UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

School of Biological Sciences

The Effects of Extremely Low Frequency Electromagnetic Fields on Insects

by

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ABSTRACT

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Flying insect species are currently in decline, including many species that provide important pollination ecosystem services. Combined exposure to various environmental stressors are associated with insect declines, including land-use change, pesticide use and climate change, but the potential biological and environmental effects of extremely low frequency electromagnetic fields (ELF EMFs) are poorly understood. ELF EMFs are pervasive in the environment, and anthropogenic ELF EMF pollution has increased greatly in recent years. Despite this, little has been done to consider the potential environmental impacts of ELF EMFs. Given that there is evidence that ELF EMFs can have biological effects, it is important to explore these biological stimuli and their potential to affect insects in the environment.

Here the biological effects of ELF EMFs on important insect species were investigated, with two-fold aims of both increasing understanding of the biological effects of ELF EMFs, and determining whether field-realisitc levels of ELF EMFs have the potential to cause envrionemental stress to insects. ELF EMF impacts were investigated with the desert locust, as an economically important agricultural pest and a species that provides utility in understanding insect neurophysiology, and the honey bee, as a globally important pollinator and a well known study species for insect cognitive behaviour. Short-term exposure to high levels of ELF EMFs was found to affect neurophysiology, and reduce locomotory function in locusts, as well as increase stress protein levels in bees and locusts, and affect honey bee cognitive behaviour. Acute exposure to ELF EMFs at levels that can be encountered regularly in the environment around man-man sources for ELF EMFs reduced honey bee performance in olfactory learning assays, affected flight behaviour, and

affected feeding and flight performance in a semi-field scenario. Further to this, some of these impacts of ELF EMFs on cognitive behaviour and flight were reduced when ELF EMFs were applied in combination with other well-known environmental stressors, neonicotinoid insecticides.

These findings give a more detailed indication of some of the physiological effects that may underpin changes in insect locomotory behaviour that occur after short-term exposure. This is the first time that powerline simulating ELF EMFs have been directly measured and applied to insects in the context of considering the ecological effects (and thus using field-realistic exposure levels) of the ELF EMFs, rather than just the biological effects. This is the first indication that short-term and acute ELF EMF exposure can affect insect cognitive behaviour, and these effects have been shown to occur at levels which can be encountered in the field by a globally important pollinator species, the honey bee. This is also the first indication that acute field-realistic ELF EMF exposure can affect insect locomotory behaviour in the environement. This research describes new effects of ELF EMFs on insect biology and establishes that ELF EMFs have a potential to affect insect ecology, such that future ELF EMF understanding must be focused in further exploring mechanisms by with ELF EMFs cause biological effects, as well as the larger scale ecological risk assessment of ELF EMF impacts from powerlines.

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Academic Thesis: Declaration of Authorship

I, Sebastian James Shepherd, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

The Effects of Extremely Low Frequency Electromagnetic Fields on Insects

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
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6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as:

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Signed: .....

Date: .....

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Definitions and Abbreviations

Acronym	Definition
$	dollars
£	British pound
€	Euro
°C	degrees Celsius
AC	alternating current
AL	antennal lobe
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
APS	ammonium persulfate
ATP	adenosine triphosphate
B	magnetic flux density
BPA	Bonneville Power Administration
BSA	bovine serum albumin
CAT	catalase
cm	centimetre
CNS	central nervous system
CO_2	carbon dioxide
CS	conditioned stimulus
d	day
DC	direct current
DDT	dichlorodiphenyltrichloroethane
DECT	Digital Enhanced Cordless Telecommunications
DN	descending interneurons
DNA	Deoxyribonucleic acid
DTT	dithiothreitol
DWV	deformed wing virus
E	electric field
EFSA	The European Food Safety Authority
ELF	extremely low frequency

EMF	electromagnetic field
EMRE	electromagnetic response element
EN	extrinsic neuron
ENA	Energy and Networks Association
EPSP	excitatory postsynaptic potentials
ETi	extensor tibiae muscle
EU	European Union
F	force
FAO	The Food and Agriculture Organisation
FERA	Food and Environment Research Agency
FETi	fast extensor tibiae motor neuron
fps	frames per second
g	gram
GHz	gigahertz
GLM	generalized linear model
GLMM	generalized mixed effect model
GSM	Global System for Mobile Communications
ha	hectare
HL-60	Human promyelocytic leukaemia cells
hr	hour
Hsp70	heat shock protein 70 kDa
HVDC	High-Voltage Direct Current
HVTL	high-voltage transmission line
Hz	Hertz
I	electric current
ICNIRP	International Commission on Non-ionizing Radiation Protection
IEA	International Energy Agency
IEEE	Institute of Electric and Electronics Engineers
IPCC	Intergovernmental Panel on Climate Change
kDa	kilodalton
kHz	kilohertz
km	kilometre
kV/m	kilovolts per metre

kVA	kilo-volt-ampere
lbs	pounds
LD_{50}	median lethal dose
LG	lateral giant interneuron
LH	lateral horn
m	metre
MB	mushroom body
MEA	Millennium Ecosystem Assessment
MHz	megahertz
min	minutes
mN	millinewton
MRI	Magnetic Resonance Imaging
ms	millisecond
mT	millitesla
nAChRs	nicotinic acetylcholine receptors
ng	nanogram
NIOSH	National Institute for Occupational Safety and Health
nm	nanometre
nT	nanotesla
ORN	olfactory receptor neuron
P	point in space
PAGE	polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PER	proboscis extension response
PN	projection neuron
ppb	parts per billion
Q_{test}	test charge particle
RM-ANOVA	repeated measured analysis of variance
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulfate
SEM	standard error of the mean

SER	sting extension response
SG	suboesophageal ganglion
SOD	superoxidase dismutase
T	Tesla
TBS	tris buffered saline
TWh	terawatt-hours
UK	United Kingdom
UMTS/3G	Universal Mobile Telecommunications Service
US	United States
US	unconditioned stimulus
USD	United States dollars
USGS	United States Geological Survey
UV	ultraviolet
v	velocity
V	Volt
V/m	Volts per metre
VLF	very low frequency
VUMmx1	ventral unpaired median neuron 1 of the maxillary neuromere
W/g	Watts per gram
w/v	weight per volume
w/w	weight per weight
WHO	World Health Organization
wk	week
WLAN	Wireless Local Area Networks
WPAN	Wireless Personal Area Networks
µg	microgram
µl	microlitre
µm	micrometre
µT	microtesla

