361/2552)

Introduction

The blow fly *Calliphora vicina* is a common species distributed throughout Europe and can play an important role as forensic evidence in crime investigations (Greenberg, 1991; Amendt et al., 2011). Published development data act as references for the growth rate and age estimation of the immature stages, leading to the establishment of a minimum post-mortem interval. As this published reference data originate from different geographic populations, it is important to determine whether or not populations of the same species show different rates of growth related to their geographical origin. Therefore, the aim of this study was to compare developmental rates of *C. vicina* from Germany and England under different temperature regimes.

Additionally, we analysed the wing venation and the mitochondrial gene cytochrome b of flies from both countries to establish morphometric and molecular tools for separating specimens from both regions.

Materials & Methods

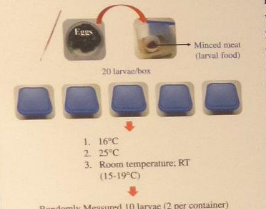

Fig 1. Experimental procedure of developmental study

Developmental study: Two colonies of *C. vicina*, one from Frankfurt (Germany) and one from London (UK), were cultured and kept at the Institute of Legal Medicine in Frankfurt, Germany. Fresh pork liver was placed into the cage of both populations. After oviposition, the eggs were incubated at 25°C and five groups of 20 freshly hatched larvae were transferred to 20 g mixed minced meat. Rearing took place under 3 temperature regimes, and samplings were taken at seven different accumulated degree hours (Fig.1).

Wing morphometrics: Flies from six different locations were analysed (Tab. 1). Right wings were prepared on glass slides, photographed, digitized and measured based on 19 landmarks following Hall *et al.* (2014) (Fig 3).

Wing size variation was estimated by the centroid size. Statistical analysis was conducted in SPSS v.17.0 software. Wing shape variation was generalized by Procrustes analysis, Canonical variate analysis (CVA), Discriminant function analysis (DA), and cross-validation test.

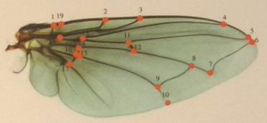

Fig 3. Right wing of *C. vicina* showing the 19 landmarks

Table 1. Geographic locations of the sampled areas and number of specimens analysed (F, females; M, males)

Populations	Locations, Code	Latitude (S)	Longitude (W)	F	M	Total
German	Frankfurt am Main, CVG1	50.1109° N	8.6821° E	32	17	49
	Dortmund, CVG2	51.5136° N	7.4653° E	4	5	9
	Laboratory colony, Frankfurt am Main, CVG3	50.1109° N	8.6821° E	50	50	100
English	Exeter, CVE4	50.7184° N	3.5339° W	14	6	20
	Haywards Heath, CVE5	50.9990° N	0.1063° W	18	2	20
	Laboratory colony, London, CVE6	51.5074° N	0.1278° W	50	50	100

Fig 2. Map of Europe (a) showing England in red area and Germany in yellow, the sampled areas in England (b) and Germany (c)

Molecular analysis: DNA was isolated from three legs per specimen (n = 49) using 5% chelex. A 784 bp long fragment of the mitochondrial cytochrome b was sequenced and these sequences were aligned and phylogenetically analyzed.

Results

Developmental study

Average larval length at every ADH (Fig 4) showed no difference between both populations (England and Germany) and between temperatures.

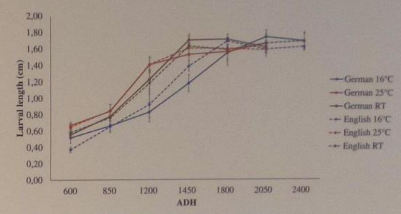

Fig 4. Comparison of developmental rate between both populations at three different temperatures

Wing morphometrics

Wing size variation in both populations showed significant difference at 0.05 level, the mean of centroid size and SD was 1271.05±5.78 (English) and 1227.05±5.83 (German). For the effect of sexes on size, both populations showed a significant difference ($p<0.05$) between male and female. The mean of centroid size and SD in English flies was 1220.56±7.19 (male) and 1307.63±7.22 (female), whereas German flies was 1157.04±6.69 (male) and 1285.65±6.31 (female). These value indicated that the wing size of females were slightly larger than males in both populations. Furthermore, comparison of wing size variation between 6 locations were performed in Table 2.

Table 2. Statistical analysis comparison of wing size variation between 6 locations

Location	CVG2	CVG3	CVE4	CVE5	CVE6
CVG1	0.005*	0.442	0.654	0.442	0.000*
CVG2		0.000*	0.004*	0.033*	0.386
CVG3			0.658	0.289	0.000*
CVE4				0.826	0.010*
CVE5					0.740

*significant difference at $p<0.05$

For wing shape variation, CVA of most geographic locations were highly significant different ($p<0.001$, $p<0.01$, and $P<0.05$), except between CVG1-CVG2, CVG2-CVE4 and CVE4-CVE5 (Fig 5). The results from DA revealed significant differences between 6 locations ($p<0.05$), except CVG2-CVE4, CVG2-CVE5 and CVE4-CVE5. The proportions of correctly classified *C. vicina* between each location was 94-100% (CVG1), 100% (CVG2), 99-100% (CVG3), 100% (CVG4), 95-100% (CVG5) and 96-100% (CVG6). Furthermore, cross-validation test showed 73-92% (CVG1), 67-89% (CVG2), 89-99% (CVG3), 35-100% (CVG4), 35-100% (CVG5) and 93-100% (CVG6).

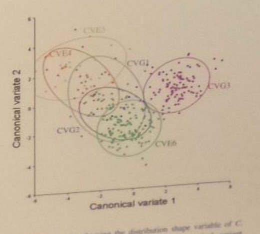

Fig 5. Scatter plot showing the distribution shape variable of *C. vicina* in each location along the first two canonical variant analysis (CV1= 57.53%, CV2= 26.85%) with 90% confidence ellipses

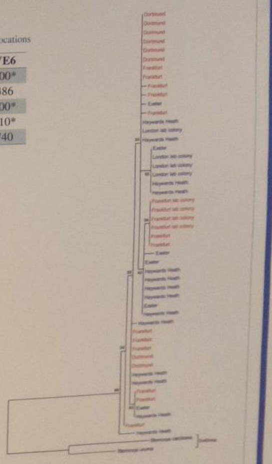

Fig 6. Molecular Phylogenetic analysis by Maximum Likelihood method. ... Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter=200.0000)). ... drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 51 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. ... There were a total of 492 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2015).

Conclusion

The developmental study of *C. vicina* from different geographic populations showed similar larval growth rates at three different temperatures. Wing shape analysis obviously discriminated Frankfurt laboratory colony from London laboratory colony, but CVA showed overlapping areas for almost every location, including English and German populations. Molecular phylogenetic analysis by maximum likelihood method confirm the wing morphometrics data. It is quite easy to distinguish the Frankfurt laboratory colony from the majority of the London laboratory colony, but not possible to separate all populations from each other. One reason could be that laboratory colonies are often established since many generations, maybe leading to a loss of variation and increased inbreeding compared to the flies from the field populations. However, future studies will increase the number of locations, specimens and broaden the range of examined temperatures.

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METHODS FOR THE INVESTIGATION OF POST-FEEDING LARVAL DISPERSAL IN UK BLOW FLIES: PRELIMINARY RESULTS

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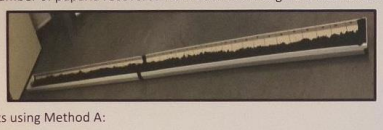
Introduction

In order to determine the minimum time since death in forensic investigations, it is essential to collect the oldest insect specimens associated with the body (Amendt et al. 2011). Often these are not found on the body itself, but have left to find a suitable pupariation site and, therefore, may be some distance from the body on which they were feeding (Lewis & Benbow 2011). A literature review revealed that much information is either contradictory or missing concerning the post-feeding larval dispersal behaviour of blow flies. This has highlighted the need for a more complete study to consider the many factors that may affect larval dispersal. Therefore, based on published experimental studies, multiple methods were explored to determine the most appropriate to study these factors. Although the results of this study are preliminary, some patterns have been observed, including the possibility of the aggregation of puparia.

Material and Methods

Three methods were tested and a total of eight experiments conducted.

Method A: Adapted from multiple studies looking at the post-feeding larval dispersal of blow flies (Greenberg 1990; Andrade et al. 2002). An acrylic channel was filled with soil, maximum 5cm deep. The soil used was taken from the Wildlife Garden at the Natural History Museum, London. Post-feeding *Calliphora vicina* larvae were introduced and left to pupariate. After one week the number of puparia recovered from each 10cm segment was recorded.



Experiments using Method A:

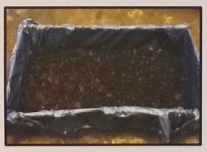
A1 and A2. The channel was 4m long, 6cm wide and 12cm deep. 100 larvae were introduced at the midpoint of the channel.

A3. The channel was 6m long, 6cm wide and 12cm deep. 320 larvae were introduced at the midpoint of the channel.

A4. The channel was 6m long, 6cm wide and 12cm deep. 100 larvae were introduced at the midpoint of the channel.

A5. The channel was 6m long, 6cm wide and 12cm deep. 100 larvae were introduced to one end of the channel.

Method B: Adapted from a study conducted concerning the post-feeding larval dispersal of blow flies (Lima et al. 2009). A rectangular arena, 2.5x1m was filled with soil, approximately 5cm deep. The soil used was taken from the Wildlife Garden at the Natural History Museum, London. Post-feeding larvae were introduced and left for one week. After one week the number of puparia recovered from each 10x10cm quadrant was recorded.



Experiment using Method B:

B1. 200 post-feeding *Calliphora vicina* larvae were introduced to the centre of the arena.

Method C: Adapted from many studies examining the post-feeding larval dispersal of blow flies (Gomes et al. 2006; Charabidze et al. 2008; Zimmer et al. 2010; Boulay et al. 2016). A circular arena, 2.5m diameter, was filled with sawdust approximately 5cm deep. Post-feeding larvae were introduced to the centre of the arena. After one week the number of puparia recovered from each 30x20cm quadrant was recorded.

Experiments using Method C:

C1. 1780 post-feeding *Calliphora vicina* larvae were introduced to the centre of the arena.

Results

Figures 1, 2 and 3 are examples of the data produced by Methods A, B and C. Of most interest was the apparent aggregation of puparia, most clearly seen in Figures 1 and 3.

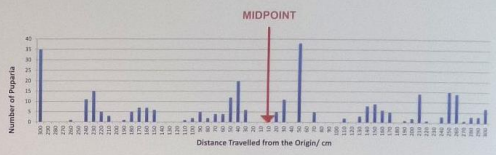


Figure 1. The distance dispersed from the midpoint by the larvae prior to pupariation in A3 (see Materials and Methods).

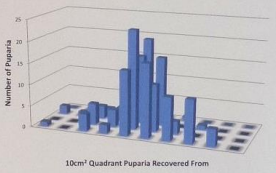


Figure 2. The number of puparia recovered from each 10x10cm quadrant in experiment B1.

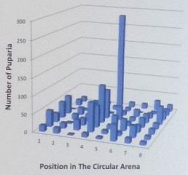


Figure 3. The number of puparia recovered from each 30x20cm quadrant in experiment C1.

To test the significance of these results, the data was analysed in frequency per sampling area.

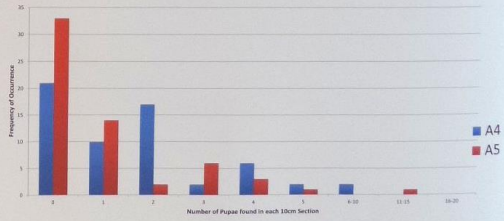


Figure 4. An example of the frequency of the number of puparia recovered from each section for experiments A4 and A5.

Aggregation was found to be highly significant in experiments A2, A3, A4, A5 and C1, $p<0.0001$ (χ^2 test) for all tests but A1.

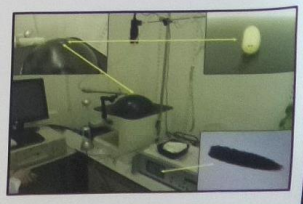
Discussion

Method A was considered best for determining distances travelled by larvae and Method C was most appropriate for measuring the distances travelled in conjunction with the directional preferences exhibited by the larvae. Method B was unsuitable as the arena was too small and had an uneven spread from the midpoint, and therefore did not have any advantages over Methods A or C.

These preliminary results provide evidence for aggregation of larvae for pupariation and this will be investigated further. Neighbourhood interactions of dispersing larvae and their perceived ability to detect other puparia in the pupariation substrate may be involved in the aggregation of puparia (Lima et al. 2009). However, other studies suggest that the larva's ability to detect neighbouring puparia and larvae in the pupariation substrate encourages them to move further to a less crowded section of substrate (Gomes & Zuben 2005).

Future Experiments

Other methods to be tested in the future will include the use of a SYNTECH ServoSphere. In response to larval movement detected by an overhead camera, the sphere rotates to maintain the larva in the camera's field of view at the top of the sphere. The movement of the sphere is recorded by the computer and, hence, enables the precise analysis of the movements of individual larvae (speed, direction and tortuosity).



Acknowledgements

Funding gratefully received from the Royal Entomological Society CPF (MMM), University of Winchester 175th Anniversary Fully Funded Studentship (MMM), University of Winchester RKE (APW), Natural History Museum (MJRH).

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USE OF CARNIVORE CARRION RESOURCES BY ARTHROPOD AND VERTEBRATE SCAVENGER COMMUNITIES IN WILD HABITATS OF SOUTHEAST SPAIN.

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Introduction

Understanding carrion decomposition and the factors affecting its variability is crucial for strengthening and improving the accuracy and reliability of forensic investigations of death; this is particularly important in forensic entomology casework as basic knowledge of carrion ecology can minimize the error in minimum post-mortem interval estimations (Tomberlin *et al.*, 2011). However, there remains much to be done on the study of arthropod communities and carrion decomposition from an ecological perspective. For instance, it is known that vertebrate scavengers accelerate carrion decomposition rates (Barton *et al.*, 2013), but studies on carrion insect succession typically exclude this factor by preventing the access of vertebrates to the experimental carcasses. Moreover, while scavenging studies of vertebrate carrion have focused on herbivore and omnivore carcasses, carnivore ones have been largely neglected. Here we present the first preliminary results from a study aimed at yielding new insights into the partitioning of carnivore carrion resources amongst the arthropod and vertebrate scavenger communities in wild habitats of southeast Spain.

