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ISOLATION OF BACTERIA AND FILAMENTOUS FUNGI FROM INSECTS SAMPLED DURING FLIGHT

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ABSTRACT

In this study, two novel techniques used for the isolation of insects in flight, A drone elevated sampler and an octanol-based midge/mosquito sampler. Insects were sampled at a height of 120 meters using a drone–towed fabric sleeve and their microbial content studied. The major point of interest behind this work is the use of a drone-towed sleeve to sample the insects. As far as can be determined, this is the first reported use of this approach to sample high flying insects in relation to a study of their microbiology. The use of a drone was shown to be ideal for the high altitude sampling of insects since it proved to be both powerful and highly manoeuvrable and there is no doubt that the drone used could have been used to sample at greater heights than the 120 m used here. The results relating to the microbiology of the insects sampled using the drone are not surprisingly similar to those obtained using other sampling methods, since the drone, of course, does not necessarily sample insects which differ from those obtained using more traditional approaches. This study contains results relating to the isolation of bacteria and fungi from insects. The work described has implications for the transfer of potential human pathogenic bacteria, notably to immunocompromised patients and also plant pathogenic fungi, in this case notably of trees.

KEYWORDS: Isolation, Insects, Fungi, Flight, Drone, octanol-based.

INTRODUCTION

Countless numbers of insect species occur around the world. With the exception of some pest species, surprisingly little is known however, about the relationship between insects and bacteria.[1],[2] Insect comprise some 53.1 percent (751,000 species) of all the known species (1.4 million) living species found on this planet. Furthermore, beetles constitute 20.5 percent of all living species (290,000) and Lepidoptera make up approximately 9.9 percent (140,000) of insects.[3],[4]

This study is devoted to an evaluation of two novel techniques used for the isolation of insects in flight. The main rationale behind this work was to determine if these techniques can be usefully employed in studies such as these. The following techniques were used for in-flight sampling.

1) A drone elevated sampler.
2) An octanol-based midge/mosquito sampler.

These are novel approaches to sampling airborne insects. The rationale behind the studies is based on the fact that 1) insects are known to be transported over long distances in the upper atmosphere and can thereby transmit a wide variety of human, animal and plant pathogens over long distance, including across continents and (2) that vast amounts of midges and mosquitoes are hatched each year (in the UK, being mainly found in Western Wales and the Scottish Highlands) and act as carriers of microbes.

The aim of the work discussed here is to evaluate the use of the two sampling approaches mentioned above to isolate bacteria, filamentous fungi and yeasts from the surface of airborne insects. Using the drone sampler, insects were isolated at a height of 120 meters (the drone had remote-activated solenoid opener/closure devise), while the octanol-based sampler isolates a large number of female midges/mosquitoes from the air when placed at ground level.

Glick (1939) collected insects from high altitudes by means of special traps fitted to various types of airplanes over Southern USA during 1926 to 1931, Some 30,000 specimens of insects were sampled from altitudes ranging from 20 to 4500 metres.[5] Eighteen orders of insects and the orders of spiders and mites were collected and represented 216 families, 824 genera, 4 new genera, 700 species, and 24 new species. The order Diptera was the most abundant order in the air, nearly three times as many specimens being taken than any other order. Coleoptera followed next after Diptera, Homoptera and Hymenoptera were sampled at 4,270 meters, the highest altitude at which insects were found, while the highest altitude at which any specimen was taken was 4,570 meters, at which a spider was caught. Not surprisingly, insect numbers decreased with sampling height. The size, weight, and buoyancy of an insect were shown to contribute directly to the height to which it is carried by air currents. Many species of the other orders represented at high altitudes were also small insects. Evidence showed that insects taken in the upper air were alive at the time of sampling. The relative distribution and abundance of insects in the upper altitudes depended on weather conditions with temperature being undoubtedly the most important The intensity of air currents is a great factor in the distribution and dispersal of insects. Most insects were taken at the lower altitudes when the surface wind velocity was from 5 to 6 miles per hour, and fewest when it was calm. The direction of the wind has influenced to a great extent the migrations of insects. In the airplane flights at Tallulah it was found that the greatest numbers of insects were taken when the surface wind direction was from the north-northeast, southeast, or southwest. Some insects were apparently moving with the wind during the spring and summer when the surface prevailing winds were from a southerly direction, and again with the wind from a northerly direction in the fall. Convection and turbulence was shown to play an important role in determining the insect population in the upper air.