Chapter 1 General Introduction

1.1 Insect ecology, declines, and global impacts

1.1.1 Relationship between insects and humans

Insects serve many crucial ecological roles that may be beneficial to humans in the form of ecosystem services, such as pollination of crops and plant species, or detrimental to humans, such as crop destruction or insect vector disease transmission. Any factor that affects the success of insects in ecosystem services, such as pollination, can affect the overall functioning of ecosystems and food security, both of which support the human population. Conversely, pest/vector species can put strains on food security and human populations. Current trends show substantial declines of beneficial insects in general, and pollinators in particular, for at least the last three decades, and most likely since the industrial revolution (Potts et al., 2010a; Willmer, 2011). For example Hallmann et al. (2017) found over a 75% decline in biomass of flying insects in 63 German nature reserves in only 27 years. *Apis* bees are currently in decline with a 59% decrease in US colonies between 1947 and 2005 (Hayes et al., 2008), and a 25% decline in European colonies between 1985 and 2005 (Potts et al., 2010b). In contrast, many pest species persistently put strains on global food security. For example, the Australian Plague Locust, *Chortoicetes terminifera*, which causes substantial crop damage in Australia, caused a plague as bad as any outbreak in history in 2010 (Deveson, 2013), with no decline in outbreak patterns since 1934, and major outbreaks occurring approximately every 2-3 years (Deveson, 2011). Consequently it is a critical global challenge to understand factors which affect insect biology, which may be utilized in the management of insect pests as well as the mitigation of pollinator declines.

1.1.2 Importance of insects as pests

Insects may have a variety of negative impacts from an anthropocentric perspective. Insects can be considered ‘pests’ by humans for reasons such as the consumption of purchased food (e.g. ants (Espadaler et al., 2004), destruction of crops (e.g. aphids, Riedell and Kieckhefer, 1995; locusts, Deveson, 2011, 2013), causing health crises as vectors (e.g. mosquitos, Catteruccia, 2007), damaging numerous ecological processes as invasive species (e.g. Asian longhorn beetles, MacLeod et al., 2002), and causing structural damage (e.g. termites, Lax and Osbrink, 2003). This can be incredibly costly for humans; for example the

diamondback moth, *Plutella xylostella*, costs the world economy $4-5 billion USD annually (Furlong et al., 2013), and mosquitos alone transmit diseases to 700 million people annually, including malaria which kills 3 million people a year (Fradin and Day, 2002). The Desert Locust, *Schistocerca gregaria*, affects 20% of the Earth's land area across North Africa and India (Skaf et al., 1990) causing significant economic damage and food security issues. For example, it cost over $500 million USD to control a plague that occurred from 2003-2005, which required treating 13 million ha of land in 22 countries with pesticides (Belayneh, 2005). During this outbreak crops were lost at levels of 80-100% in affected areas (Brader et al., 2006). It is important to understand the disruption of biological processes in pest insects to aid the control of phenomena with such negative impacts on humans. Factors which can lead to desirable biological effects in target insects, such as increased mortality, reduced mobility/viability/health, or taxes (attractive or repulsive behavioural effects), can aid efforts to deal with particular pest organisms, making this an important area to investigate.

1.1.3 Importance of insects as pollinators

90% of all flowering plants are pollinated by animals (Johnson et al., 1998) and one third of all the food consumed by humans is directly dependent on animal pollination, with the additional carnivorous portion of the human diet indirectly dependent on pollination through the crops used to feed many of the animals that humans eat (Willmer, 2011). At least 120000 animal species, but likely up to 300000, are responsible for this pollination effort, of which some species are bats and birds, but the vast majority are insects (Willmer, 2011). Roubik (1995) reported that of at least 800 animal pollinated cultivated plants globally, approximately 73% of these were pollinated by bees, 19% by flies, 5% by wasps, 5% by beetles, and 4% by lepidopterans, whilst only 6% were pollinated by bats and 4% by birds (note that these do not add up to 100% due to some overlap). Further to this, Roubik (1995) described that this study was in the tropics, and that it is likely that in more temperate climes animal pollination will be even more dominated by insects. It is clear, therefore, that insects play a critical role in pollination. In the United Kingdom alone insect crop pollination is estimated to be worth ~£400 million per year (POST, 2010), while globally it is estimated to be worth ~€153 billion per year (Gallai et al., 2009).

Bees are a particularly important taxa when it comes to pollination. There are at least 25000 species of bees within the clade Anthophila, that use both nectar and pollen as food sources and are consequentially obligate visitors of flowers, making them very important

pollinators (Willmer, 2011). Honey bees (genus *Apis*) are particularly important from an applied ecological perspective due to their relationships with many food crops and biological processes (Willmer, 2011). Honey bees are capable of increasing yield in 96% of all animal-pollinated crops (Potts et al., 2010a). As a result honey bees are extremely valuable in crop production. For example, the value of honey bees to US agriculture is estimated to be between $15-20 billion USD per year (Calderone, 2012). As a result the declines in honey bee colonies (Hayes et al., 2008; Potts et al., 2010b), as well as declines of flying insects (Hallman et al., 2017), are concerning from an economic and food security perspective. These concerns are supported by the finding that plants that are dependent on specific insect pollinator species that have declined are also declining (Biesmeijer et al., 2006), suggesting a causal link between declines in these insects and reduced quality in the pollination ecosystem services they provide.

1.1.4 Anthropogenic change

There is an inextricable link between environmental changes from anthropogenic activities, especially since the industrial revolution (1800's), and adverse effects on ecosystems (Millennium Ecosystem Assessment (MEA), 2005) such as biodiversity loss (Butchart et al., 2010; Costello et al., 2013). There are many examples of anthropogenic practices and technologies causing environmental change and impacting upon ecosystems, including climate change (Parmesan, 2006), air pollution (Zvereva and Kozlov, 2010), agricultural intensification including the increased use of agrochemicals (Geiger et al., 2010), land-use change (Reidsma et al., 2006), human movement and trade leading to increased infectious disease and invasive species spread (Hulme, 2009) and light pollution from artificial lighting (Gaston et al., 2013).