Iberian Peninsula

Study areas

Material and methods

Data were collected from 20 red fox (*Vulpes vulpes*) carcasses, placed at two mountainous areas ("Sierra Noroeste" and "Sierra Espuña"; Mediterranean climate) in the Region of Murcia (SE Spain) from mid-winter to early spring 2016 (February-March). Within each area, inter-carcass distance was >1km. Carcasses were attached to trees or rocks by a wire camouflaged with soil and vegetation, allowing access to both insect and vertebrate scavengers. Insects were collected manually whereas vertebrate scavenger activity was monitored during the first seven days by camera-traps (Bushnell Trail Scout and Bushnell Trophy Cam) placed close to each carcass and programmed to take photos every minute provided they detected movement.

Carcasses were examined to collect insects on four occasions: 3, 7, 29-35 and 56-65 days after placing carcasses. All specimens were kept in ethanol 70%, and, in the case of the larvae and eggs of Diptera, they were fixed in hot water (>80ºC) for 30–60 seconds prior to preservation in ethanol. To estimate the relative abundance of immature stages of Diptera, as well as adults and pre-adult stages of Coleoptera, in each carcass we visually recorded the number of individuals per family according to the following categories: N0 (family not detected), N1 (only one individual), N2 (2-10 individuals), N3 (11-100 individuals), and N4 (>100 individuals).


Camera - trap

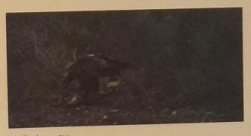

Blow flies

Results

Vertebrate scavengers

In Sierra Espuña, we recorded five vertebrate scavenger species: red fox, wild boar, stone marten (*Martes foina*), wild cat (*Felis silvestris*), and golden eagle (*Aquila chrysaetos*) (mean: 1.2 spp/carcass). In Sierra Noroeste, red fox carcasses were visited by three vertebrate scavenger species: red fox, wild boar (*Sus scrofa*), and magpie (*Pica pica*) (mean: 0.3 spp/carcass). However, only one carcass was partially consumed (by golden eagle in Sierra Espuña).


Sus scrofa


Aquila chrysaetos

Arthropod scavengers

Diptera, Coleoptera and Hymenoptera were the three insect orders identified during the decomposition process of the studied red fox carcasses, including 11 families (Diptera other than Calliphoridae family are pending of identification; see Fig. 1).

Diptera. Blow flies (Calliphoridae) were the first colonisers in all carcasses both in "Sierra Espuña" and "Sierra Noroeste", being *Calliphora* the most abundant genus, with both *Calliphora vicina* and *C. vomitoria* species developing on the carcasses (Fig. 2).

Coleoptera. Seven families were detected in the study areas. Staphylinidae and Silphidae were especially abundant in Sierra Espuña and Sierra Noroeste, respectively. The relative abundance of Histeridae, Dermestidae, Cleridae and others (Fig. 2) was similar in both areas.

Hymenoptera. At the first two visits, families Vespidae, Braconidae and Formicidae were collected in both study areas. The presence of Hymenoptera during the subsequent visits was variable: Vespidae was detected at third check only at Sierra Espuña and Formicidae at fourth visit in both study areas (Fig. 2).

Figure 1. Diversity of families

	SIERRA ESPUÑA	SIERRA NOROESTE
CHECK 1	MINIMUM - MAXIMUM: 1 - 4 MEAN: 3.9 TOTAL: 5	MINIMUM - MAXIMUM: 1 - 4 MEAN: 1.7 TOTAL: 4
CHECK 2	MINIMUM - MAXIMUM: 2 - 5 MEAN: 3.7 TOTAL: 8	MINIMUM - MAXIMUM: 2 - 6 MEAN: 4.5 TOTAL: 10
CHECK 3	MINIMUM - MAXIMUM: 1 - 7 MEAN: 4.5 TOTAL: 8	MINIMUM - MAXIMUM: 3 - 6 MEAN: 3.7 TOTAL: 7
CHECK 4	MINIMUM - MAXIMUM: 3 - 6 MEAN: 4.4 TOTAL: 9	MINIMUM - MAXIMUM: 1 - 6 MEAN: 4.2 TOTAL: 7

Figure 1. Minimum, maximum, mean and total number of families detected in red fox carcasses during consecutive visits. Calliphoridae is the only Diptera family included (the rest of specimens belonging to other families are pending of identification).

Figure 2. Relative abundance of necrophagus insects

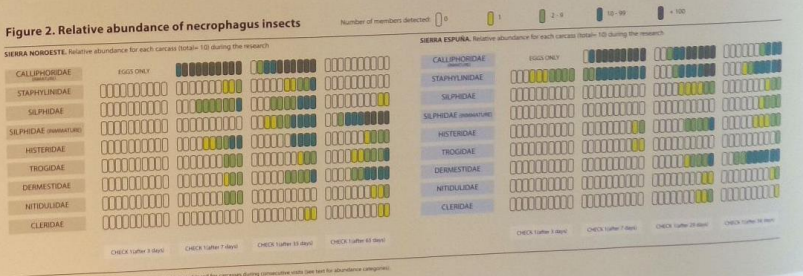


Figure 2. Relative abundance of Diptera and Coleoptera families detected in red fox carcasses during consecutive visits (see text for abundance categories).

Discussion

According to previous studies (Moleón *et al.*, in prep.), vertebrate scavengers, especially mammals, barely consumed red fox carrion. This favoured a greater carrion availability and persistence and, consequently, a more prolonged and wide use of this trophic resource by necrophagous insects. The predominance of adults and larvae of genus Calliphora (Diptera) coincided with another study developed in Murcia in the same season, which used chicken carcasses (Arnaldos *et al.*, 2001). However, in that study, *Calliphora* post-feeding larvae were detected between days 12–25 with no re-colonisation of the carcasses, while in our case new egg-layings were recorded during the successive visits and third-instar larvae were collected until day 56 and 35 in Sierra Espuña and Sierra Noroeste, respectively. These differences could be due to the different characteristics of carcasses (e.g., size and species) and should be considered in minimum post-mortem interval (minPMI) estimations.

In our study, within the family Staphylinidae (Coleoptera), there was a clear difference in species composition between areas, mainly due to the presence of smaller species in Sierra Espuña in comparison to Sierra Noroeste (Fig. 2). This result suggests possible differences in species distribution within the same region. If so, it could be used to detect eventual post-mortem relocations of carcasses and, thus, be of clear forensic utility. In the case of Silphidae, differences between areas were particularly evident in the fourth visit: in Sierra Espuña, silphid larvae were absent or virtually absent whereas calliphorid third-instar larvae were very abundant. Contrarily, in Sierra Noroeste, the number of silphid larvae feeding on carcasses was particularly high whereas no calliphorid larvae were observed (Fig. 2). Atypical cases of large masses of larvae of Silphidae consuming soft tissues have been reported when calliphorids were absent (Matuszewski *et al.*, 2010).

Although it has been suggested that calliphorid third-instar larvae are their preferential hosts (Reznik *et al.*, 1992), Hymenoptera parasitoids of Braconidae family were detected only during the first two visits, coinciding with the presence of first- and second-instar larvae.

Further studies on the interactions between invertebrate and vertebrate scavengers in natural conditions, conducted in different environments and seasons, and using carcasses of different species, should provide promising results to be applied in forensic science.

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ANATOMY OF A BUBBLE:

Understanding early pupal metamorphosis of blow flies and its significance in forensic entomology studies

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Introduction

The processes taking place during the intra-puparial period of blow flies are of special interest to forensic entomologists as this period lasts more than 60% of the total immature development; hence, it can be crucial for estimating a minimum post-mortem interval ($_{min}$PMI). Although the intra-puparial period is usually referred to as the "pupal stage", it actually includes three developmental stages—prepupa, pupa and pharate adult—with different durations. The pupa is, however, the stage which shows the most dramatic changes during metamorphosis, from an apparent headless and leg-less larva to a winged pharate adult fly. The current study aims to enhance our understanding of this fascinating process through the visualisation of the key morphological changes using X-ray images and micro-CT scanning reconstructions of prepupae and pupae of the blow fly *Calliphora vicina* Robineau-Desvoidy.

Material and methods

White prepupae (i.e. irreversibly contracted post-feeding larvae) reared under a constant temperature of 24°C ± 0.8ºC were considered as the time zero for this study. At 0, 3, 4, 6, 13, 18, 24 and 28 hours, 9–10 puparia were placed in a Nikon Metrology HMX ST 225 micro-CT scanner and imaged with an X-ray beam of 110kv and 203µA, passed through a 0.1 mm aluminium filter. Two 28 hours-old puparia were imaged every minute during 3.5 hours, in order to register the major morphological changes occurring during that time. Additionally, 5 puparia were collected at six-hour intervals during the first 48 hours after pupariation, killed in hot water, stained with 0.5 M iodine and scanned using the same micro-CT system (exposure: 500 ms; voltage: 110 kV; current: 100 µA). The resulting projections were reconstructed using CT-Pro 2.1 (Nikon Metrology, Tring, UK). Slice stacks in the three principal planes (cross, horizontal and sagittal) were rendered for each specimen using VG Studio Max 2.2 (Volume Graphics GmbH, Heidelberg, Germany) and segmented for 3D visualisations using SPIERS 2.20 (Sutton et al., 2012).

Results and Discussion

Micro-CT has been shown to be a powerful, non-destructive tool for visualising blow fly intra-puparial development for forensic purposes (Richards *et al.*, 2012). Here we use it to reveal the major, dramatic morphological changes, which occur during the early stages of metamorphosis. Despite the wide use of the term 'pupa' for any fly individual during its intra-puparial development, the actual pupal stage only lasts for a relatively short period of time, i.e. approximately between the first 7.5–30% of the total intra-puparial period (Figure 1). The pupal stage is delimited by apolyses, i.e. the separation of the epidermal cells from the old cuticle. Thus, before the completion of the larval-pupal apolysis the insect should be called *the prepupa*, and after the pupal-adult apolysis it should be called *the pharate adult* (Figure 1). The misinterpretation of these terms and concepts in developmental studies and analyses may lead to errors in $_{min}$PMI estimations (Martín-Vega *et al.*, in press).

The most observable event during the prepupal and pupal stages is the development of a gas bubble in the central part of the body, within the apoptotic larval tissues (Figure 1) and between the two main dorsal tracheal trunks, displacing the adult midgut to the ventral side (Figure 2). The gas bubble does not appear until 3 hours after pupariation (at 24ºC) and then grows increasingly in volume until reaching its maximum at approximately 24 hours after pupariation (Figures 3 and 4). In most imaged pupae the volume of the gas bubble stayed at maximum values during the following 4 hours, but then rapidly decreased until the total disappearance of the bubble (Figure 3). Indeed, X-ray images taken at 1 minute intervals showed that the total shrinkage of the gas bubble takes less than 5 minutes, as its contents move to the posterior end (Figure 4), likely along one or both dorsal trunks. The gas bubble plays an essential role in the transformation of the amorphous cryptocephalic pupa into the headed phanerocephalic pupa (Figures 1 and 4), as the gas moves between the puparium and the cryptocephalic pupa creating a space into which the head everts (Figure 4). The current study unveils blow fly metamorphosis and its explosive pace for the first time (Hall *et al.*, in prep.), as the cataclysmic eversion of the head lasts for only a very few minutes (Figure 4). A correct understanding of blow fly developmental biology and its concepts is pivotal for strengthening forensic entomology research and for avoiding confusions resulting in inaccurate $_{min}$PMI estimations.

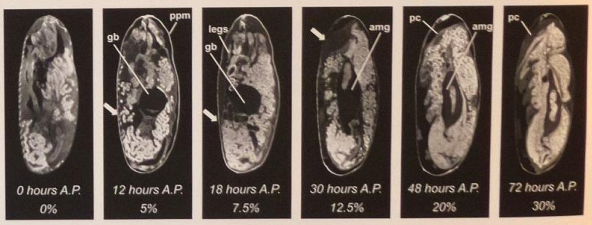

Figure 1. Micro-CT virtual sagittal sections of the blow fly *Calliphora vicina* Robineau-Desvoidy at different times after pupariation (A.P.) at a constant temperature of 24ºC. Percentages in the figures refer to the total intra-puparial period, i.e. until adult emergence. At pupariation (0 hours A.P.), the prepupa is still attached to the puparium, i.e. to the third-instar larval cuticle. 12 hours A.P., larval-pupal apolysis is taking place but it is still not complete, as the epidermis is still attached to the puparium in some areas of the abdominal region (arrow). 18 hours A.P., larval pupal apolysis is complete as the epidermis has detached from the puparium over the body (arrow); the legs and wings have partially everted and the prepupa has become the cryptocephalic pupa. 30 hours A.P., the head has everted (arrow) although it will maintain a hyaline appearance until the migration of the fat bodies. The cryptocephalic pupa has transformed into the phanerocephalic pupa. 48 hours A.P., the adult epidermis has detached from the pupal cuticle only in some areas; the pupal-adult apolysis is still not complete. 72 hours A.P., the pupal adult apolysis is complete as the pupal cuticle has detached over the body; the insect is now a pharate adult, i.e. no longer a pupa. Abbreviations: amg, adult midgut; gb, gas bubble; pc, pupal cuticle; ppm, puparium.

Figure 2 (above). False-colour 3D reconstruction of puparium with gas bubble (blue) 24 hours after pupariation. Colour key. Green, apoptotic larval hindgut; purple, malphigian tubules; red, dorsal tracheal trunks; yellow, adult midgut.

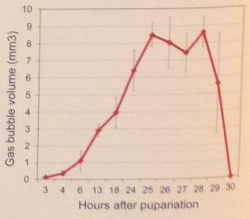

Figure 3 (right). Average volume ± STD of the gas bubble (n = 9–10) at different times after pupariation at a constant temperature of 24°C. Volumes were calculated as ellipsoids.