As has already been noted, millions of metric tons of insects exist in the Earth's atmosphere at any given moment, most of which comprises insects involved in high-altitude, wind-borne migration, often at heights several hundred meters above ground level, where they take advantage of the strong winds found in this region to cover considerable distances, frequently tens or even hundreds of kilometres.[6],[7] This vast aerial "bioflow" has major implications for ecological, physiological, and genetic studies of insects, and added applications relevant to pest management, conservation, and environmental change programs.[8] In the past, the study of insect migration has relied primarily on data from long-distance flights, catches in light traps and other ground-based observations. Maintaining sampling platforms in the air) is however, expensive and impracticable over long periods. The insect fauna flying at high altitude can now be monitored continuously and for long time periods, using autonomous vertical looking radar systems (VLR systems). Combined with aerial sampling technology and sources of bio-meteorological information, these systems have considerable potential for area-wide monitoring of economically important pests and could clearly be used for pest management and forecasting systems.[9] VLR is clearly a powerful new tool that will revolutionize the study of insect migration and provide us with significant new information on both pure and applied entomology.

MATERIALS AND METHODS

Fungi media

Czapek Dox Agar media was prepared by suspend 50 g Czapek Dox agar then dissolved in 1000 ml Distilled water in a flask with magnetic spin-bar until the solution dissolved than transfer class flask with covered or lid into autoclave at 120°C for 30 min for sterilization. Then the solution was poured in Petri dishes carefully when solution temperature decline to 60-55 °C. Preliminary identification is based on the colour the isolated Fungi colony developed when samples are grown on Czapek Dox agar medium.

Impingers

Impingers use a liquid medium for particle collection, sampled air being drawn by a suction pump through a narrow inlet tube into a small flask containing the collection medium; speeds up the air towards the surface of the collection medium, the flow rate being determined by the diameter of the inlet tube.

Impactors

Impactor samplers use a solid or adhesive medium, such as agar gel, rather than a liquid for particle collection.

Drone samplers

The drone sampler consisted of a piece of muslin sleeve (length) (used for a pipe cover) sealed at one end and held open by a circular piece of wire (diam.). The sampling sleeve was attached to the drone which possessed a thin plastic circular cover which, on command from the ground, could open and close the circular end (i.e. aperture) of the sampling sleeve; small flying insects were caught inside the sleeve and sampled on its return to the surface. The drone was fitted with a camera, an altimeter and GPS (Fig1). The done was launched as single event in open field near Bakewell**,** Derbyshire to a high 120 meters and horizontal distance of 500 meters (to comply with current regulations) above the town for 3 minutes.

A **B**

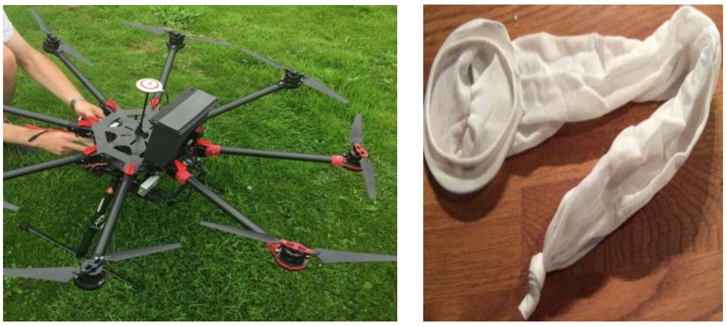

Fig. 1: (A).Drone used to capture airborne insects and (B). muslin sleeve (length).