For insects many human-derived changes have had large impacts and have been made to directly manage insect populations, such as developments of major insecticidal products including traditional pesticides such as dichlorodiphenyltrichloroethane (DDT) (Turusov et al., 2002), systemic insecticides such as neonicotinoids (Simon-Delso et al., 2015) and even transgenic crops expressing insecticidal proteins, such as *Bacillus thuringiensis* (Romeis et al., 2006). In addition, many human-derived activities have had unintended effects on insect populations, such as artificial light pollution dynamically affecting insect distributions and predator prey interactions (Longcore and Rich, 2004). In some cases human-derived activities that have been implemented specifically to control insect pest populations have been linked to unintended impacts on beneficial insect species such as honey bees, including

contributing to pollinator declines (e.g. the use of systemic insecticides) (Goulson, 2013; Rundölf et al., 2015). Many other human activities have been associated with declines in pollinators and specifically honey bees, such as habitat fragmentation (Wilcock and Neiland, 2002; Goverde et al., 2002), land-use/agricultural change (Ollerton et al., 2014; Goulson et al. 2015), climate change (Kerr et al., 2015; Goulson et al., 2015), environmental pollutants (Lusebrink et al., 2015; Farré-Armengol et al., 2016) and even the anthropogenic spread of pollinator parasites (Goulson and Hughes, 2015; Wilfert et al., 2016). The current consensus is that this cocktail of human-derived environmental stressors/pollutants in combination is leading to declines in honey bees and other pollinators (Potts et al., 2010a; Ollerton et al., 2014; Goulson et al., 2015).

Despite their unintended impacts on beneficial insects, and sometimes because of their impacts on pest insects and larger ecosystems, many of these human practices and technologies that lead to environmental effects are crucial to modern societies. There are major global challenges to mitigate the effects of anthropogenic change on insects and wider ecosystems such that their benefits can still be utilised. In a modern environment, best practice to mitigate the impacts of anthropogenic activities on ecosystems involves (i) identifying anthropogenic activities that may lead to adverse ecological effects, (ii) identifying knowledge gaps regarding effects of anthropogenic activities, (iii) improving understanding of anthropogenic effects on ecosystems through increased/improved research outputs, (iv) developing and implementing solutions to anthropogenic related ecological problems (e.g. alternative practices, development of technologies, and constructive policies) (MEA, 2005; World Health Organization [WHO], 2007a; Intergovernmental Panel on Climate Change [IPCC], 2015).

1.1.5 Electromagnetic fields and anthropogenic change

Electromagnetism is a ubiquitous fundamental physical force with which life has evolved, and as a result has important actions in the activities of organisms (Delgado, 1985). Electromagnetic forces provide the physical interaction that holds biological molecules together, defining the key properties of biological molecules and their interactions (Berg et al., 2006). Consequently a large quantity of biological processes are driven by forces governed by the rules of the electromagnetic fundamental interaction e.g. neuronal signalling, muscle contraction and cardiac rhythm (Hall, 2015). At the behavioural level many different organisms can detect environmental electromagnetic stimuli (Kirschvink et al., 2001; Wiltschko and Wiltschko, 2005).

Anthropogenic pollution of the environment with electromagnetic fields (EMFs), which causes deviations in this fundamental physical force from natural levels, has increased dramatically over the last century as human utilization and reliance on technologies that emit different types of EMFs has escalated. For example, electricity from power plants became commonplace in the late 1800's. In 1990 global energy consumption had reached 101000 terawatt-hours (TWh), and by 2012 this had increased to 155000 TWh, a 53% increase in 22 years (IEA, 2014). This increase in electricity consumption, and the consequent need to increase electricity generation and transmission, has resulted in a great increase in pollution of the environment with extremely-low frequency electromagnetic fields (ELF EMFs). In the 1900's radio and television innovation increased the emission of radiofrequency EMFs. Microwave and radio-frequency EMF pollution increased dramatically from the 1990's with the proliferation of different mobile phone and internet technologies including GSM (Global System for Mobile Communications) and UMTS/3G (Universal Mobile Telecommunications System/3rd Generation) wireless phones and base stations, WLAN (Wireless Local Area Networks), WPAN (Wireless Personal Area Networks) and DECT (Digital Enhanced Cordless Telecommunications) as sources for microwave/radiofrequency EMFs (Balmori, 2009). These technologies are now found commonly in a modern environment producing microwave and radiofrequency EMFs that animals may be exposed to. As a result there are calls to improve our scientific understanding of the biological effects of electromagnetic fields, and to determine their potential impacts on health and the environment (WHO, 2007a). With the extreme propagation of EMF polluting technologies, particularly in the last 20-30 years, EMF pollution represents a critically important anthropogenic activity to research to determine its impact on ecosystems, including potential effects on insects and the important ecosystem processes they are involved in.

1.2 Electromagnetism and its properties

To determine the potential effects of increased anthropogenic EMF pollution on the environment, including its potential biological effects, it is critical to understand what EMFs are, and their properties, including the physical mechanisms by which they interact with materials.

1.2.1 Physical fields

The term ‘field’ is used in physics to represent the fundamental interactions (or fundamental forces) in nature for ‘actions at a distance’ e.g. gravitational fields, electric fields, and magnetic fields. A field is an area of space in which one object exerts force on other objects, and it extends outwards indefinitely (McMullin, 2002). The strength of this force exerted at a point in the field is a function of the inverse square of the distance of that point from the centre of the original object (the strength of the force exerted on a point weakens as distance from the central object increases) (Feynman, 1964). Consequentially at certain distances the intensity of a particular field becomes negligible.

1.2.2 Electric fields

Electric fields are a major component of the electromagnetic fundamental interaction. Electric fields produce both attracting and repelling forces on objects with charge. A charge is a physical property of matter with a positive or negative sign. Negatively charged objects have more electrons than protons, whereas positively charged objects have more protons than electrons (objects with equal protons and electrons have neutral charge). Coulomb’s law explains that electric charges exert forces on one another in a direction along the line between the charges, such that charges with the same sign (e.g. two positive charges) will repel each other, whereas charges with opposite signs attract each other (Feynman 1964). This area of space around a charged object is an electric field, and if an object with the same charge were introduced to the field it would be repelled from the centre, in the same way that if an object with opposite charge were introduced it would be attracted to the centre (Purcell and Morin, 2013). An electric field (E) determines the force (F) experienced by a motionless positively charged test particle (Q_{test}) such that electric field strength at a point in space is defined as $E = F/Q_{test}$ (Christensen and Durney, 1999). Electric field strength is measured in Volts/metre (V/m). As a consequence of the definition of an electric field, a charge Q placed in an electric field E will have a force of $F = QE$, such that as electric field

strength (V/m) increases, so will the force exerted on the charge Q, highlighting the principal interaction that electric fields have by exerting forces on charged particles placed within the field (Christensen and Durney, 1999).