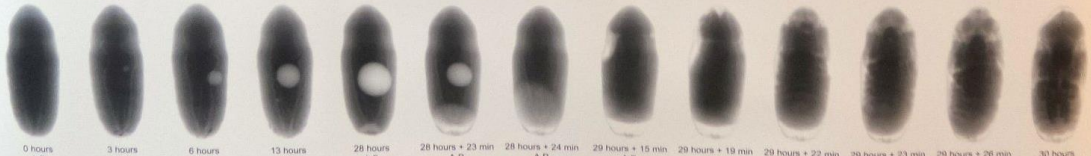

Figure 4. X-ray images taken at different times after pupariation (A.P.). Note how a small gas bubble is already formed 3 hours A.P., showing its maximum volume 28 hours A.P. The bubble disappears 28 hours and 24 minutes A.P. as the gas moves to the posterior end of the puparium and then around the pupa. Head eversion starts 29 hours and 19 minutes A.P. and is completed within just a few minutes. During the next minutes the abdomen sways while the legs fully extend.

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Acknowledgments

We are grateful to Farah Ahmed, Amin Garbout, Rebecca Summerfield and Dan Sykes (Imaging and Analysis Centre, NHM) for their help and assistance. The first author is supported by an EC funded Marie Curie Intra-European Fellowship (FP7-PEOPLE-2013-IEF n: 624575).

Estimation of post-mortem interval based on puparia of *Phormia regina* (Meigen) and larvae of *Necrodes littoralis* (L.) – A case report from Poland

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Background information

On 16th of July 2015, corpse of an adult man in advanced decomposition was found in the rubble located in an open area of the suburb of Śrem (Poland) (Fig. 1). The postmortem interval (PMI) established by the forensic pathologist was from 3 to 6 weeks. Entomological evidence was collected by the assistant prosecutor.

Insects collected

Various species and developmental stages were sampled. Among them, puparia of *Phormia regina* from the soil samples were the most developmentally advanced specimens of blowflies (Fig. 2). Moreover, third instar larvae of *Necrodes littoralis* were collected directly from the corpse (Fig. 3).

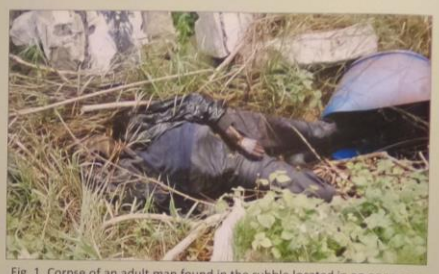

Fig. 1. Corpse of an adult man found in the rubble located in an open area.

PMI estimation based on puparia of *Phormia regina*

Estimation of pre-adult development time

↓

Thermal summation method and ADD calculation
(average daily temperatures from the nearby weather station)

32.2 days in 16.0°C	min 716.3 hrs	mean 789.8 hrs	max 863.3 hrs (in 16.1°C)
↓	↓	↓	↓
148.1 ADD	140.3 ADD	154.7 ADD	169.1 ADD
after Marchenko (2001)	after Anderson (2000)		

Lower threshold: 11.4°C	Uncorrected temperatures	Temperatures corrected /+ 1°C	Temperatures corrected/+ 2°C
Minimum PMI, time elapsed from oviposition till adult emergence (days)	34-36	29-32	22-25
PMI, minPMI + 2 days of pre-oviposition interval	36-38	31-34	24-27

PMI estimation based on larvae of *Necrodes littoralis*

Estimation of pre-appearance interval (PAI)

↓

after Matuszewski & Szafałowicz (2013) and Matuszewski & Mądra-Bielewicz (2016)

↓

estimated *c*: $PAI=10.47827+e^{[(8.80464)+(-0.41974)*T]}$;
fixed *c*: $PAI=8.3+e^{[(7.574822)+(-0.322031)*T]}$

Estimation of development interval

↓

Thermal summation method and ADD calculation after Dekeirsschieter (2012)

↓

Body length of the third instar larva 16.83 mm

↓

13 days in 18°C = 104 ADD

Lower threshold: 10.0°C	Uncorrected temperatures	Temperatures corrected/+ 1°C	Temperatures corrected/+ 2°C
Minimum PMI, time elapsed from hatching of 1st instar larva till appearance of 16.8 mm 3rd instar larva (days)	17-18	14-15	12-13
PMI, minPMI + PAI	37-40	31-34	28-29

Fig. 2. Puparia of *Phormia regina*.

	Uncorrected temperatures	Temperatures corrected /+ 1°C	Temperatures corrected/+ 2°C
	36-38	**31-34**	**24-27**
Phormia regina	June 9-June 11	June 13-June 16	June 20-June 23
	37-40	**31-34**	**28-29**
Necrodes littoralis	June 7-June 10	June 13-June 16	June 18-June 19

Fig. 3. Third instar larva of *Necrodes littoralis*.

Conclusion

Time elapsed since death was:

1. 36-40 days, therefore death occurred between 7 and 11 June 2015 (uncorrected temperatures) or
2. 31-34 days, therefore death occurred between 13 and 16 June 2015 (temperatures corrected by 1°C) or
3. 24-29 days, therefore death occurred between 18 and 23 June 2015 (temperatures corrected by 2°C).

Taking into consideration all calculations made, it can be estimated that death occurred between **7 and 23 June of 2015** and most probably between **13 and 23 June of 2015.**

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The history of medicolegal entomology in Brazil

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Introduction

With its enormous territory and wide variation in abiotic and biotic factors, which allow for a wide variety of carrion-associated entomofauna, Brazil has great potential for studies in medicolegal entomology. In addition, high crime rates in some Brazilian metropoles account for many corpses and a consequent demand for forensic-entomology tools.

Inspiration

This report on the development of medicolegal entomology in Brazil was inspired by two major sources: first, the comprehensive paper by JRPL and colleagues (2008), which marked the centenary of the first medicolegal studies in this country. Second, the chapter by TCM and WACG (2015) in "Forensic Entomology: International Dimensions and Frontiers", edited by JK Tomberlin and ME Benbow. In this chapter, the authors provided a general overview of the history, accomplishments, and challenges of forensic entomology in South America, especially in Brazil.

The beginning

Medicolegal entomology in Brazil began with the work of Oscar Freire (**Fig.1**) in 1908. Freire presented the first Brazilian collection of necrophagous insects, as well as the results of his research on arthropod fauna associated with human remains and carcasses of small animals, to the Medical Society of Bahia (Northeast Brazil). In November 1908, Roquette-Pinto (**Fig.2**) published a paper entitled "Nota sobre a Fauna Cadaverica, no Rio de Janeiro". In this paper, the author stated that estimating the date of death using the succession of insects on a corpse, as proposed by Mégnin, would produce inaccurate outcomes in tropical zones.

Fig.1. Oscar Freire

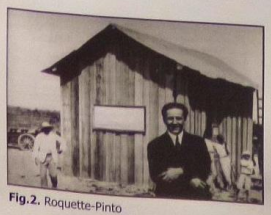

Fig.2. Roquette-Pinto

Financial support: FAPESP

Milestones

1911: Hermann Luederwaldt (**Fig.3**) published studies on beetles of forensic importance in São Paulo;

Fig.3. Luederwaldt in the garden of the Museum of Zoology (University of São Paulo)

1914: Freire published *Algumas Notas para o Estudo da Fauna Cadaverica na Bahia*, with observations on Muscidae, Calliphoridae, and Sarcophagidae;
1919: Belfort Mattos published a doctoral thesis on the genus *Sarcophaga* in São Paulo;
1923: Freire's posthumously published *Fauna Cadaverica Brasileira* provided a comprehensive inventory of insects collected from carrion;
1926: Luederwaldt published *Observações Biologicas sobre Formigas Brasileiras Especialmente do Estado de São Paulo*, with important contributions to the field of forensic myrmecology;
1941: Pessôa (**Fig.4**) and Lane (**Fig.5**) published an article on necrophagous beetles, mainly Scarabaeidae.

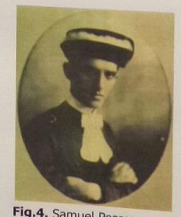

Fig.4. Samuel Pessoa

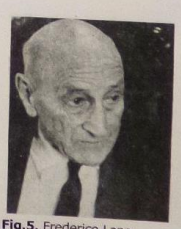

Fig.5. Frederico Lane

After four decades (1940-1980) with virtually nothing published on forensic entomology *sensu stricto* in Brazil, Monteiro-Filho and Penereiro (1987) reported on the decomposition of, and insect succession on, small rodent carcasses in a secondary forest in the state of São Paulo. Since then, research on medicolegal entomology in the country has continued.

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Application of wing morphometric and terminal restriction fragment length polymorphism (T-RFLP) analysis for species identification of forensically important flies

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Institute of Legal Medicine Frankfurt am Main

Introduction and Objective

Correct species identification is an initial step in forensic entomology e.g. for accurate post-mortem interval estimation as each species has its own developmental rate. Several identification techniques based on morphological and molecular characters. However, the classical use of morphology requires expert knowledge for correct identification, especially immature stages are quite difficult to identify on species level because of a lack of comprehensive keys for all life stages. In this study, we applied the use of wing morphometric as well as terminal restriction fragment length polymorphism (T-RFLP) techniques to facilitate species identification of forensically important blow flies for non-experts in entomology.

Wing morphometric analysis

Materials and Methods

Right wing of 12 blow fly species (Chrysomya chani, Chrysomya megacephala, Chrysomya nigripes, Chrysomya pinguis, Chrysomya rufifacies, Chrysomya villeneuvi, Lucilia cuprina, Lucilia papuensis, Lucilia porphyrina, Lucilia sinensis, Hemipyrellia ligurriens, and Hemipyrellia pulchra) were removed, mounted with Permount mounting medium, and photographed using a digital camera attached to a stereomicroscope at 1.5X magnification.

All images were built tps file using TpsUtil V.1.64 software and digitized 19 landmarks followed Hall et al. (2014) (Fig. 1) using TpsDig2 V.2.22 software. Each wing was digitized twice in order to reduce the measurement error.

Fig. 1 Nineteen landmarks plotted on the right wing followed Hall *et al.* 2014.

Morpho J software

Generalized Procrustes analysis, Canonical variate analysis (CVA) using for species discrimination, Discriminant function analysis (DFA), and cross-validation test (CV) using for assignment the specimen to the correct species and sexes. The significance of species discrimination and sexual shape dimorphism were performed in a permutation test with 10,000 replications.

Results

Species discrimination

- CVA: wing shape of all species (see fig. 2 for names and abbreviations) were highly significant different (P<0.001, P<0.01, and P<0.05).
- DFA: wing shape between most species were highly significant different (P<0.0001 and P<0.01), except HL/HP, HP/LC, HP/LPA, HP/LPO, HP/LS, LC/LS, and LPO/LS (P>0.05). The proportions of correctly classified specimens in each species was generally high ranged from 98.1% (CM) to 100% (CC, CP, CN, CR, CV, LC, LPA, LPO, LS, HL, and HP).
- CV: The accuracy of correctly classified specimens in Chrysomya species ranged from 90.6% (CN) to 100.0% (CC, AV), in Lucilia species ranged from 71.9% (LPA) to 83.3% (LPO), and in HL and HP were 87.5% and 33.3%, respectively.

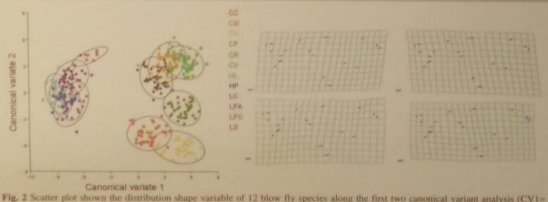


Fig. 2 Scatter plot shown the distribution shape variable of 12 blow fly species along the first two canonical variant analysis (CV1= 52.86%, CV2= 17.61%) with 90% confidence ellipses. Transformation grids illustrate the shape changes from overall average along CV1 and CV2 axes in both positive (left) and negative (right) directions. CC: *Chrysomya chani* (n=40); CM: *Chrysomya megacephala* (n=53); CN: *Chrysomya nigripes* (n=32); CP: *Chrysomya pinguis* (n=39); CR: *Chrysomya rufifacies* (n=47); CV: *Chrysomya villeneuvi* (n=39); HL: *Hemipyrellia ligurriens* (n=32); HP: *Hemipyrellia pulchra* (n=3); LC: *Lucilia cuprina* (n=29); LPA: *Lucilia papuensis* (n=32); LPO: *Lucilia porphyrina* (n=18); LS: *Lucilia sinensis* (n=8).

Sexual shape dimorphism

DFA: wing shape between males and females of all species were highly significant different (P<0.0001 and P<0.05), except HP (only female). The proportions of correctly classified specimens for males in all species were 100.0%, while correctly classified specimens for females were 85.7% (LPO) and 100% (CM, CC, CP, CN, CR, CV, LC, LPA, LS, and HL).

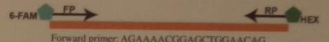
CV: The accuracy of correctly classified specimens for males ranged from 62.1% (CP) to 100.0% (CC, LPO, LS), while the accuracy of correctly classified specimens for females ranged from 50% (CP) to 100% (CC).

T-RFLP analysis

Materials and Methods

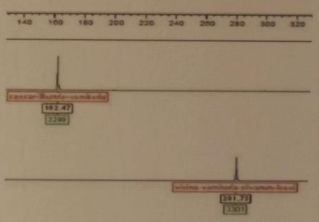
DNA was extracted from the head of adults (*L. caesar* and *L. illustris*) or middle part of 2nd and 3rd instar larvae (*Lucilia ampullacea*, *Lucilia sericata*, *Calliphora vicina*, *Calliphora vomitoria*, and *Phormia regina*) using 5% Chelex-suspension.

A fragment of the mitochondrial COI gene (452 bp) was amplified using two differently fluorescence-labeled forward and reverse primers.



Forward primer: AGAAAACGGAGCTGGAACAG
Reverse primer: TCAGGAAAGAAGGAAACATTTG

Labeled PCR amplicons were digested with a combination of five restriction enzymes (BpmI, BsaBI, BstEII, PstI and RsaI).



Labeled terminal restriction fragments (TRFs) were mixed with DNA size standard (CC5 ILS 500) and detected by capillary electrophoresis using an ABI3100 genetic analyzer.

Each species has a specific combination of the two generated fragments (blue and green). Analysis and automatic species assignment was performed using GeneScan and Genotyper software.

Results

Table 1 Predicted and observed lengths of terminal restriction fragments (TRFs) of the mitochondrial COI gene (452 bp) of each blow fly species using a combination of five restriction enzymes.