Octanol-based midge samplers

A commercial midge sampler (Predator Dynamic) (Fig. 2) was used to catch large quantities of airborne midges and mosquitoes. It contains a strong vacuum fan which sucks the insects into the trap and dehydrates them.

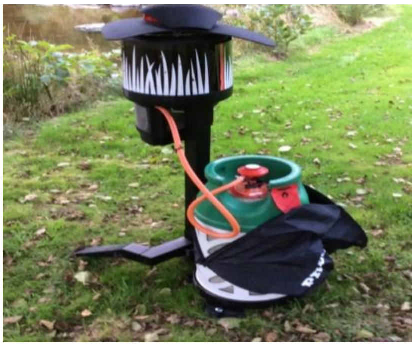

Fig. 2: The Predator midge collector.

Bacterial isolation

The total bacterial load of the midges and high flying insects was obtained by macerating the whole body in a small amount of sterile ¼ strength Ringers' solution, plating onto Nutrient Agar and then incubating overnight at 25^{0}C. Identification was achieved by the use of 16SrRNA and classical methods following Bergey's Manual.

Fungi isolation

Using a cotton wool swab, moistened with sterile distilled water to isolates fungi from the surface of midges. The swab was then spread onto the surface of the isolation Czapek Dox Agar media.

Table 1: Insects and associated bacteria isolated using a drone from a height of 500 meters. Bacteria (identified using classical methods).

Insects	***Hoverfly (Eristalis intracarius)***	***Vinegar fly (Drosophila funebris)***	***Aphid (Pemphigus burarius)***
Bacteria	*Acetobacter aurantius*	*Azotobacter vinelandii*	*Bacillus licheniformis*
	Actinomyces israelii	*Bacillus licheniformis*	*Bacillus megaterium*
	Bacillus brevis	*Bacillus megaterium*	*Enterococcus faecium*
	Bacillus licheniformis	*Bacillus mycoides*	*Psedomona aeruginosa*
	Bacillus megateroium	*Enterococccus durans*	*Rothia dentocariosa*
	Bacillus mycoides		*Streptococcus sanguis*
	Wolbachia		*S. sobrinus*

In contrast, no Bacilli were isolated from the midge samples sampled at ground level (Table 2), the reasons for which are not immediately apparent.

Table 2: Bacteria isolated from midges-identified to species level using classical methods.

Insects	**Midges**
Bacteria	*Pseudomonas aeruginosa*
	Wolbachia sp.
	Moraxella catarrhalis
	Staphylococcus pasteuri
	Acinetobacter baumannii
	Stenotrophomonas maltophillia
	Comommonas terrigena
	Flavobacterium columnare
	Chryseobacterium indologenese
	Citrobacter brakkii
	Ehrlichia ewinii

Of interest is the isolation of *Stenotrophomonas maltophillia*, a bacterium which is increasingly being recognized as an important pathogen of immunocompromised patients. *Stenotrophomonas maltophillia. Stenotrophomonas* infections have been associated with high morbidity and mortality in severely immunocompromised and debilitated individuals.[10] Risk factors associated with this pathogen include: HIV infection, malignancy, cystic fibrosis, the use of catheters, recent surgery and trauma. It is also enhanced by the use of broad spectrum antibiotics

Table 3: Fungi isolated from midges-identified using classical methods.

Insects	Midges
Fungi	*Aspergillus niger*
	Penicillium brevicopmatcum
	Penicllium citrinum
	Penicllium chrysogenum
	Aternaria tenuis
	Fusarium oxysporum
	Fusarium solanum

Fungi were also isolated from the midges. Table 3 shows that the isolates are common spore-forming Deuteromycetes, all of which are commonly found on most environmental samples.

Fertilizer Potential of collected midge biomass

The vast quantity of midges which can be collected by octanol-based midge collectors, like the one used here, opens up the potential of using midge biomass as a fertilizer.