1.2.3 Magnetic fields

Magnetic fields are another major component of the electromagnetic fundamental interaction. When charges move they exert a force on one another that is not along the line between the charges. Moving charges produce an electric current (I), which in turn produces a magnetic field that encircles the current dependant on its direction (Feynman, 1964). Unlike electric fields, magnetic fields do not produce a force on stationary charges, but they do produce a force on moving charges (as well as the force produced by the electric field). The force on a moving charge (Q_{test}) which is moving at a velocity (v) at a point in space (P) has a magnitude of $F = BvQ_{test}$, where B is known as the magnetic flux density and represents the magnetic field (Fig. 1, A-B) (Christensen and Durney, 1999). The direction of the force is perpendicular to v and B. Magnetic flux density is measured in units of Tesla (T – e.g. 100 millitesla = 100 mT). As magnetic flux density increases the force exerted on moving charges increases (Feynman, 1964), making magnetic flux density a critical factor to consider in determining the effects of EMF on materials.

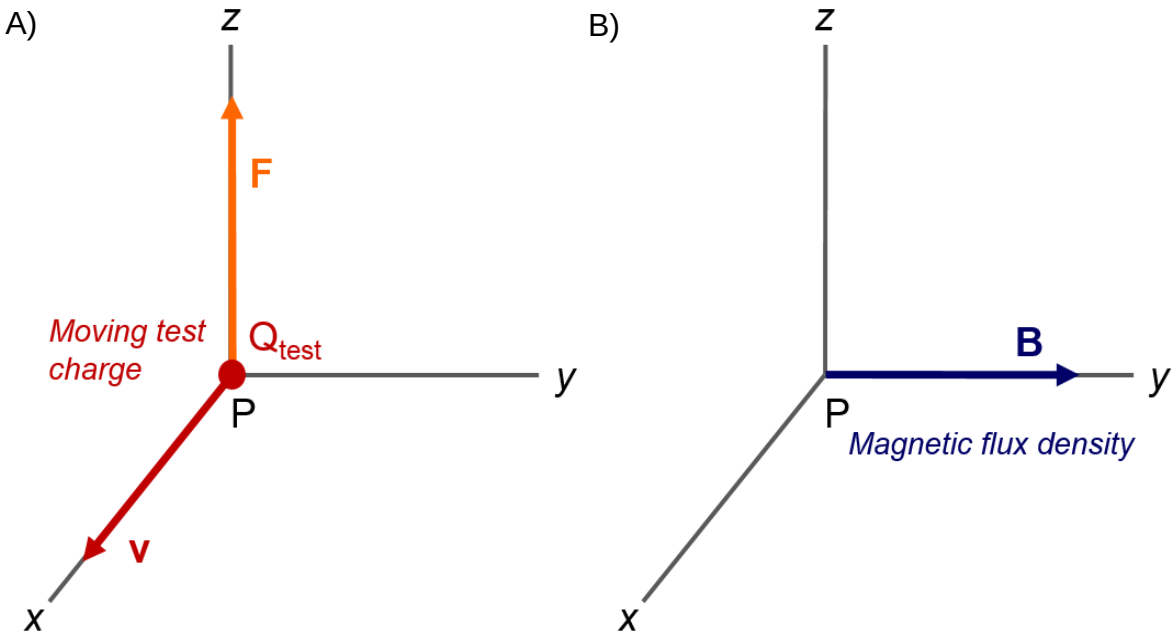

Figure 1. Representation of forced exerted on a moving charged particle in a magnetic field A) Force (F) exerted on Q_{test} having velocity (v) at a point (P) in space (F being perpendicular to v). B) Magnetic flux density (B) at a point (P), that causes F (B is perpendicular to v and F) (Figure adapted from Christensen and Durney, 1999).

1.2.4 Electromagnetic fields

The complex dynamic interaction between magnetic and electric fields exerting forces on charged particles is referred to as the electromagnetic interaction, and is one of the four fundamental interacting forces of nature along with the strong interaction, the weak interaction, and gravity (Feynman and Wilczek., 2017). In 1861 James Clerk Maxwell published the equation deriving Ampère's circuital law, describing that magnetic fields circulate around electric currents and time-varying electric fields, as well as Faraday's law, describing that electric fields circulate around time-varying magnetic fields (Maxwell, 1861; Christensen and Durney, 1999). This intrinsic interaction of electric fields and magnetic fields results in their reference together as electromagnetic fields (EMFs) (Christensen and Durney, 1999).

1.2.4.1 Time-varying EMFs and electromagnetic radiation

A time-varying magnetic field occurs when the direction of moving charge (i.e. current) changes direction with time (e.g. with an oscillating charge), thus changing the direction of the resultant magnetic field. In this way a changing electric field gives rise to a changing magnetic field (Ampère's circuital law, 1.2.4), and this changing magnetic field will give rise to a changing electric field (Faraday's law, 1.2.4), creating an electromagnetic wave that propagates through space with electric and magnetic components. The oscillations in this electromagnetic wave match the oscillation of the source charge. The propagation of electromagnetic waves through space is a phenomenon known as electromagnetic radiation. The electric and magnetic field components of electromagnetic radiation exert forces on charged particles, and the direction of the exerted force on charge particles alternates with the oscillations of the electromagnetic waves.

1.2.4.2 The electromagnetic spectrum

Electromagnetic radiation is common in a modern environment as many man-made devices generate time-varying EMFs. For example, alternating current (AC) is commonly used in electrical transmission which generates time-varying EMFs that rise and fall sinusoidally with time (in contrast transmission with direct current (DC) generates a static magnetic field). The rate of the sinusoidal variation in the generated time-varying EMF is referred to as frequency, and is measured in Hertz (Hz), with a 50 Hz field varying sinusoidally 50 times per second (20 ms cycle period). Mains electricity around the world is usually transmitted at 50 Hz or 60 Hz, producing a 50 or 60 Hz EMF (WHO, 2007a). Along

with magnetic flux density, frequency is a critical factor to consider with regards to how EMFs will affect materials.

The way in which electromagnetic waves with differing frequencies interact with physical material varies greatly, and as a result the particular types of electromagnetic waves are classified based on their frequencies in the electromagnetic spectrum (Table 1). Broadly speaking waves are classified by frequency into ionising (approximately > $10^{16\ Hz}$) and non-ionising (approximately < $10^{16\ Hz}$) radiation, based on the wave's ability at higher frequencies to dissociate electrons from atoms (thus ionising them) (Christensen and Durney, 1999). Further distinction to different types of electromagnetic radiation comes from the different anthropogenic utilizations e.g. extremely low frequency (ELF, 3 Hz - 3 k Hz), radio waves (3 kHz – 300 GHz), and x-rays ($10^{19\ Hz}$ - $10^{22\ Hz}$) (Christensen and Durney, 1999).

Table 1. Classification and example sources of different types of electromagnetic fields, including static fields, as well as the specific classifications of time-varying electromagnetic fields on the electromagnetic spectrum.