Species	No. of specimen	Terminal restriction fragment length (TRFs) (bp)				Restriction enzymes				
		Blue peak (6-FAM-labelled)		Green peak (Hex-labelled)						
		Predicted	Observed	Predicted	Observed	BmpI	BsaBI	BstEII-HF	PstI-HF	RsaI
*L. caesar**	68	277	273.0±0.08	89	84.4±0.34	x	x	x		
*L. illustris***	69	277	273.1±0.04	166	162.9±0.37	x	x			
L. ampullacea	11	317	312.6±0.08	89	84.8±0.37			x	x	
L. sericata	26	317	312.5±0.09	135	135.1±0.20				x	
C. vicina	36	286	282.0±0.19	113	109.4±0.19		x			x
C. vomitoria	21	286	282.0±0.22	166	162.8±0.15		x			
P. regina	56	363	363.5±0.22	89	84.3±0.45			x		

*Total specimens of *L. caesar* were 74, including 68 of unambiguous (91.9%); 4 of ambiguous (5.4%) were the same TRFs to *L. illustris*, and green peak fragment of 2 samples shown at 173.98±0.04.
**Total specimens of *L. illustris* were 72, including 69 of unambiguous (95.8%) and 3 of ambiguous (4.2%) were the same TRFs to *L. caesar*.

Conclusion

- For wing morphometric analysis, wing shape of *Chrysomya* species were clearly separated from *Lucilia* and *Hemipyrellia* species (Fig. 2). Within *Chrysomya* species, the proportions of correctly classified from both DFA and CV were very high (>90.6%), while between *Lucilia* and *Hemipyrellia* species, wing shape largely overlapped (Fig.2) with a low percentage of corrected identification (33%-87.5%). Therefore, using landmark-based characterizations of wing morphology could separate each of *Chrysomya* species, but is not suitable for species discrimination of *Lucilia* and *Hemipyrellia* species. In addition, wing shape of all species clearly revealed sexual dimorphism. However, the accuracy of correctly classified for each sex was low in some species. Due to a small number of specimens, a further study will be carried out with more specimens to test the reliability of wing shape for species discrimination. Wing morphometric analysis is simple and a low cost technique, but it requires non-damaged wings for analysis. Furthermore, it's a time-consuming process in locating the landmarks for large scale study and the reliability of this technique might be low due to a low percentage of CV and the error/bias from manual digitization of each landmark point.
- For T-RFLP analysis, the present findings support its application as a reliable technique for species identification. T-RFLP analysis could even be used for the separation of the sister species *L. caesar* and *L. illustris*, although some specimens shared the same TRFs of both species. In comparison to PCR-RFLP, T-RFLP is rapid, sensitive, highly reproducible, and more accurate. However, it may be possible to misidentify different species when they are sharing the same TRFs. Therefore, the evaluation of molecular marker and restriction enzymes to discriminate between sequences of all flies with possible occurrence should be done carefully.

Reference

Hall, M.J.R., MacLeod, N. and Wardhana, A.H. (2014). Use of wing morphometrics to identify populations of the old world screwworm fly, *Chrysomya bezziana* (Diptera: Calliphoridae): a preliminary study of the utility of museum specimens. *Acta Tropica*, 138S, S49-S55.

Acknowledgements

Royal Golden Jubilee Ph.D. Program (PHD/0118/2556), Institute of Legal Medicine, Goethe-University Frankfurt, Department of Parasitology, Faculty of Medicine, Chiang Mai University, and Nina Feddern (Institute of Legal Medicine, Bern, Switzerland).

THE STUDY AND APPLICATION OF UNDERWATER DECOMPOSITION

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INTRODUCTION

While decomposition and insect succession on land has been studied in detail, the taphonomic processes and in particular insect succession underwater are not well documented. Whilst faunal succession in water is known to be different to that on land (Anderson 2002), few studies have attempted to quantify this difference. Insect succession is also known to vary according to geographic location (Campobasso *et al.* 2001, Amendt *et al.* 2004) therefore it is recommended that forensic entomologists collect their own data for the particular area they are working in. It is common to find human remains in water through accidents (such as swimming or boating accidents), body dumps following murders, and even suicide by drowning. Since Portsmouth is a coastal area with easy access to open water in several areas, this research aims to investigate and compare insect succession on land, in fresh water, and in salt water in order to improve the accuracy of post-mortem interval estimation in the

AIMS AND OBJECTIVES

1. To identify the sequence of invertebrate succession (if any) on pig (*Sus scrofa)* cadavers on land, in fresh water, and in salt water.
2. To place the research into context with other similar research and with practice by conducting interviews and questionnaires with forensic practitioners and others in relevant occupations.
3. To inform investigative strategy and police procedure on estimation of mPMI of cadavers found in outdoor aquatic environments.

Figure 1: Image showing the transparent lidded plastic boxes which house the experiments.

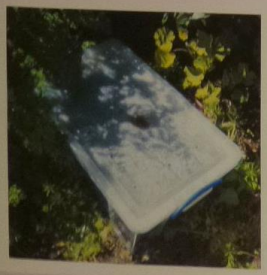

Figure 2: Holes were drilled in the lids of the plastic boxes to facilitate insect access whilst preventing scavenging by larger birds and mammals.

Figure 3: Image showing a rabbit carcass floating on the surface of the water.

PILOT STUDY

The experiments were set up in plastic boxes (Figs. 1-2). One box was filled with a layer of sediment and water retrieved from a local freshwater stream while the other was filled with a layer of sand and water from the sea at the south end of Portsea. A fresh gutted rabbit carcass was then placed inside each box (Fig. 3). Data loggers were placed nearby and used to monitor temperature and humidity. The carcasses were monitored and samples taken daily for the first 18 days then every other day until the carcasses sank below the surface of the water. Carcasses were then inspected visually every 1-2 weeks with samples taken whenever possible, until algal growth and products of decomposition prevented observation. At this point, the rabbits were removed from the water, samples were taken, and the decomposition state was recorded (Fig. 4). Samples in each case consisted of a water sample, insect samples from the surface of the carcass, the water, or the air. Water temperatures were also taken using an infrared thermometer and photographs were taken to record the state of decomposition. Monitoring of the carcasses in this way will continue until they have fully skeletonised. The skeletal remains will then also be monitored for a period of time.

Figure 4: Removal of the rabbit carcasses from the plastic boxes using a butterfly net.

PRELIMINARY RESULTS

The dominant species encountered in both the salt water and freshwater environments was *Calliphora vicina,* which has appeared throughout the decomposition period so far (Fig. 5). Appearances of *C. vomitoria* were limited to the early post-mortem period and species of Hymenoptera only appeared in the later stages after or at the point of full submersion of the carcasses.

- Neither carcass passed through the bloat stage due to being eviscerated.
- The carcass placed into the sea water initially appeared to be decomposing at a faster rate, with visible decay appearing on the head early.
- The carcass placed into the fresh water sank several weeks prior to the other, inhibiting insect access to the remains.
- Once removed from the water, it was found that the head of the salt water carcass had fully skeletonised while the body remained more intact. Both carcasses exhibited skin slippage and minor adipocere formation.

The water samples will be analysed for changes in the microfauna and diatom composition across the decomposition period using microscopy.

NEXT STEPS

On 24th March 2016, a pond was created in a woodland area near Wickham, North of Portsmouth. The pond was filled with tap water before being left to mature. At the end of May, a fresh piglet carcass (*Sus scrofa)* will be placed into the pond and allowed to decompose naturally in the manner previously described for the rabbit carcasses. The pond will be covered by a large piece of sturdy mesh which will be weighted down in order to prevent scavenging by large mammals or birds. Sampling and monitoring of temperature and humidity will take place as in the pilot study, with sampling initially taking place daily wherever possible, with the frequency of sampling gradually decreasing over the decomposition period.

Following a pilot study to assess how long the piglet carcasses take to decompose in the marine environment, nine piglet carcasses will be suspended inside crab traps from a raft at three different depths (shallow, mid-depth and sea floor) . The crab traps are pots made from sturdy plastic mesh which will prevent the carcasses from floating away and which will exclude larger scavengers in order to help retain the remains for the duration of the study. Sampling will take place in the manner previously described, with daily sampling wherever possible (depending on availability of the boat used to access the raft).

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Campobasso, C. P., DiVella, G., Introna, F. (2001) *Factors affecting decomposition and Diptera colonization,* Forensic Science International, 120, pp. 18-27

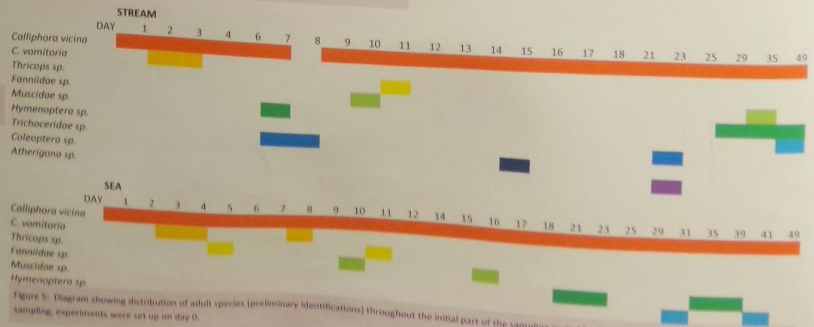

Figure 5: Diagram showing distribution of adult species (preliminary identifications) throughout the initial part of the sampling period for the pilot study. Day 1 denotes first day of sampling, experiments were set up on day 0.

University of Portsmouth

Institute of Criminal Justice Studies

Molecular Identification of Forensically Important Calliphoridae and Sarcophagidae Species Using ITS2 Nucleotide Sequences

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▪ Background & Purpose

The application of insect evidence to forensic investigations is mainly based on the estimation of postmortem interval and the identification of insect species from samples that are collected from the crime scene. Due to the limited number of expert taxonomists, species identification is one of the major barriers for crime scene investigators to utilize forensic entomology. Therefore, the molecular identification of species, using mitochondrial cytochrome c oxidase subunit I (COI) gene, has been suggested as an alternative strategy. However, in some cases, these maternally inherited markers cause confusion; hence, nuclear DNA markers such as internal transcribed spacer 2 (ITS2) are also required as supporting tools.

▪ Results

Eleven Calliphoridae and 5 Sarcophagidae fly species collected in Korea were successfully distinguished using internal transcribed spacer 2 (ITS2) nucleotide sequences.

	Cl	Gr	La	Vi	Mg	Pi	Re	Am	Ca	Il	Se	Al	Hm	Me	Pg	Si
Cl																
Gr	0.120															
La	0.059	0.094														
Vi	0.036	0.118	0.060													
Mg	0.159	0.175	0.144	0.153												
Pi	0.169	0.182	0.154	0.163	0.019											
Re	0.198	0.228	0.178	0.192	0.131	0.113										
Am	0.301	0.343	0.277	0.272	0.247	0.248	0.287									
Ca	0.310	0.358	0.289	0.283	0.262	0.266	0.300	0.091								
Il	0.313	0.361	0.292	0.286	0.260	0.265	0.298	0.090	0.003							
Se	0.283	0.316	0.265	0.270	0.239	0.241	0.310	0.183	0.178	0.182						
Al	0.294	0.295	0.267	0.260	0.262	0.255	0.283	0.289	0.331	0.334	0.322					
Hm	0.292	0.281	0.271	0.261	0.258	0.250	0.285	0.261	0.318	0.321	0.303	0.037				
Me	0.280	0.286	0.264	0.252	0.261	0.253	0.264	0.286	0.295	0.298	0.310	0.028	0.050			
Pg	0.258	0.274	0.238	0.236	0.243	0.243	0.252	0.315	0.324	0.322	0.314	0.087	0.108	0.090		
Si	0.270	0.282	0.242	0.253	0.239	0.233	0.280	0.270	0.310	0.314	0.290	0.030	0.048	0.033	0.060	

Table 1. The average interspecific sequence distances between 11 Calliphoridae and 5 Sarcophagidae fly species. The interspecific distances were all above 0.019, except for that between the sister species, *L. illustris* and *L. caesar* (0.003).

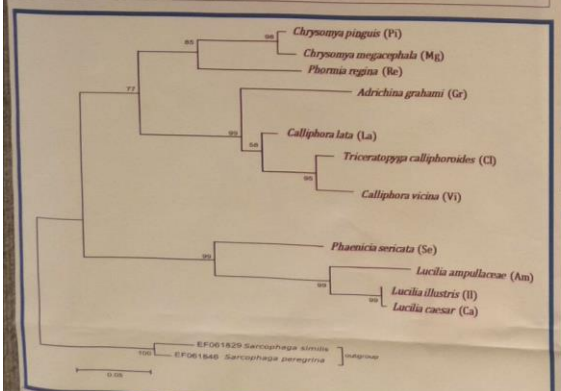

Figure1. The phylogenetic tree of Calliphoridae fly species The phylogenetic tree of Calliphoridae fly species showed no species- or subfamily-level paraphyly, although the distinction of *L. illustris* and *L. caesar* was supported by a very short branch length and relatively low bootstrap value (58%). Two other sister species, *Chrysomya megacephala* and *Chrysomya pinguis* were clearly distinguished.

▪ Conclusion

- Eleven Calliphoridae and 5 Sarcophagidae fly species collected in Korea were successfully distinguished using ITS2 nucleotide sequences.
- The sister species, *L.illustris* and *L.caesar* were also distinguished, despite the very low level of interspecific diversity.
- However, when comparing with European samples, the sister species *L.illustris* and *L.caesar* couldn't be clearly distingushed. Therefore, further studies on European samples are required.

▪ Materials & Methods

◆ ***Fly collection***

- Calliphoridae and Sarcophagidae fly specimens were collected in Seoul from 2005 to 2014 using pork liver bait. A total of 143 individual flies (108 Calliphoridae and 35 Sarcophagidae) were identified.

◆ ***DNA extraction, polymerase chain reaction (PCR), and sequencing***

-Firstly, all specimens were photographed. DNA extraction was performed using Exgene™ tissue SV mini kit. DNA quality was checked on 1% agarose gel and concentration was measured using a DU650 or DU730 UV spectrophotometer. Fragments of approximately 439 bp were amplified by PCR using a 2720 Thermal Cycler. PCR conditions consisted of an initial denaturation step at 95°C for 11 min, followed by 35 cycles at 95°C for 30 s, 50°C for 30s, and 72°C for 1 min, and then a final elongation step at 72°C for 15 min. The bidirectional cycle sequencing reactions were performed using BigDye v3.1 Cycle Sequencing Kit according to the manufacturer's instructions. A pair of primers was used for PCR and direct sequencing (5'-GAACTGCAGGACACATGAAC-3' and 5'-CCTCCCCTCATTAATATGCT-3') and the sequencing products were analyzed using an ABI3730xl Genetic.