RESULTS

Three types of insects were obtained at a height of 120 meters using the drone–towed sampler, namely a Hoverfly, a Vinegar Fly and an Aphid (Table 1). It is likely that these insects were carried to this height by a combination of flying and uplift on wind currents. Table 1 also shows the bacteria obtained from these sampled insects. The bacteria are commonly isolated environmental organisms, showing a preponderance of spore forming Bacilli, an expected finding considering the high level of resistance to adverse conditions shown by Bacilli-endospores.

Octanol- based midge collectors are used in areas of the world which have very large midge and mosquito populations, i.e. wet, relatively warm mountainous areas, such as N. Western Scotland, North Wales and British Columbia and Nova Scotia, Canada. Most of the large amounts of biomass caught by individual traps are likely to be casually dumped, sent to landfill or incinerated.

Table 4: Concentration of ammonium and nitrate (μg ml^{-1} dry weight) extracted from soil amended with midge-biomass (Means of triplicates, all treatment values significantly different from control, p=0.05).

Days	Amended soil Ammonium	Amended soil Nitrate	Control (un-amended soil) Ammonium	Control (un-amended soil) Nitrate
0	25	10	20	10
7	60	25	25	15
14	105	30	30	10
21	110	45	25	12
28	125	60	30	20

The results of this study suggest however, that such biomass could have fertilizer potential. Table.4 shows that the addition of fresh midge biomass to an agricultural loam led to a substantial increase in the concentration of the two main fertilizer sources of nitrogen, i.e. ammonium and nitrate, with ammonium predominating. It is likely that dried biomass would provide even larger amounts of fertiliser-nitrogen. This practice would have the advantage of reducing transport costs, but of course the amount of such dried material would be limited by the high costs of heat-drying. For this reason, only wet midge biomass was evaluated here for its fertilizer potential. Such biomass could be applied to soils directly or after a period of composting and could be used alone or together with waste plant materials. One could envisage large amounts of such biomass being produced by individuals or perhaps council-run midge collectors (and co-operatives) and, as a result, relatively large amounts of material could be made locally available to farmers and the public. Transport costs might however, limit the wide-spread collection and use of midge biomass on an industrial scale. Certainly however, an individual octanol-based collector, when located in a high midge area, could supply useable nitrogen fertiliser to homes, allotments, and even small to medium sized fruit and commercial fruit and vegetable growers. The production costs of midge biomass could be offset by local authorities, hotels or other tourist locations, where the waste is produced when attempts are being made to reduce the tourist-nuisance potential of vast numbers of midges or mosquitoes. The fact that this study shows that the midge of biomass collected here does not contains major pathogenic bacteria means that its use need not be limited by safety reasons and, as a result, there is no obvious need for it to undergo expensive sterilization; heat-based sterilization could however, be advantageous in producing a concentrated product, capable of being economically transported, which could be bagged and sold by garden–supply shops.

DISCUSSION

The UK supports some 40 species of biting midge, but only five are thought to regularly attack people, with the Highland midge, *Culicoides impunctatus*, being the most troublesome, and only the bloodthirsty female causing problems. This midge is particularly common around dawn and dusk and in the Highlands and north-west Wales, where damp conditions provide it with perfect breeding grounds. Individual midges are almost invisible to the human eye, at about a millimetre long. The male feeds on plants and nectar, while his mate requires blood in order to form her eggs. Midges become aware of humans when they detect carbon dioxide on the breath and a swarm can inflict about 3000 bites each hour using a distinctive feeding technique. While mosquitos pierce the skin and suck up blood through a syringe-like mouthpiece, midges cut the skin, and then lick up the resultant pool of blood. A midge's saliva stops the blood in the wound from clotting allowing it keep on drinking indefinitely. It is the saliva which irritates the human body and leads to skin reactions and swelling at the site of a bite. Some people appear immune to midge bites; women tend to react more badly than men to the bites and the tendency to be targeted is hereditary. Midges also attack cattle, deer, sheep, cats, dogs, rabbits and mice, and spread bluetongue, a debilitating disease affecting sheep and cattle caused by a virus belonging to the family Reoviridae. Midges prefer damp, sheltered conditions, woodland and forest areas, avoid breeze, and unlike most other insects, prefer dark-coloured clothing to light.[11]