Frequency (Hz)			**Magnetic field classification**	**Example Sources**	
				Natural	*Anthropogenic*
		None	Static	Earth's geomagnetic field	Bar magnets, MRI
Electromagnetic Spectrum	*Non-ionizing radiation*	10^0-10^2	Extremely low frequency (ELF)	Natural disturbance of geomagnetic field, lightning	Power lines, household appliances, industry
		10^2-10^5	Very low frequency (VLF)	Lightning	Computers, some radio and communications
		10^5-10^8	Radiowaves	Cosmic background, lightning	Mobile phone and cellular towers, television/radio signals, wireless internet, radar, microwave ovens
		10^8-10^{12}	Microwaves and Radiowaves		
		10^{12}-10^{14}	Infrared radiation	Sunlight (50%)	Lidar, optical communications
		10^{14}-10^{16}	Visible light	Sunlight (40%)	Artificial lighting
	Ionizing radiation	10^{16}-10^{18}	Ultraviolet radiation	Sunlight (10%)	Artificial lighting
		10^{18}-10^{22}	X-rays	Astrophysical phenomena	Medical imaging, radiopharmacology
		$>10^{22}$	Gamma rays	Cosmic rays, thunderstorms	Nuclear radioactive decay

1.2.4.3 Sources of electric and magnetic fields

Sources for electric and magnetic fields are ever present in the environment as the electromagnetic interaction is a fundamental ongoing process, with charged particles interacting consistently at background levels. There are, however, more major naturally occurring and anthropogenic sources for different types of electric and magnetic fields that impact upon the electromagnetic interaction. Static electric fields are prominent in the environment, for example they range from 120-150 V/m in the atmosphere, but can reach 20 kV/m under thunderstorms and 150 kV/m within thunderstorms (Marshall et al., 1995). Anthropogenic sources for static electric fields include DC electrical power lines for long-

range bulk power transmission. Under a DC transmission line that generates a 30 kV/m static electric field, a 22 μT static magnetic field would be generated. In comparison the naturally occurring static geomagnetic field typically ranges from 30-70 μT (it can reach up to 100 μT) (Simon, 1992). Employees that use DC electric equipment can experience high static magnetic fields. For example, workers in aluminium production are exposed to fields ranging from 4-50 mT (Kowalczuk et al., 1991). In the future the general public may be exposed to 50 mT static magnetic fields with the introduction of magnetically levitating trains (Simon, 1992). MRI patients are exposed static magnetic fields up to 2.5 T and MRI operators are frequently exposed to 5 mT static fields (Feychting, 2005).

Natural sources for time-varying EMFs include solar radiation and lightning discharges, and the range of anthropogenic sources for time-varying EMFs is vast (Table 1). Alternating current is used almost universally for electrical devices and electrical power transmission, and a multitude of devices use elements of electromagnetic radiation to function (mobile phones, microwaves, radios, x-rays etc.). Public exposure to alternating fields generally arises from such devices, as well as electrical appliances, household wiring, and AC transmission and distribution lines. Many household appliances produce magnetic fields, and these can be as strong as 50-150 μT, however the strength of these fields decreases rapidly as one moves away from the field source (WHO, 2007a). Around power stations fields peak as high as 270 μT. High exposures are experienced by those with an occupational use for EMF's in industry, for example industrial welders, who may experience alternating EMFs up to 130 mT (Simon, 1992). For some anthropogenic sources, the EMF is an essential functional component of the utility that produces it, whereas in others it is a by-product. For example, in mobile phone communications radio waves are generated as an essential function of transmitting communication signals whereas the extremely low frequency (ELF) EMFs produced by powerlines are simply a by-product of the transmission of electricity. As a result, in a modern environment there has been an extreme proliferation of electromagnetic radiation ranging most of the electromagnetic spectrum, exerting forces on charged particles in all materials, including biological materials, with very little increase in the scientific understanding of the potential biological effects of this change.

1.3 Electromagnetic fields

1.3.1 Mechanisms by which EMFs interact with physical material

To improve our scientific understanding of the biological effects of EMFs, potentially leading to ecological/health consequences, it is critical to understand how EMFs interact physically with materials. As described previously, as part of the fundamental electromagnetic interaction, electric and magnetic fields exert forces on charged particles. This is complicated by the fact that charged particles within a material will also be a source for electric and magnetic fields. In many electrically neutral objects their state is often a result of the positive and negative charges being so close together that the fields they produce cancel on a macroscopic scale. Applied electric and magnetic fields, however, can exert forces on these internal charges, causing them to separate so that the macroscopic fields they produce no longer cancel. These fields are additive to the applied field and produce a new internal field, which further affects internal charges (Feinman, 1964). In this manner an applied EMF can lead to a variety of physical effects when interacting with a material.

The interaction of electric and magnetic fields on charged particles is described macroscopically in terms of three main effects: induced polarisation, alignment of existing electric dipoles, and movement of 'free' charges. Induced polarisation means that when an electric field is applied (or induced by a time-varying EMF) the positive charge within a material moves in one direction, and the negative charge in the opposite direction, causing charge separation, and inducing a dipole within the material (Fig. 2) (Christensen and Durney, 1999).

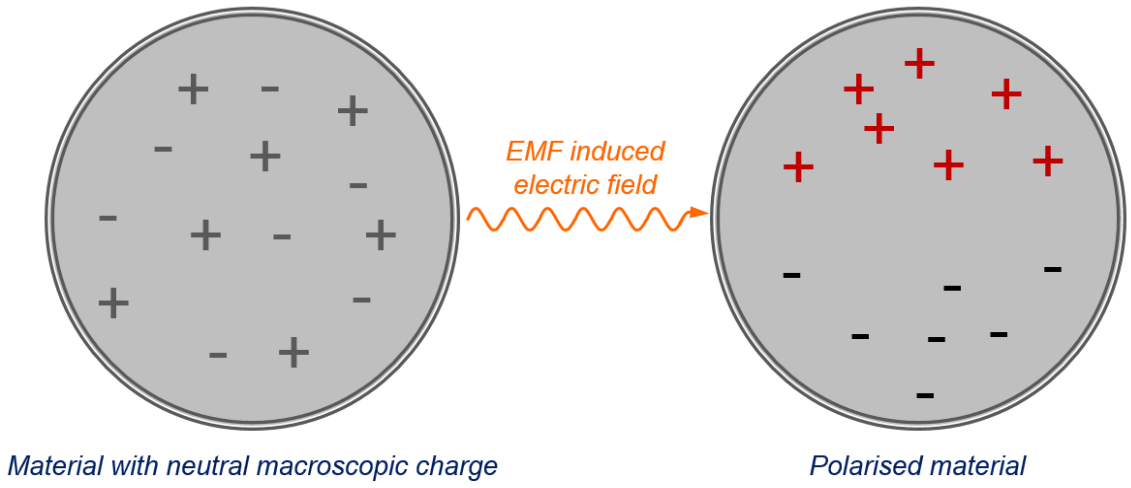

Figure 2. Induced polarisation of a material by an induced electric field.