◆ ***Phylogenetic analysis and sequence comparison***

- Phylogenetic trees were generated by the maximum likelihood method with 1,000 replicates of bootstrapping based on the Tamura-Nei model using MEGA6 software. The heuristic search trees were obtained by the neighbor-joining method to a matrix of pairwise distance estimated using the maximum composite likelihood (MCL) approach.

11 Calliphoridae

	Species	Distance
Triceratopyga calliphoroides	Cl	0.000
Adrichina grahami	Gr	0.000
Calliphora lata	La	0.000
Calliphora vicina	Vi	0.000
Chrysomya megacephala	Mg	0.000
Chrysomya pinguis	Pi	0.000
Phormia regina	Re	0.001
Lucilia ampullaceae	Am	0.000
Lucilia caesar	Ca	0.000
Lucilia illustris	Il	0.000
Phaenicia sericata	Se	0.000

5 Sarcophagidae

	Species	Distance
Parasarcophaga albiceps	Al	0.000
Sarcophaga haemorrhoidalis	Hm	0.000
Helicophagella melanura	Me	0.001
Parasarcophaga similis	Si	0.000
Boettcherisca peregrina	Pg	0.001

Table 2. The sequence distances of ITS2 locus between the same species The sequence distances of ITS2 locus between the same species were 0.000 for 13 fly species and 0.001 for 3 fly species The nucleotide sequences of ITS2 locus between Calliphoridae and Sarcophagidae fly species generally had high interspecific and low intraspecific distances.

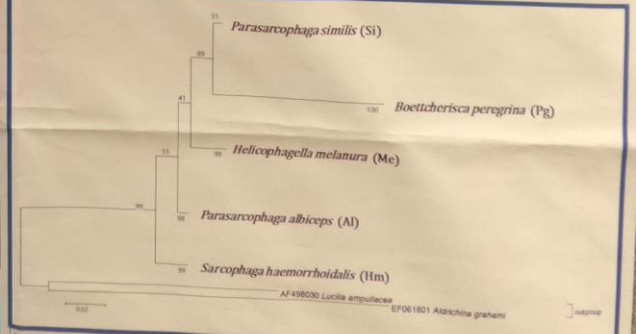

Figure2. The phylogenetic tree of Sarcophagidae fly species The phylogenetic tree of Sarcophagidae fly species demonstrated no species-level paraphyly .

▪ Acknowledgements

- This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) and funded by the Ministry of Education, Science, and Technology (2013R1A1A1012223) and the Korea University Research Fund (K1300061).

Calliphora vicina development under WIN 55,212-2 cannabionoid influence

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ABSTRACT

The blue bottle fly *Calliphora vicina* (Robineau-Desvoidy, 1830) is one of the most frequent species in the Basque Country (North of Spain) and an early colonizer of human remains.

We reared it under laboratory conditions to detect the potential influence of cannabinoids in combination with an anesthetic in the development of the maggots. Maggots were reared in rat carcasses that received an overdose of WIN 55,212-2 (synthetic cannabinoid) and Chloral hydrate (anesthetic). A control was euthanized with $CO2$ to prevent any chemical influence in blowfly development.

Three parameters were evaluated, development time, maggot size during development, and adult size at the end of it. We observed assortment in maggot size at the beginning of the development (D1-D4) in carcasses with drug; maggots increased their size after D5, and it did not affect to the final size of the adults. With this research we confirm that drug consume can influence insects development and may introduce an error in the estimation of the postmortem interval (PMI) based on the age of the maggots.

Therefore, **drugs should not be underestimated when PMI is based on period of insect activity (PIA)**

Introduction

Entomotoxicology studies the presence of drugs and toxins in insect tissues, even if conditions do not allow detecting them in cadaveric tissues. It also contributes to analyze possible effects of these substances in arthropod development, as they can alter and modify the postmortem interval (PMI) estimation.
Insects, as any other living being can be affected in their development and lead to miscalculations if this aspect is obviated.
Due to the increase of drug-related deaths (mainly heroin and cocaine) we chose a synthetic cannabinoid for the first study of entomotoxicology developed at the UPV/EHU.
The main objective of this research has been to determine whether the consumption of soft tissues with the presence of drugs metabolized by rats (*Rattus norvegicus* Berkenhout, 1769) can affect the development of the blue bottle *Calliphora vicina* (Robineau-Desvoidy, 1830) (Diptera, Calliphoridae).

Figure 1.
Cage for adults (left) and rearing containers (right)

The main objective of this research has been to determine whether the consumption of soft tissues with the presence of drugs metabolized by rats (*Rattus norvegicus* Berkenhout, 1769) can affect the development of the blue bottle *Calliphora vicina* (Robineau-Desvoidy, 1830) (Diptera, Calliphoridae).

Blowfly colonies *Calliphora vicina* (Robineau-Desvoidy, 1830) (Diptera, Calliphoridae) were based on field captured flies collected with selective traps and raised under controlled conditions in dependencies of Forensic Entomology Service, SGIKER UPV/EHU.

Materials y methods

Experimental model: RHC1-5: 5 laboratory rats (males) treated the synthetic cannabinoid WIN 55,212-2 and perfused with chloral hydrate before euthanasia;
RH01-5: second group of 5 rats only treated with chloral hydrate;
R001: the control received no drug or anesthetic and was sacrificed with CO_2.

Breeding conditions: Populations were maintained under controlled conditions in a rearing chamber Radiber AGP-1400- HR with light-dark cycles (L:D) 12:12; relative humidity (RH) of 75% and environment temperature of 20ºC.

Measurements: All developmental stages (eggs, larvae, pupae and adults) were measured using a stereo microscope Nikon SMZ1500.

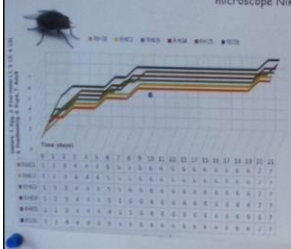


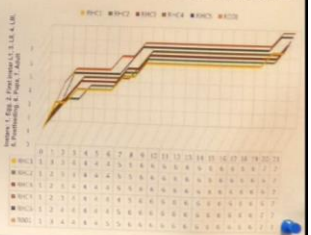
Figure 2. *Calliphora vicina* development throughout the study (21 days) in rats treated with chloral hydrate (RH01-5) versus control (R001).

A delay of 24 hours is observed in RH0 populations (RH02, RH04 and RH05) regarding control (R0) during LI>LII and LII > LIII, and from postfeeding LIII towards pupa in RH01, RH02 and RH05 populations.

Figure 3. *Calliphora vicina* development throughout the study (21 days) in rats treated with cannabinoid and chloral hydrate (RC01-5) versus (R001) control.

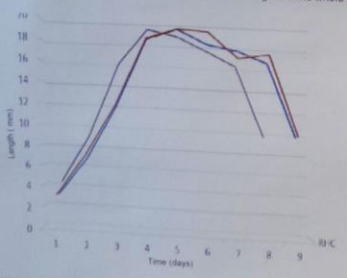
A delay of 24 hours is appreciated during LI>LII compared to control population (R001)

Figure 4. Mean development times of treated populations (RHC and RH0) *versus* control R001.



Population treated with cannabinoid and chloral hydrate shows clear time lapses regarding control, with delays of 24-hour at all instars. Population treated with chloral hydrate only shows delay during early stages.

Figure 5. Mean lengths of larvae, pupae, females (F) and males (M), of treated blowflies (RHC and RH0) against control population (R00) throughout the whole study (21 days).



We obtained smaller LIII from maggots reared on treated rats respect to the control population (R0).

Conclusions

Calliphora vicina development reared on an experimental rat model treated with synthetic cannabinoid and anesthetized with chloral hydrate prior to euthanasia confirmed how the pattern of development (body length and time) of the blowfly *C. vicina* (Diptera Calliphoridae) is altered udner the combined effect of both tested substances (synthetic cannabinoid and chloral hydrate). Development alteration is strengthened under the combined presence of both substances in soft tissues and can affect postmortem interval estimation (PMI).

Therefore, it is important not to obviate the possible presence of drugs in cadaveric tissues as its influence on insects' development may affect the life cycle, and introduce errors in the estimation of postmortem interval based on the period of activity of the insects collected during a crime scene investigation.

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IDENTIFICATION OF SOME FORENSICALLY IMPORTANT BEETLES (COLEOPTERA: SILPHIDAE) BASED ON COI GENE IN INDIA

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INTRODUCTION

1. Insects feeding on carrion form a distinct faunal succession associated with the various stages of decomposition .
2. Coleopterans tend to be associated with the later stages of decomposition, which is very important in terms of the dry bones of the body. But most researches have focused on flies, and beetles (Coleoptera) have been underemphasized.
3. The majority of forensically useful beetles lack morphological features specific to particular instars and in order to estimate postmortem interval from immature insects, it is necessary to accurately determine which instars are present on the corpse.
4. Silphidae is one of the forensically important families of beetles. They perform vital ecosystem functions by promoting the breakdown and recycling of organic matter into terrestrial ecosystems

MATERIAL & METHOD

1. All the collection was done by using bait traps Fig.1) .Specimens were picked by forceps and preserved in 95% alcohol in the field for further identification. Identification was done with the help of Stereo zoom microscope (Model No. RI-90-01) using relevant keys and photography was done with the help of Canon EOS 1200D.

2. For molecular purpose, CTAB DNA extraction method (Doyle & Doyle,1991) was used.

3. For amplification COI gene was used with universal primers (Table 1)

GENE	PRIMERS	DIRECTION	SEQUENCES	REFERENCE
COI	LCO1498	FORWARD	5' GGTCAACAAATCATAAAGATATTG G 3'	Herbert *et al.*, 2003
	HCO2198	REVERSE	5'TAAACTTCAGGGTGACCAAAAAATCA 3'	

Table 1. Gene and Primers used in this study

Fig. 1 Bait traps

Family	Genus	Species	Date of collection	Location	Coordinates
Silphinae	Necrophila	Necrophila ioptera	26-v-2014	Kullu, H.P.	31°57' 35.8"N 77°07' 21.8"E
		Necrophila rufithorax	20-v-2014	Gagret, H.P.	31°39' 47.8"N 76°04' 02.6"E 31°34' 24.8"N 76°03' 08.9"E
			30-v-2015	Fatherkot, Punjab	32°01' 11.14" N75°04' 58.54" E
		Necrophila cyaniventris	22-vi-2015	Kullu, H.P.	31°57' 35.8"N 77°07' 21.8"E
	Necrodes	Necrodes littoralis	19-vi-2014	Gagret, H.P.	31°39' 47.8"N 76°04' 02.6"E
			15-vi-2015	Kullu, H.P.	31°57' 35.8"N 77°07' 21.8"E
		Necrodes nigricornis	20-ix-2014	Gagret, H.P.	31°39' 47.8"N 76°04' 02.6"E
	Thanatophilus	Thanatophilus minutus	25-v-2014	Kullu, H.P.	31°57' 35.8"N 77°07' 21.8"E
Nicrophorinae	Nicrophorus	Nicrophorus nepalensis	29-v-2015	Kullu, H.P.	31°57' 35.8"N 77°07' 21.8"E

Table 2. Collected specimens of forensically important beetles along with their geographical coordinates.

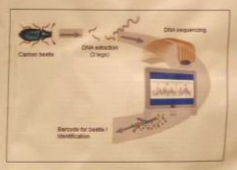
Thanatophilus minutus *Necrodes nigricornis* *Necrodes littoralis* *Necrophila rufithorax*

Necrophila cyaniventris *Necrophila ioptera* *Nicrophorus nepalensis*

RESULTS

1.Seven species of Silphidae were collected and identified during the sampling period, one Nicrophorinae: *Nicrophorus nepalensis* and six Silphinae: *Necrophila* (*Calosilpha*) *ioptera*, *Necrophila* (*Deutosilpha*) *rufithorax*, *Necrophila* (*Calosilpha*) *cyaniventris*, *Necrodes littoralis*, *Necrodes nigricornis* and *Thanatophilus minutus* from different localities of India (Table 2).

2. Out of 7 collected samples, 6 were successfully amplified with the mentioned primers. Rest of the work is in progress.



Molecular work plan

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ACKNOWLEDGEMENT—The current research was funded by Department of Science & Technology, government of India. The authors are grateful for help to Pavel Jakubec (Czech University of Life Sciences, Prague) and Javier Pérez Valcárcel for their aid in identification of the specimens.

Forensic flies of the Hungarian Natural History Museum

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History:

- forensic entomology is an important ecosystem service
- well-identified comparative materials are essential for forensic studies
- HNHM has one of the largest Diptera collection in Central Europe
- 1.000.000 specimens (specimens identified at least Family level and 75% of the specimens identified to species level)

Collecting trips:

1958 – Montenegro, Ferenc Mihályi
1967 – Finland, Ferenc Mihályi
1963, 1969 – Slovakia, Ferenc Mihályi
1974 – Austria, László Papp, Zsuzsa Bajza
1974 – Afghanistan, László Papp
1982 – Bulgaria, Ágnes Dely-Draskovits
1989 – India, László Papp
1990, 2013 – Czech Republic, László Papp
1994 – England, László Papp
2000 – USA, Mihály Földvári
2000 – Taiwan, László Papp
2003 – Taiwan, László Papp, Mihály Földvári
2004 – Thailand, László Papp, Mihály Földvári
2005 – Bulgaria, Mihály Földvári
2009 – South Africa, László Papp , Mihály Földvári
2009 – Vietnam, László Papp
2010 – Vietnam, László Papp, Gábor Lengyel, Zoltán Soltész
2012 – Thailand, László Papp

Important forensic fly families:

- Phoridae
- Sepsidae
- Heleomyzidae
- Piophilidae (incl.Thyreophoridae)
- Milichiidae
- Sphaeroceridae
- Scatophagidae
- Anthomyiidae
- Fanniidae
- Muscidae
- Calliphoridae
- Sarcophagidae

Zsuzsa Petrovics, Zoltán Soltész, László Papp

Current research activities:

taxonomy and systematics

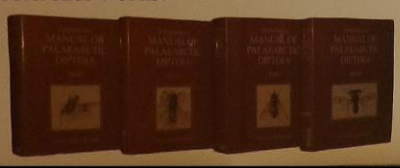
communities of small, ephemeral sources

blood sucking vector flies

pollination

forensic flies

Significant reference works:



Checklist and records of the most common sarcophagous Diptera from north Algeria

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INTRODUCTION

Knowledge of the carrion-breeding species presented in a location during a particular time is important and necessary to apply forensic techniques. Algeria is situated in north Africa, bordered by the Mediterranean Sea in the north. Data regarding the occurrence of sarcophagous insects are not available in Algeria, therefore, the aim of this work was to identify and inventory the most important sarcophagous Diptera in northern Algeria to improve fundamental knowledge regarding the distribution of these species.