Chemical solutions are available to deter midges, including insect repellents containing DEET, or the natural alternative, citronella, a product of lemongrass extract which can be bought as a spray or infused into candles. These work by blocking the insect’s odour receptors on the antennae and mouthparts, thereby confusing the midge so that it avoids the person. Homemade repellents include bog myrtle which grows wild in the Highlands, and thyme. There are also several traps on the market, including the Predator (i.e. the trap employed here), which it is claimed, simulates a large smelly cow and attracts midges by replicating breath, heat, body odour and movement - then catching the creatures on sticky paper. In trials during 2010, a single Predator trap collected 800,000 midges over a five-day period. Midges cost Scotland's tourist industry an estimated £286m per year and midges are threatening the economy of the Lake District and North Wales and as far south as Cornwall. However, midges play a crucial role in the Scottish ecosystem, providing food for bats, birds and even carnivorous plants like sundews and butterworts, and they may have been partially responsible for restricting the development of the Highlands, and thereby maintaining this area as a remarkable wilderness.[11]

Of particular interest was the isolation of *Wolbachia* from the trapped midges. *Wolbachia* is a bacterial genus which infects arthropods, including insects and nematodes and is therefore one of the most common parasites in the biosphere.[12] It sets up a mutualist, rather than parasitic relationship with its host, some of which cannot survive and reproduce in its absence. It is estimated that some 25 to 70 percent of all insects are potential hosts. The genus was first identified in 1924 by Hertig and Wolbach in the common house mosquito and is now of considerable interest, not least as a potential biocontrol agent.[13] *Wolbachia*. Bacteria can infect many different organs, but most notably the testes and ovaries. They are ubiquitous in mature eggs, but not mature sperm and as a result, only infected females pass the infection on to their offspring.

Wolbachia has been linked to viral resistance in *Drosophila* and mosquito species, flies infected with the bacteria being more resistant to RNA viruses, including the West Nile virus and can also confer insecticide resistance.[14] In species of *Phyllonorycter blancardella* (leaf miners), *Wolbachia* bacteria help produce green islands on yellowing tree leaves, allowing the hosts to continue feeding while developing into their adult forms and larvae treated with an antibiotic which kills *Wolbachia*, lose this ability and as a result only 13% emerge as adult moths. In parasitic filarial nematodes which cause elephantitis, *Wolbachia* has become an obligate endosymbiont and supplies the host with the chemicals required for its reproduction and survival; elimination of the *Wolbachia* symbionts by antibiotics therefore prevents nematode reproduction, and eventually results in death. Some *Wolbachia* that are infect arthropods and mediate iron metabolism under nutritional stress, and can also help the host to synthesize vitamin-B.

Wolbachia species infect a variety of isopods, including spiders, mites and filarial nematodes including those causing River Blindness and elephantitis in humans and heat worms in dogs. The elimination of *Wolbachia* from filarial nematodes generally results in either death or sterility of the nematode; as a result, these diseases can be controlled using the antibiotic doxycycline to kill the bacterium (Li *et al*., 2014). *Wolbachia* can also be used to control dengue and malaria and a recent study has shown that *Wolbachia* can prevent the spread of Zika virus in mosquitos in Brazil.

CONCLUSION

This study contains results relating to the isolation of bacteria and fungi from insects. The work described has implications for the transfer of potential human pathogenic bacteria, notably to immunocompromised patients and also plant pathogenic fungi, in this case notably of trees. The use of, what appear to be, novel insect collectors, i.e. a drone-carried sampler and the Predator midge collector has been described; further studies will determine if these methods can be added to, and improved upon, the large number of insect collectors already in use.

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