Dipoles may also already exist within a material, however their orientation may be random causing the net field they produce to be zero. Upon the application of an electric field (or induction by a time-varying EMF) pre-existing dipoles can align due to the exerted force of the electric field on positive ends of the dipoles in one direction, and the exerted force of the field on the negative ends of the dipoles in the other direction (Fig. 3). This reduces the randomization of the dipoles such that the net electric field produced by the dipoles is no longer zero (Christensen and Durney, 1999).

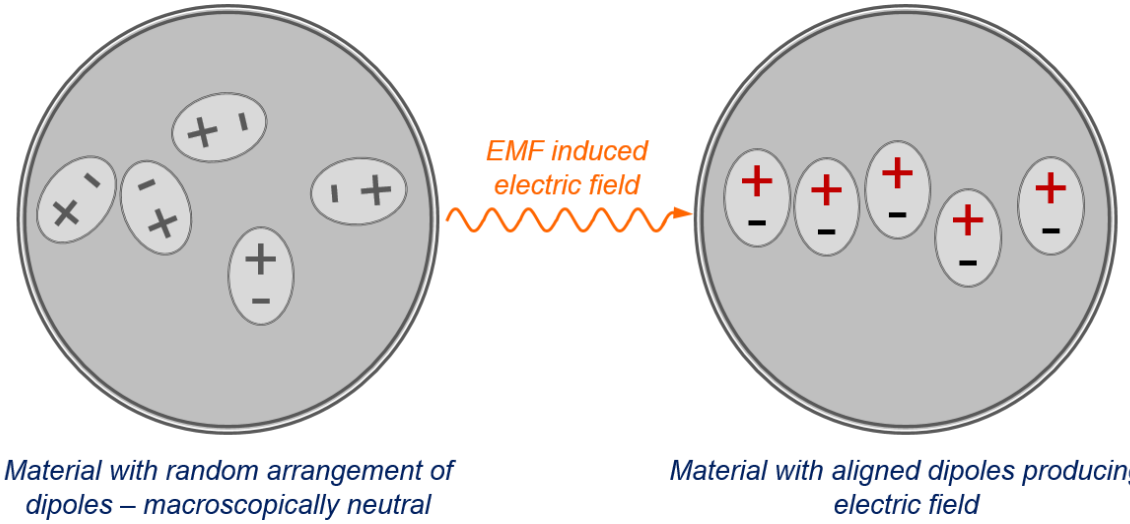

Figure 3. Alignment of existing electric dipoles in a material by an induced electric field.

Electrons and ions within a material are considered ‘free’ charges as they are loosely bound and can move in response to an applied (or EMF induced) electric field. These charges move a short distance, collide with other particles and then move in a different direction which accumulates to an average macroscopic velocity in the direction of the electric field (Fig. 4). The movement of these charges constitutes what is called conduction current (Christensen and Durney, 1999).

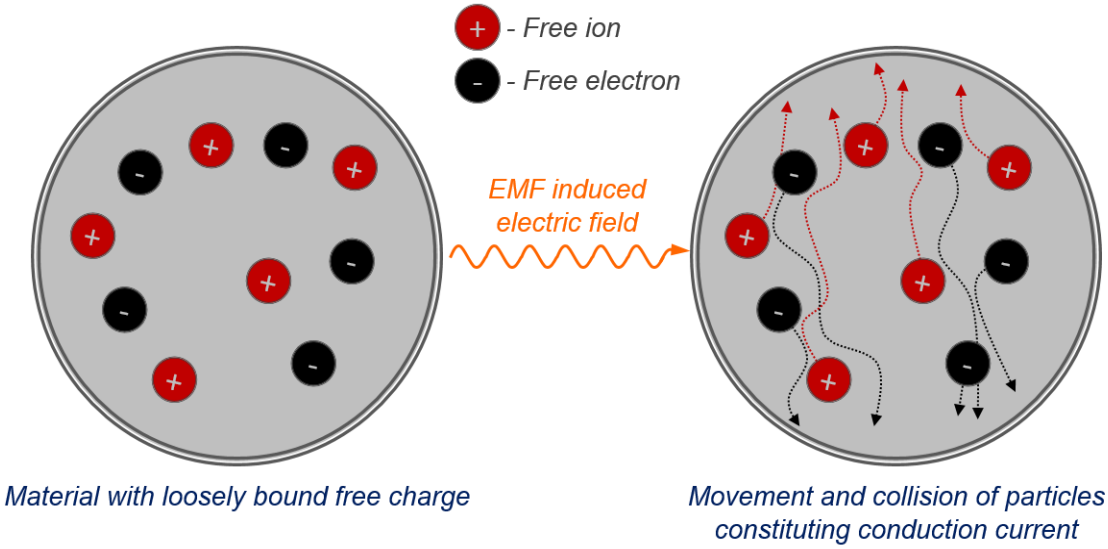

Figure 4. Forces on free charges in a material and resultant conduction current produced by an induced electric field.

1.3.2 Electromagnetic mechanisms for biological effects

1.3.3 Potential for biological effects

All of the basic physical mechanisms of electromagnetic interactions with materials apply to biological materials. The scale of electromagnetic interactions with biological systems through bioelectrodynamic processes is enormous (Zhou and Uesaka, 2006). Organisms have evolved for billions of years under the selection pressures of electromagnetic stimuli. Electromagnetic environmental stimuli in the form of light is a driving force in the biochemical process of photosynthesis (Hohmann-Marriott and Blankenship, 2011) and the transduction of electromagnetic waves by photoreceptors is the physical mechanism of vision (Arshavsky, 2002). Many organisms use Earth's static geomagnetic field for orientation, or have been linked to magnetoreceptive capabilities, including both vertebrates (Wiltschko and Wiltschko, 1972; Light et al., 1993) and invertebrates (Collett and Baron, 1994; Jacklyn and Munro, 2002). There are also different mechanisms of magnetoreception including direct detection through either magnetite (Fe_3O_4) ferromagnetic crystal deposits (Kirschvink et al., 2001) or cryptochrome molecules (Liedvogel and Mouritsen, 2010). The physical laws of electromagnetic forces control a multitude of processes on a biochemical level. Numerous biological processes rely on charge including ions in cellular signalling (e.g. K^+ Na^+ Cl^- and Ca^{2+}) (Halgamuge et al., 2009). Many dipolar molecules, or molecules with dipolar moments, have critical biological functions dependent on their electric polarity (e.g. DNA, water, α/β-tubulin heterodimers in microtubule filaments) (Srobar, 2013), and

some biological molecules have ferric properties that make them particularly electromagnetically active (e.g. iron in Haemoglobin) (Atef et al., 1995). These features and processes of biological systems are all candidates for potential biological effects of EMFs.