MATERIAL AND METHODS

The specimens were collected between 2011 and 2015 on human corpses, animal carcasses and baited traps. Sample collection was performed according to procedures described by Amendt et al. (2007). Flies were morphologically identified using keys of Smith (1986) and Szpila (2010, 2012). Identification was confirmed at least by two specialists.

RESULTS

The most common identified species are depicted in Table I. Identified specimens belong mostly to the Calliphoridae family (fig. 1-3) followed by the Sarcophagidae (fig. 6) and the Muscidae families (fig. 4 and 5). seasons.

Table I: Sarcophagous Diptera species recorded between 2011 and 2016 from different regions of northern Algeria.

family	species	season
Calliphoridae	*Chrysomya albiceps* (Wiedemann, 1819)	spring, summer .
	Lucilia sericata (Meigen, 1826)	spring, summer , winter, autumn.
	Lucilia silvarum (Meigen, 1826)	spring, summer.
	Calliphora vicina (Robineau-Desvoidy, 1830)	winter, automne, spring.
	Calliphora vomitoria (Linnaeus, 1758)	Winter, spring.
Sarcophagidae	*Sarcophaga africa* (Wiedmann, 1824)	spring, summer , winter, autumn
	Sarcophaga carnaria (Linnaeus, 1758)	spring, winter, autumn.
Muscidae	*Muscina stabulans* (Fallén, 1817)	spring, summer , winter, autumn
	Hydrotaea (ophyra) capensis (Wiedemann, 1818).	spring, summer , winter, autumn.
	Musca domestica (Linnaeus, 1758)	spring, summer , winter, autumn.

Fig. 1. *Lucilia sericata* (Meigen, 1826) (Diptera : *Calliphoridae*).

Fig.2. *Chrysomya albiceps* (Wiedemann, 1819) (Diptera : *Calliphoridae*).

Fig. 3. *Calliphora vicina* (Robineau-Desvoidy, 1830) (Diptera: *Calliphoridae*).

Fig. 4; *Hydrotaea (ophyra) capensis* (Wiedemann, 1818) (Diptera : *Muscidae*)

Fig. 5. *Muscina stabulans* (Fallén, 1817) (Diptera : *Muscidae*).

Fig. 6. *Sarcophaga africa* (Wiedemann, 1824) (Diptera : *Sarcophagidae*).

CONCLUSION

This study presents the first report on necrophagous Diptera of northern Algeria. Data from this study provide basic information on carrion insects' fauna in Algeria. They also form a basis for similar studies in different geographical and climatic regions of Algeria.

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MEGASELIA SCALARIS (DIPTERA: PHORIDAE) ACTIVITY AT DIFFERENT TEMPERATURES IN LONG AND SHORT PHOTOPERIODS

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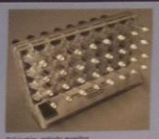
Abstract

Circadian clocks have evolved to synchronize physiology, metabolism, and behaviour to the 24-hour geophysical cycles of the Earth. The understanding of the circadian clock mechanism is a crucial element of forensic entomology because it is able to control routines such as locomotor activities, location of food sources, feeding, mating, ovipositing, and emergence times. Colonization of carrion and human cadavers by insects allows for the minimum Post Mortem Interval (mPMI) to be estimated. The prevailing opinion is that flies that are the first colonizers of a cadaver are not active during the night-time and therefore do not oviposit during this time. Determining the prevalence – if any – of nocturnal activity in forensically important flies, is fundamental for an accurate estimation of the mPMI.
Previous studies demonstrated that the scuttle fly *Megaselia scalaris* (Diptera: Phoridae) has nocturnal activity during the night and in dark conditions under 12:12 LD photoperiod in controlled condition.
In this paper we present the effects of longer 16:8 and shorter 8:16 photoperiods on the activity of this fly at 15, 20 and 25°C. Independently to the experimental temperature we demonstrated that after being entrained in long and short photoperiods, flies recover a 24hr cycle if maintained in dark conditions, confirming the role of the circadian clock in the activity of this fly. Experiments showed that the total amount of activity depend on temperature and that the evening peak is more pronounced than the morning peak. As in previous work, these experiments were also performed using Trikinetics technology, commonly used in *Drosophila* studies, which allows for factual data rather than observational data as reported in many articles.

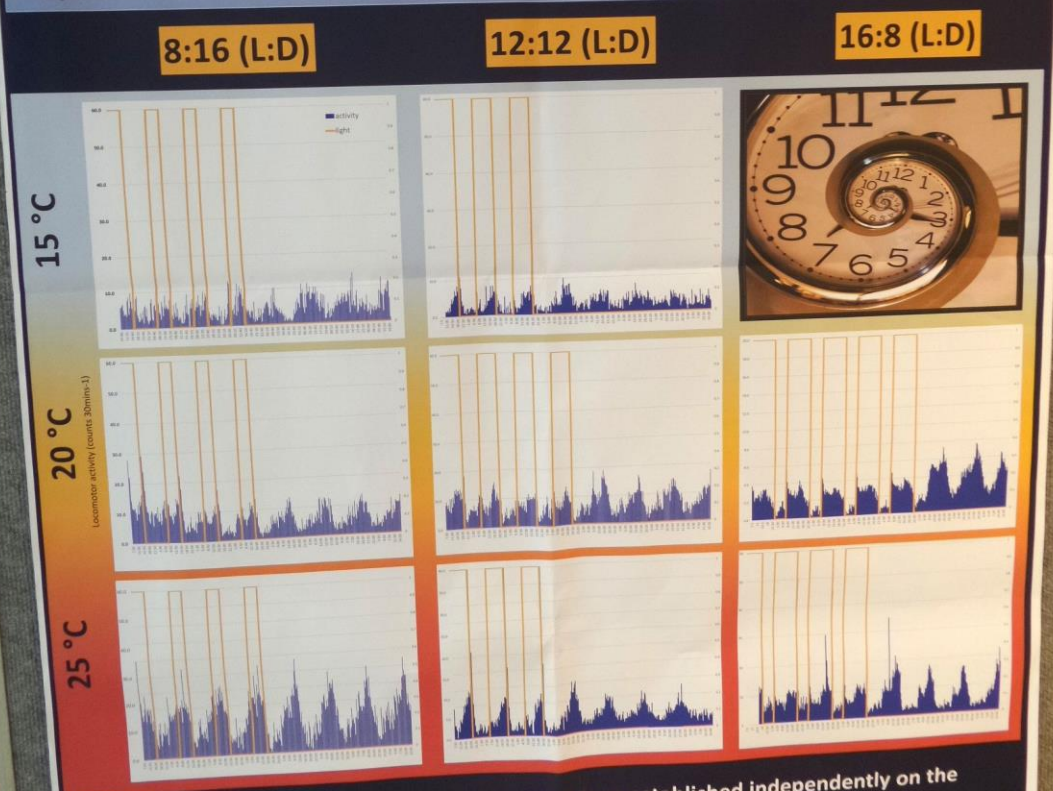
Megaselia scalaris adult fly.

Methods

Locomotor activity of *M.scalaris* was measured using Trikinetics *Drosophila* activity monitors (DAM) The monitors contain 32 tubes. Each tube is made from Pyrex glass with a diameter of 5 mm with a length of 65 mm; each tube houses an individual fly. An infrared beam (IR) is present across the tube midpoint, so when the fly travels through the middle of the tube, the IR beam is interrupted and an activity count is recorded by the systems host computer using the DAM system date acquisition software package (Trikinetics). Simultaneous and continuous monitoring of temperature, light intensity and humidity were made with recorders placed adjacent to the activity tubes. In order to understand if *M.scalaris* behaviour was under (circadian) clock regulation, we performed experiments running over eight days in which flies were entrained under LD 12:12, 16:8 or 8:16 conditions at the temperature of 15, 20 and 25 ºC for 3 days and then maintained in complete dark for five days .



Trikinetics activity monitor



In *Megaselia scalaris* the circadian rhythm (~24) is re-established independently on the training photoperiod. Temperature is only partially affecting the rhythmicity.
...what happen to a population inside a coffin? Can temperature function as a "Zeitgeber" in absence of light?

EFFECTS OF ETHANOL ON THE DEVELOPMENT OF *MEGASELIA SCALARIS* (DIPTERA: PHORIDAE)

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Abstract

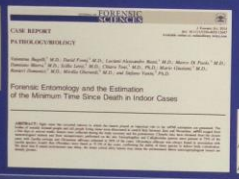
In Forensic Entomology the estimation of the age of insects is used for estimation of the minimum post mortem interval (mPMI). Insect development rate is mainly temperature dependent. However, several studies have demonstrated that drugs and other chemicals can affect the growth of larvae feeding on the dead body, leading to incorrect mPMI estimations. The presence of a high level of ethanol is associated with several deaths.

The aim of this study was to investigate the effect of ethanol on the development of *Megaselia scalaris*, a common species in indoor cases both in Europe and in the USA. This species is very important for mPMI estimation in indoor cases, as observed by the authors and reported in the literature.

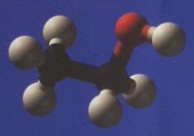
Larvae of *M. scalaris* were reared on pork liver (commercial pet food, pate') with four different concentrations of ethanol (0=control, 1ml, 2ml, 4ml on 32 g of maggot food). Ethanol affected the developmental time (larval eclosion delayed, larval development accelerated), but it did not affect the larval size (ANOVA, p=0.432) and the pupal size (ANOVA, p=0.946).

In conclusion this experiment demonstrated that ethanol has an effect on the immature developmental time of *M. scalaris* but not on the immature stage length, with consequences on the mPMI estimation.

.."Scuttle flies (Phoridae, *Megaselia scalaris*) were found in 3 cases (37.5% of the analyzed cases), confirming the ability of this species in indoor body colonization. The same percentage (36%) of *M. scalaris* in indoor cases has been reported by Goff (1991) that published a comparison between indoor and outdoor colonization.."..



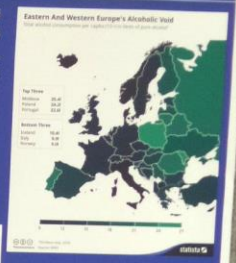
Forensic Entomology and the Estimation of the Minimum Time Since Death in Indoor Cases



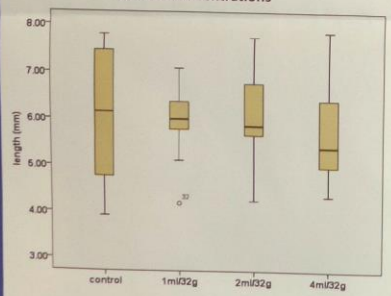
Ethanol, commonly called alcohol, ethyl alcohol, and drinking alcohol, is the principal type of alcohol found in alcoholic beverages. It is produced by the fermentation of sugars by yeasts and it is a neurotoxic, psychoactive drug, and one of the oldest recreational drugs. It can cause alcohol intoxication when consumed in **sufficient** quantity.

There is little data available about its detection from insects as reported in the following table.

		Insect species	Developmental stage	Analytical procedure	References
Alcohol	Ethanol	Calliphoridae, Sarcophagidae	Larva	HS GC–FID	Definis-Gojanovic et al, 2007
Alcohol	Ethanol	*Phormia regina*	Larva	HS GC–FID	Monthei, 2009

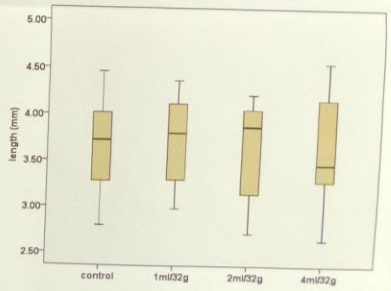


Length of III instar larvae bred on food with different ethanol concentrations



	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.722	3	.907	.922	.432
Within Groups	114.093	116	.984		
Total	116.814	119			

Length of pupae obtained from larvae bred on food with different ethanol concentrations



	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.084	3	.028	.124	.946
Within Groups	26.372	116	.227		
Total	26.457	119			

M. Definis-Gojanovic, D. Sutlovic, D. Britvic, K. Bozo Drug analysis in necrophagous flies and human tissues Arh. Hig. Rada Toksikol., 58 (2007), pp. 313-316

D.K. Monthei Entomotoxicological and Thermal Factors Affecting the Development of Forensically Important Flies Faculty of Virginia Polytechnic Institute, Blacksburg (2009) pp. 1-133

A COMBINED PROTOCOL FOR IDENTIFICATION OF MAGGOTS OF FORENSIC INTEREST

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INTRODUCTION

In Forensic Entomology the correct species identification is crucial in order to provide an accurate estimation of the mPMI (*minimum Post Mortem Interval*). Until now a morphological approach based on phenotipic keys has been used for species identification. In the last years a molecular approach has been developed and it is now frequently used especially for the identification of immature stages of insects due to the lack of identification keys. In the majority of cases the molecular identification is based on a destructive approach and, for this reason, depending on the legal system, it requires a specific authorization of the authority in charge of the case, especially when only a few larvae or insect fragments are available.
A combined protocol that allows the preservation of the larval exoskeleton and of the unused tissues in the same vial is here proposed. Their storage in ethanol, as preservative solution, will allow the repetition of both the molecular and morphological analyses and will reduce the risk of loss of the evidence.