1.3.4 How EMF exposure leads to biological effects

Since a variety of biological processes depend on electromagnetic forces it is likely that electric and magnetic fields will have biological effects. The question is how these effects may come about. The way in which an electromagnetic field interacts with a biological material can be broadly described as a gradual chain of events from initial exposure to the EMF, in terms of the magnitude of any outcomes/effects of exposure (Fig. 5). First it is necessary to consider whether the forces produced by a particular field are transduced by biological tissue e.g. potentially modifying molecules/membranes. Second, does this transduction confer a cellular signal? If cellular signalling is affected then does this confer a biological response (i.e. physiological changes or behavioural changes)? Then if there is a biological response does this lead to cellular dysfunction or other large-scale organismal changes, and if so are these permanent, or temporary, and are they beneficial or negative for the organism (Valberg et al., 1997). It is important to note that an EMF would not need to cause cellular dysfunction to be considered as having a biologically important effect, since an EMF leading to a behavioural response (without cellular dysfunction) could still have large ecological implications.

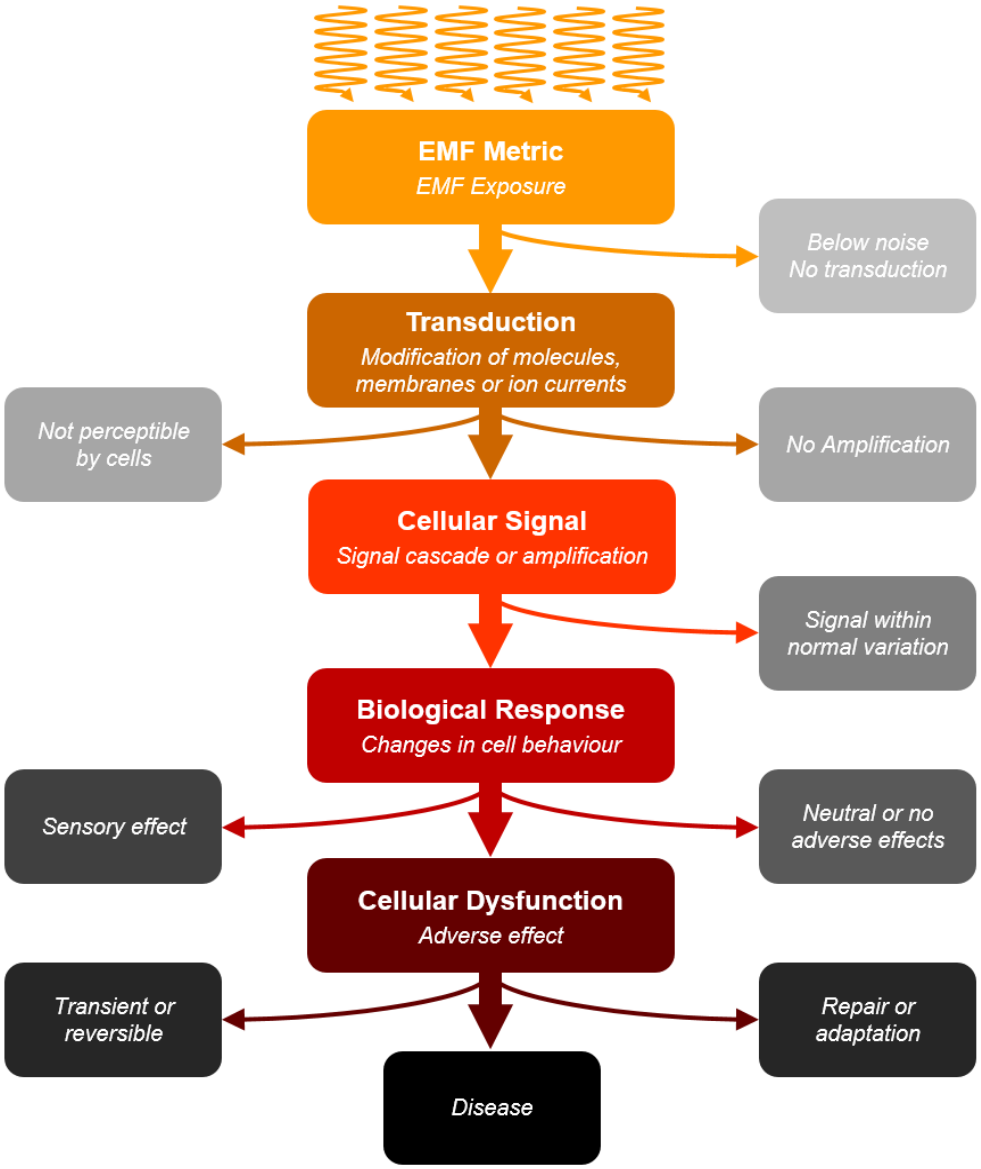

Figure 5. A simplified event chain depicting the potential pathways from EMF exposure downstream to varied potential biological outcomes (adapted from Valberg et al., 1997)

The way in which different components of the electromagnetic interaction (electric or magnetic), their state (static or time-varying), or even different classification within these groups (e.g. gamma radiation or radio waves), interact with biological material varies tremendously. To explain these mechanisms and examples clearly some of these effects have been categorized based on key differences.

1.3.4.1 Biological interactions of electric fields

Electric fields are easily perturbed by living organisms. Electric fields induce a surface charge on an organism in the field, with the net field inside the organism effectively equalling

zero (Repacholi and Greenebaum, 1999). Affecting an organism's surface charge can still have a biological effect if this modified surface charge results in a transuded signal in the organism. For example, in insects charged external mechanoreceptors (e.g. antennae, wings) would experience the forces incurred by a disrupted surface charge from an applied electric field due to Coulomb's law (Greggers et al., 2013). If these forces are sufficient to induce a signal in the organism in question then there is certainly potential for external fields to have a biological effect. Newland et al. (2015) found that fruit flies, *Drosophila melanogaster*, avoid static electric fields in a Y-tube choice chamber, where these fields produce a force that physically lifts the wings of the flies, which is likely to cause the avoidance behaviour as intact wings on the flies are necessary for the response to occur. Alongside this behavioural response there are chronic neurochemical responses by the flies to the static electric field through increased biogenic amine levels (Newland et al., 2015). These results show how forces on the surface of organisms from static electric fields can occur, as well as transduced biological effects. Another example is in bees where it has been shown they are able to perceive external electric fields and detect the surface charge of other bees, which may comprise an element of signalling between individuals within a hive in the waggle dance (Greggers et al., 2013). The electric fields in this study were mechanically generated to simulate natural electric fields generated by interacting bees, but show in detail how a natural process of electric field detection occurs, providing evidence of a potential route for biological effects from electric fields.