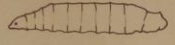
Calliphora vomitoria

Lucilia sericata

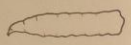
Musca domestica

Megaselia scalaris

Calliphoridae (~ 1.7-1.3 cm)



Muscidae (~ 10 mm)

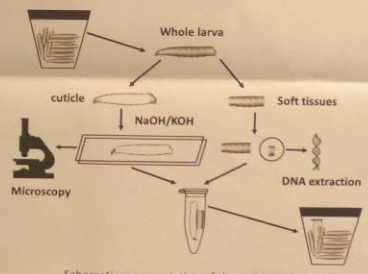


Phoridae (~ 6.5 mm)

MATERIALS AND METHODS

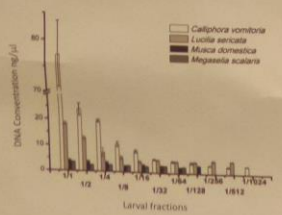
Post-feeding third instar larvae were collected in triplicate for each of the four forensically important fly species used for this experiment. *C. vomitoria*, *L. sericata*, *M. domestica* and *M.scalaris*. The outer cuticle was removed, diaphanised in a NaOH/KOH solution and stored in ethanol. After skin removal, soft tissues were cut using a surgical scalpel and then were progressively halved until the smallest fragment was obtained. **The smallest obtained soft tissue larval fraction was 1/1024 for *C. vomitoria*, 1/512 for *L. sericata*, 1/128 for *M. domestica* and 1/64 for *M. scalaris***

Diaphanisation methods
(a. Perforation; b. Szpila method; c. Presented method)



Schematic rapresentation of the presented method

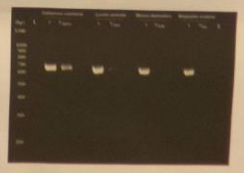
DNA quantification of each larval fractions of the four analysed species



RESULTS

DNA was positively extracted from all the fractions of all the species. A region of mtCOI gene 658 bp long was amplified and sequenced in order to perform the molecular identification of the species. Obtained results were in line with the previous morphological identification.

DNA extracted from *Calliphora vomitoria* larvae



DNA amplification of the maximum and minimum larval fractions

CONCLUSIONI

In the majority of the cases the molecular identification is based on a destructive approach related to the lost of the main morphological features of the samples. In this work the possibility to perform a molecular identification several times on the same specimen (larva) in a systematic way is demonstrated. At the same time the anatomical characters of the insects exoskeleton are preserved in order to allow the repetition of the morphological analysis, especially for the immature stages of Diptera. The reduced risk of sample loss provide the opportunity to repeat the analysis whenever requested by the authorities in charge of the legal process.

EFFECT OF FUR ON THE MICROBIAL AND ENTOMOLOGICAL COMMUNITIES ON RABBIT CARCASSES: FINAL RESULTS

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Abstract

Different factors, both intrinsic and extrinsic, have been reported affecting the decomposition of a carrion/body. These factors mainly interact with the speeds of the biological and chemical reactions happening after death. The biological reactions are mainly due to the activity of microorganisms and insects. Pigs (*Sus scrofa domesticus*) have been used as a model for human studies and the obtained results have been applied to other mammals without considering the effect that fur can have on the decomposition process and on the insect or microbial colonization. In order to investigate this point, rabbits (*Oryctolagus cuniculus*) with fur and without were used in two sets of experiments in Huddersfield in 2014 (summer) and in 2015 (spring). Entomological data for the first experiment revealed the presence of Diptera Calliphoridae (*Lucilia sericata*, *Calliphora vicina*, *Protophormia terranovae*), Muscidae (*Ophyra* sp), Sphaeroceridae (*Leptocera caenosa*, *Coproica vagans*, *Coproica hirticula*, *Coproica hirtula*), and Piophilidae (*Allopiophila vulgaris*); Hymenoptera Pteromalidae (*Nasonia vitripennis*); Coleoptera Cleridae (*Necrobia rufipes*) and Dermestidae (*Dermestes lardarius*). Differences in colonization time were observed only in spring: animals without fur were colonized two days before animals with fur. No significant differences were observed in summer. The microbial community was investigated using BIOLOG EcoPlate™ and by pyro-sequencing (spring data under analysis). The functional diversity of the bacterial community on all carcasses showed a big variability dependent on the stages of decomposition and the sampling region (mouth, skin, soil-carrion interface). The content of water seems to play the most important role in the bacterial community growth, whereas the presence or absence of fur does no affect the functional diversity (TWO WAY ANOVA: fur-no fur p=7.121; body regions p=0.00; interaction p=0.952). At the beginning of the sampling the bacterial community activity is very high in the mouth area, whereas the community activity at the interface soil-carrion is negligible. This community increased its diversity during the decomposition process through to the end of the experiment (4 months). The community on the exposed skin is a function of the drying process with a belt shape: limited diversity at the beginning and at the end of the decomposition process and a maximum during the active decomposition. At the phylum level four main *phyla* of bacteria were found among analyzed carrion. During Active Decay of the decomposition process Proteobacteria was the most abundant phylum (68.8%) followed by Bacteroidetes (33.17%), Firmicutes (13.89%) and Actinobacteria (3.49%). Over the decomposition Proteobacteria decreased becoming the second most abundant phylum (24.47%) during the Advanced Dry stage. Bacteroidetes increased becoming the most abundant phylum (66.83%). Also Actinobacteria increased (18.38%) while the amount of Firmicutes didn't change significantly. The analysis at the family level was able to highlight differences at the temporal scale but as well between carrion with and without fur.

Methods

A suitable experimental site was identified within the University on the roof of the school of Applied Sciences. This site has a single entrance and fence on both sides and was chosen based on the fact that there had been no recently decomposition events on that site in order to avoid cross contamination or previous saprophagous insect attraction.

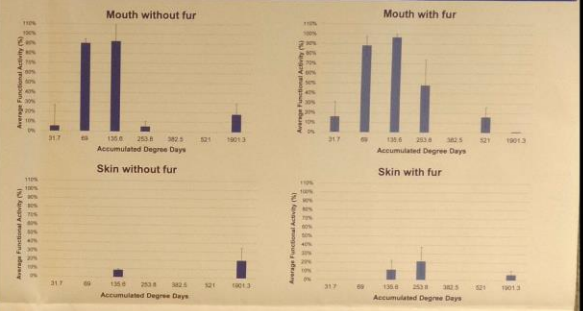
Experiment location (roof of the School of Applied Sciences, University of Huddersfield)

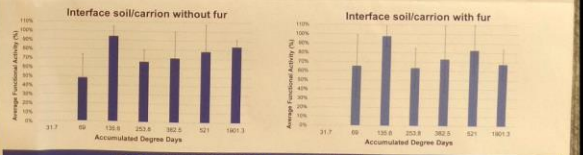
Materials

- Six frozen Rabbits (1.95 -2.60 kg) were used (3 with and 3 without fur).
- Plastic boxes with lids.
- Sterile Sand.
- Data logger.
- Digital weight scale.
- Single Swabs.
- Biolog EcoPlate .

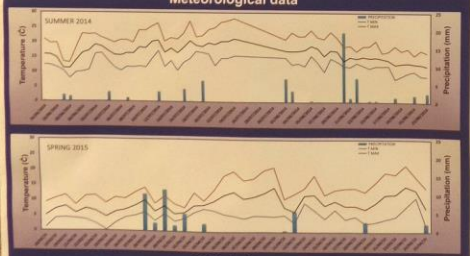
The Biolog EcoPlates contain 96 wells, three replicates of 31 different common carbon sources compounds and one water-only control well [(31+1)x3]. It provides for a functional fingerprint at community level.

Functional Activity results in summer

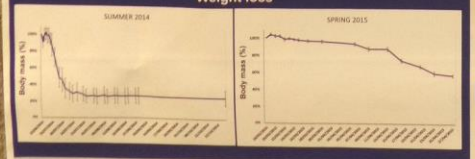




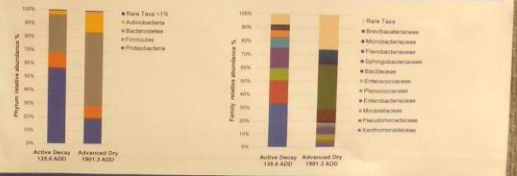
Meteorological data



Weight loss



Post mortem microbial taxa distribution in summer

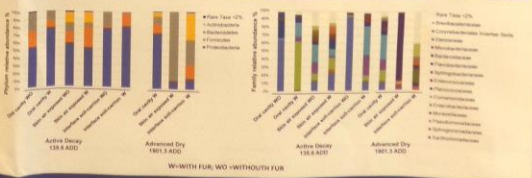


Entomological results

Order	Family	Species
Coleoptera	Dermestidae	*Dermestes lardarius*
Hymenoptera	Pteromalidae	*Nasonia vitripennis*
Diptera	Calliphoridae	*Calliphora vicina*
		Lucilia sericata
		Protophormia terraenovae
	Sphaeroceridae	*Leptocera caenosa*
		Coproica vagans
		Coproica hirticula
	Muscidae	*Hydrotaea* sp.
	Piophilidae	*Allopiophila vulgaris*

Allopiophila vulgaris

The effect of fur and body regions on taxa distribution



W=WITH FUR; WO =WITHOUTH FUR

Conclusions

The decomposition process, the insects colonization of the carcasses and the *post mortem* microbial communities mainly depend on the tissue water content. The average microbial functional activity is strictly related to the body regions of the carcasses and it does not depend on the presence of fur in both seasonal trials (summer and spring). The fur just delayed the insect colonization in spring experiment. In addition, the lowest activity was observed on the exposed skin samples due to its dry nature. Four bacterial *phyla* were identified as the main bio-markers of the decomposition (Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes) and their distribution significantly changed over time and space. No differences were observed in the *phyla* distribution depending on presence/absence of fur, while different patterns were observed at family level. These results show a temporal variation of the *post mortem* microbial community: this potential microbial clock could be applied in Forensic Veterinary field but....further research is needed.

NON-INVASIVE MOLECULAR APPROACH FOR ADULT FLY IDENTIFICATION

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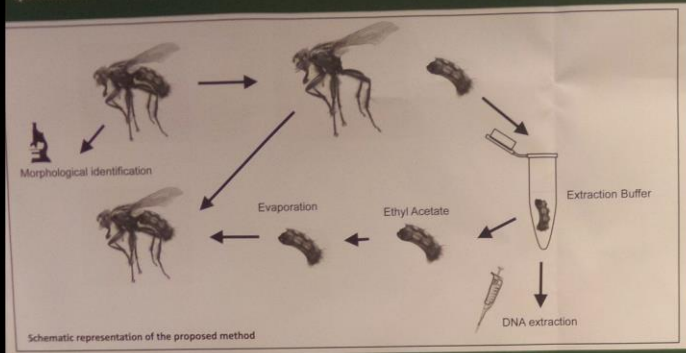
Introduction

A correct species identification is the key point for all the fields dealing with studies that involve insects. For these purposes, alongside the common morphological identification, a molecular approach, based on the characterization and analysis of specific mitochondrial or nuclear regions is becoming increasingly frequent. Despite the good results that can be reached, the molecular characterization is frequently identified as an invasive technique that often leads to the destruction of the sample. Twelve specimens of Sarcophagidae belonging to a private collection (SV) were tested for the non-invasive DNA extraction protocol.



Schematic representation of the proposed method

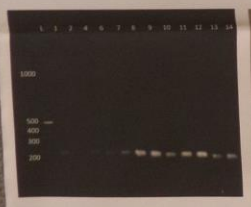
Materials & Methods

The abdomen of each fly was detached from the thorax and used for the molecular analysis. After the lysis step, each abdomen was dried on an absorbent paper, washed in Absolut Ethanol, passed in Ethyl Acetate before being re-attached to the rest of the pinned specimen in order to restore the original adult fly.

Gene	Length	Primers
COI	658 bp	FW : LCO1490 - Folmer et al., 1994 (5'-GGTCAACAAATCATAAAGATATTGG-3')
		RV : HCO2198 - Folmer et al., 1994 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3')
ND5	445 bp	FW : Zehner et al., 2004 (5'-CCAAAATATTCWGATCAHCCYTG-3')
		RV :Zehner et al., 2004 (5'-GGATTAACTGTTTGTTATWCTTTTCG-3')
COI short	211 bp	FW : 5'-GTAATTGTAACAGCTCATGC-3'
		RV : 5'-AACCAGTACCAGCTCCGTTT-3'

Results

Samples that showed a clear band of the appropriate molecular weight were purified and sequenced for species identification. DNA was successfully amplified from most samples using primers for ND5 (445 bp) and COI genes (211bp). 658 bp sequence of the COI gene was amplified only from "fresh" samples in contrast PCR performed with primers designed for a COI smaller inner region (211bp) showed positive results as well for old specimens.



Amplification of the 211bp COI smaller inner region

* = Agreement
C = Control
O.N.= Overnight

Sample	Year	Estraction	Amplify Gene	Specie
1	2015	Qiagen (O.N.) 40µl Prot K	ND5	*Sarcophaga variegata**
2	2011	Qiagen (O.N.) 40µl Prot K	ND5	/
3	2015	Qiagen (O.N.) 40µl Prot K	ND5	*Sarcophaga subvicina**
4	2011	Qiagen (O.N.) 40µl Prot K	ND5	*Sarcophaga crassipalpis**
5	2015	Qiagen (O.N.) 40µl Prot K	ND5	*Calliphora vomitoria** (C)
6	2011	Qiagen (O.N.) 40µl Prot K	ND5	*Sarcophaga crassipalpis*
8	2002	Qiagen (O.N.) 40µl Prot K	ND5	/
9	2013	Qiagen (O.N.) 40µl Prot K	ND5	*Sarcophaga variegata**
10	2013	Qiagen (O.N.) 40µl Prot K	ND5	*Sarcophaga variegata**
11	2004	Qiagen (O.N.) 40µl Prot K	ND5	/
12	2003	Qiagen (O.N.) 40µl Prot K	ND5	/
13	2004	Qiagen (O.N.) 40µl Prot K	ND5	*Sarcophaga carnaria**
14	2002	Qiagen (O.N.) 40µl Prot K	ND5	/

Sample	Year	Estraction	Amplify Gene	Specie
1	2015	Qiagen (O.N.) 40µl Prot K	COI	*Sarcophaga variegata**
2	2011	Qiagen (O.N.) 40µl Prot K	COI	/
3	2015	Qiagen (O.N.) 40µl Prot K	COI	/
4	2011	Qiagen (O.N.) 40µl Prot K	COI	/
5	2015	Qiagen (O.N.) 40µl Prot K	COI	*Calliphora vomitoria** (C)
6	2011	Qiagen (O.N.) 40µl Prot K	COI	/
8	2002	Qiagen (O.N.) 40µl Prot K	COI	/
9	2013	Qiagen (O.N.) 40µl Prot K	COI	/
10	2013	Qiagen (O.N.) 40µl Prot K	COI	/
11	2004	Qiagen (O.N.) 40µl Prot K	COI	/
12	2003	Qiagen (O.N.) 40µl Prot K	COI	/
13	2004	Qiagen (O.N.) 40µl Prot K	COI	/
14	2002	Qiagen (O.N.) 40µl Prot K	COI	/

Sample	Year	Estraction	Amplify Gene	Specie
1	2015	Qiagen (O.N.) 40µl Prot K	COI short	*Sarcophaga variegata**
2	2011	Qiagen (O.N.) 40µl Prot K	COI short	*Sarcophaga cruentata*
3	2015	Qiagen (O.N.) 40µl Prot K	COI short	*Sarcophaga subvicina**
4	2011	Qiagen (O.N.) 40µl Prot K	COI short	*Sarcophaga crassipalpis**
5	2015	Qiagen (O.N.) 40µl Prot K	COI short	*Calliphora vomitoria** (C)
6	2011	Qiagen (O.N.) 40µl Prot K	COI short	/
8	2002	Qiagen (O.N.) 40µl Prot K	COI short	*Sarcophaga variegata*
9	2013	Qiagen (O.N.) 40µl Prot K	COI short	*Sarcophaga variegata**
10	2013	Qiagen (O.N.) 40µl Prot K	COI short	*Sarcophaga variegata**
11	2004	Qiagen (O.N.) 40µl Prot K	COI short	*Sarcophaga variegata*
12	2003	Qiagen (O.N.) 40µl Prot K	COI short	*Sarcophaga carnaria*
13	2004	Qiagen (O.N.) 40µl Prot K	COI short	*Sarcophaga carnaria**
14	2002	Qiagen (O.N.) 40µl Prot K	COI short	*Sarcophaga teretirostris*

Conclusion

This study examines a way to non-invasively identify species through their genomic DNA. Based on first preliminary results, molecular analysis allows the correct identification of the specimens without impair their morphological structures useful for further studies. The results clearly demonstrate the need of working with sequences of different length depending on the age of the sample. In this work samples from 2015 till 2002, respectively 8 months to 14 years old, were analysed: long sequences were amplified only for younger samples whereas short sequences were obtained for the majority of the specimens. Further test with different extraction buffers and different gene targets will be performed in the next month.
Validation of the molecular identification will be perform in a blind way.

An experimental approach to charred entomological evidence identification

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Introduction

Despite the great importance of entomology in the determination of time of death, there is very little data in the literature concerning its application to the frequently encountered scenario of burnt bodies. In addition, a commonly asked question in court is whether it is possible to determine if a body has been burnt before or after decomposition. In order to answer this question some experiments were performed using pupa and puparia of *Calliphora vomitoria, Calliphora vicina, Lucilia sericata* (Diptera, Calliphoridae) and *Megaselia scalaris* (Diptera, Phoridae) in order to estimate their burn point. A hot-stage microscope with a digital imaging system was used to record pictures and reflected light intensity profiles of puparia heated at 10 °C min -1 to 700 °C under static air.

Method

One set of experiments was performed using puparia of *Calliphora vomitoria, Calliphora vicina, Lucilia sericata* (Diptera, Calliphoridae) and *Megaselia scalaris* (Diptera, Phoridae). A hot-stage microscope consisting of a Eurotherm heat source control with a Olympus S7-CTV, which allowed a digital imaging system to record pictures and reflected light intensity profiles (*Fig 3 -30*) of puparia heated at 10 °C min^{-1} to 650 °C under static air was used. The puparia were placed in the furnace with the spiracles facing the lens. The temperature was ramped up at 10°C per minute (linear heating) under air until the desired temperature of 650°C was reached. Photographs were taken at intervals of 25°C, starting at 50°C. A second set of experiments was performed with the same protocol on pupae of *Calliphora vomitoria, Lucilia sericata* and *Megaselia scalaris*. All sample types were burned six times.

Fig 1: Hot Stage Microscope used to burn the puparia.

Fig 2: The furnace where the sample was placed to be burned.

Results

The pictures (recorded at 25 °C intervals) showed that the puparia are completely destroyed between 475 and 550 °C (Fig. 3 - 18). The pupa however were not entirely destroyed at this temperature (Fig. 19 – 30). No significant differences were detected between species (p=0.09). Interestingly, the shape of the posterior region and the spiracles were not affected by the heating until immediately prior to complete combustion allowing potential identification of the samples (*Fig 5,9,13,17,21,25,29*). The graphs indicate where the colour change in the samples occurred as they were heated, measuring the reflective light intensity of the sample against it's temperature *(Fig. 31-37)*.

***Calliphora vomitoria* puparium**

Fig 3: Starting image - Puparia spiracles at 50°C.

Fig 4: Puparia at 225°C when charring began.

Fig 5: Puparia spiracles at 400°C where the puparia is fully charred.

Fig 6: End image - Puparia at 575°C.

Fig 31: Temp Vs. relative light intensity for *Calliphora vomitoria* puparia

***Calliphora vicina* puparium**

Fig 7: Starting image – Puparia spiracles at 50°C.

Fig 8: Puparia at 225°C when charring began.

Fig 9: Puparia spiracles at 425°C where the puparia is fully charred.

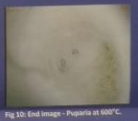

Fig 10: End image - Puparia at 600°C.

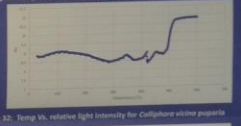

Fig 32: Temp Vs. relative light intensity for *Calliphora vicina* puparia

***Lucilia sericata* puparium**

Fig 11: Starting image – Puparia spiracles at 50°C.

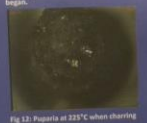

Fig 12: Puparia at 225°C when charring began.

Fig 13: Puparia spiracles at 400°C where the puparia is fully charred.

Fig 14: End image - Puparia at 600°C.

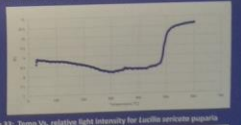

Fig 33: Temp Vs. relative light intensity for *Lucilia sericata* puparia

***Megaselia scalaris* puparium**

Fig 15: Starting image – Puparia at 50°C.

Fig 16: Puparia at 225°C when charring began.

Fig 17: Puparia spiracles at 350°C where the puparia is fully charred.

Fig 18: End image - Puparia at 600°C.

Fig 34: Temp Vs. relative light intensity for *Megaselia scalaris* puparia

***Calliphora vomitoria* pupa**

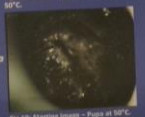

Fig 19: Starting image – Pupa at 50°C.

Fig 20: Pupa at 225°C when charring began.

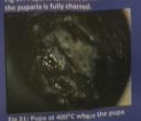

Fig 21: Pupa at 400°C where the pupa is fully charred.

Fig 22: End image - Pupa at 575°C.

Fig 35: Temp Vs. relative light intensity for *Calliphora vomitoria* pupa

***Lucilia sericata* pupa**

Fig 23: Starting image – Pupa at 50°C.

Fig 24: Pupa at 225°C when charring began

Fig 25: Pupa at 350°C where the pupa is fully charred.

Fig 26: End image - Pupa at 575°C.

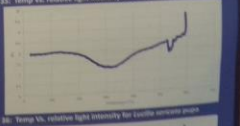

Fig 36: Temp Vs. relative light intensity for *Lucilia sericata* pupa

***Megaselia scalaris* pupa**

Fig 27: Starting image – Pupa at 50°C.

Fig 28: Pupa at 225°C when charring began

Fig 29: Pupa at 425°C where the pupa is fully charred.

Fig 30: End image - Pupa at 575°C.

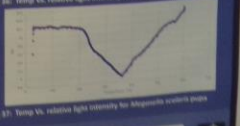

Fig 37: Temp Vs. relative light intensity for *Megaselia scalaris* pupa

Conclusion

Puparia and pupae combustion show a different pattern over 300°C. This effect can be related to both the organic matter present in the metamorphosing pupae and to the different amount of water. In all the experiments, posterior spiracles and main morphological features are visible until 500°C.

THE AWARDS

THE AWARDS

iFLY: A Mobile Forensic Entomology Application For Capturing Site Specific Data

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Abstract

The use of mobile computing applications in the field of forensic entomology is an emerging area that revolutionizes the accuracy of capturing data in the field, allowing for reliable data gathering, scene visualization, archiving and computations in remote locations. Moving from paper record keeping to mobile computing poses challenges and opportunities in developing the type of the data collected, and expands our ability to understand spatial relationships between species in ways not previously easy to achieve. Additionally, this transition presents an opportunity for the integration of multiple user datasets for metadata analysis.

We present iFly™, an iOS® application for the iPad® that provides a central input format that is based upon the body of the carrion being investigated. This application creates a mobile platform for capturing data on-site, allowing for the integration of environmental data directly into the site database and allows for specimen tracking with ease, a feature not previously obtainable from traditional techniques. Central to iFly™ is the use of integrated visual keys for aided identification of mature specimens that automatically executes nodal decisions and properly attributes source for the dichotomous key utilized. It also allows for the assessment of total body score for decomposing remains, and the automated calldown of weather data from national weather centers, with the ability to identify and specify the weather stations and data date ranges needed. We plan to implement this program beginning in the United States, and then continuing to other regions as resources and partnerships are available.

Methods

Using the programming tools Xcode (http://developer.apple.com/technologies/tools/) and SQLite Manager (www.sqlite.com) we integrate traditional information recorded in the field with an anatomical model to provide a visual referencing system not previously possible in the traditional paper medium (Figures 1, 3-6). This system will integrate automated data acquisition by utilizing application programming interfaces (API) from the National Oceanic and Atmospheric Administration (Figure 3; NOAA; www.NOAA.gov) and Agrinomics (Celsius™ line of iPad integrated thermocouples (www.agrinomics.com), or Variable Inc.'s NODE sensor system (www.variableinc.com), allowing direct integration from sensor to the reference database (not shown). Information is organized in an easy-to-access format (Figure 2).

Results

FIGURE 1. Specimen Choice Screen. The first task of the investigator is to establish the baseline anatomical model being interacted with (A; pig, human male or human female). Once a specimen is chosen, then as insects are sampled from the body you highlight the region they were taken from (B). Region of origin is tracked throughout the database (see figure 4 for an example of region-identified specimens). The individual number of specimens can be imputed later so that individual specimens can be worked with and data recorded from them. NOTE: this feature is not finished in the interactive example on site.

FIGURE 2. Front Screen. Once a specimen type (see figure 1) is chosen the user is taken to this screen. From here, each icon represents a different type of data that the user can interact with.

FIGURE 3. Weather Data control screen. Individual weather stations are mapped based upon feedback from NOAA. The spread around the identified centerpoint can be made wider or more narrow using the diameter controls (A; 5 levels of control). Weather stations are chosen and joins the list of downloaded data on the left side of the screen (B). Individual downloaded weather station datasets can be viewed in the pop-up screen when highlighted (C). Under this feature dates for weather station reporting can also be changed.

FIGURE 4. Species Identification control screen. Individual specimens are legged by region on a scrollable list. Touching an individual specimen brings up biometric data (caught live/dead, weight, length and temperature of location caught) as well as audio and written notes. You enter the visual key by selecting the "Fill with visual key" text. The Nodes portion of the screen will capture nodal decisions from the visual key. NOTE: some of these features are not fully implemented in the interactive example on site.

FIGURE 5. Multiple Nodal Decisions. Some nodes contain multiple possible decisions to determine that node. Where this happens, a figure icon appears below the nodes, indicating how many possible morphological features could be used in making this nodal decision (in this example, three exist). By touching the different figure icons, the user is confronted with the different morphological feature combinations (here organized side by side as A, B, and C respectively). Once a decision is made, that specific sub-node is recorded. Because of this, it is possible to still understand exactly what decision was made when multiple possibilities exist.

FIGURE 6. Visual Species ID screens. The interface of the visual key system is designed to be intuitive and clutter-free. The images themselves are the buttons the user presses to move to the next decision node, allowing the images to take center stage and be as large as possible. This is important for decision-making clarity since the features are sometimes hard to see if the image is too small. At each node (A, B, C, and D respectively in this example) the user is confronted with a dichotomous decision that is not biased by including the species names.

Conclusions

This presentation highlights two major features of iFly: the weather station data acquisition and built-in visual key. The weather station data acquisition system represents an easy way to visualize weather stations in the region, choose those stations and download recorded weather data.

The built-in visual key is proof of concept that easy to use, trackable visual-based decisions are possible on a remote platform. The Cutter & Dahlem key (http://www.nku.edu/~dahlem/ForensicFlyKey/Homepage.htm) is specific to just Northern Kentucky, so future work on this should increase the number of species beyond the limited selection currently available. It is possible to increase the number of groups represented in this key, so including Sarcophagidae (flesh flies) and other forensically important dipteran species would be helpful. Further expansion beyond just dipteran species would also be useful. Coleoptera are ripe for this type of inclusion.

Beyond research, the integration of the visual key feature allows undergraduate and even high school students to utilize the software to identify forensically important species. This means that iFly could potentially be used as a tool for ephemeral ecology or forensic entomology education.

Acknowledgments

Development of the species ID interface would not have been possible without the programming expertise of Eileen Lake and Michael Hill of CERIS. I am especially thankful for their ability to interpret the intent behind my madly scribbled "rough" sketches and descriptions, even when I lack the programming skill to explain what the feature needs to do beyond "this would be so cool." Further, the icons on the front screen were designed by Ben Prickel and add to the aesthetic of the app immensely.

Finally, all adult dipteran images used in this presentation are taken from the Cutter & Dahlem website. As such, this project would not have been possible without the efforts of both Regina Cutter and Gregory Dahlem. Trevor Stamper is deeply indebted to Lauren Weidner for her support and feedback on iFly and for traveling to present this information.