1.3.4.2 Biological interactions of magnetic fields

Unlike electric fields, magnetic fields are not easily perturbed by the surface of an organism, and consequently there are several mechanisms by which magnetic fields can interact with living tissues. First, there is 'magnetic induction' where magnetic fields exert forces on moving ions in solution (Matthes, 1994) that in turn gives rise to induced electric fields and currents (Repacholi and Greenebaum, 1999). Second there is the 'magnetomechanical effect', where magnetic fields produce torques on charged molecules and ferromagnetic materials (Matthes, 1994). This is an example of how the Earth's geomagnetic field is proposed to interact with magnetite particles in bees/birds/other organisms in geomagnetic orientation (Kirschvink et al., 1992). Third are 'electronic interactions', where magnetic fields alter energy levels and spin orientation of electrons (Matthes, 1994). Many chemical reactions require intermediate electron states and magnetic fields could affect the transition of an electron from a higher to lower state. Many biologically relevant electron transitions are so short that magnetic-field effects via this

mechanism may be negligible, however it has been suggested that magnetic fields may affect hydrogen bonding, altering nuclear energy levels in DNA molecules, therefore affecting the stability of DNA (Repacholi and Greenebaum, 1999).

1.3.4.2.1 Static magnetic fields

There are clear variances in how different types of magnetic fields will affect biological material. Static magnetic fields require very high intensities to have effects on a biological level. For example, it is widely accepted that for static magnetic fields to induce changes in enzyme structures (leading to altered metabolic reaction rates) to have a clear health effect, magnetic flux densities would have to be greater than 10 T (Azanza and Delmoral, 1994). It is accepted that 10 T is the cut-off point for static magnetic fields to have biochemical effects, with the exception of reactions involving free radicals as observable changes in the rate of free radical reactions is expected in static fields as low as 10 mT (Grissom, 1995).

1.3.4.2.2 High-frequency alternating fields

Biological effects of alternating magnetic fields are able to occur at much lower intensities than static magnetic fields. Where unidirectional effects would have occurred with static fields, alternating fields have a fluctuating effect due to the constantly changing direction of the field (and consequentially the force it exerts on objects within it). The oscillating nature of alternating EMFs makes them highly biologically reactive. At very high frequencies (and thus high energy intensities) the oscillatory nature of these alternating EMFs makes them capable of ionisation, meaning that they can remove electrons from atoms and molecules, which can have disastrous biological effects. For example, at high levels they can cause cancers through DNA strand breaks and formation of highly reactive free radicals leading to genetic lesions (Pierce et al., 1996; Moysich et al., 2002). At lower frequencies alternating EMFs have lower energies but are much closer to biologically relevant signals, and therefore may be more likely to cause completely different effects than higher frequency fields.

1.3.4.2.3 Mid-frequency alternating fields

Radiofrequency EMFs are an example in the mid-range of the electromagnetic spectrum, where an alternating field may be more likely to have an effect by occurring at a specific biologically relevant frequency. Larmor frequency is a critical frequency for magnetic moments, and is related to the resonance frequency in the theorised radical-pair

mechanism in the abilities of organisms to achieve the sense of magnetoreception (Ritz et al., 2004). Larmor frequency is equal to 0.028 x *B*, such that B is the magnetic flux density of Earth's geomagnetic field in a given location. In a particular example by Vacha et al. (2009) they calculated the Larmor frequency in their laboratory to be 1.2 M Hz. At this frequency it is hypothesised that the precise characteristics of that field make it much more likely to disrupt an organism's ability to detect earth's geomagnetic field via radical-pair based magnetoreception, and indeed Vacha et al. (2009) found that extremely weak fields around 12-18 nT at 1.2 M Hz will disrupt the magnetoreception of the American Cockroach with a 'deafening' effect. At different frequencies, however, a different magnetic flux density would be required to elicit the same biological effect (e.g. at 2.4 M Hz the threshold is 18-44 nT). This is supported by other examples including Engels et al. (2014) who found that a range of radiofrequency fields from 10 k Hz – 5 M Hz disrupted magnetic orientation in European robins.

1.3.4.2.4 Low-frequency alternating fields

At even lower frequencies than radiofrequency EMFs, other biologically relevant signals can be conferred. A key effect of alternating EMFs is that they can induce electric fields in biological tissues. The magnitude of this effect is potentially very large as membrane compartmentalization is extremely common within living organisms, not just for every cell membrane within an organism, but also the membranes of organelles (including mitochondria, chloroplasts, nuclei, Golgi, and endoplasmic reticulum). Free ions (such as K^+ Na^+ Cl^- and Ca^{2+}) and charged molecules have essential biological interactions with these membranes, often moving between channels and existing either side of the membranes in different concentrations and at biologically critical gradients (Halgamuge et al., 2009). The concentration of ions and charged molecules either side of a membrane affects the net voltage they confer on the membrane, which in turn can affect signalling via membrane proteins sensitive to voltage (Panagopoulos et al., 2002a). This voltage regulation, as well as mechanical stimulation by ion pressure, are two mechanisms by which ions can affect the activation of membrane channel proteins to affect signalling (Halgamuge et al., 2009). As EMFs can induce forces on ions within cells these mechanisms are strong candidates for different models of EMF interactions with living tissues that could lead to cellular signalling, and consequently biological effects.

The *forced vibration model* is a key potential mechanism for biological effects of alternating EMFs interfering with ions and the excitability of biological tissue. In this model

an induced oscillating field will cause the free ions, which maintain essential ion gradients/charge differentials, in proximity to a membrane to vibrate (at the frequency of the time-varying field) (Panagopoulos et al., 2002a; 2002b). This forced vibration can cause irregular opening of voltage-gated channels in the membrane, which can lead to cellular signals and, consequently, biological responses. To generate a force on a voltage sensor equal to that of a 30 mV change in transmembrane potential, an induced field must cause an ion displacement of approximately 0.01 nm (Halgamuge et al., 2009). This means, therefore, that the strength of the effect of an applied field is inversely dependent on the frequency of the applied field. In other words if the frequency of an applied field is increased, then the time variance between field directional changes decreases, allowing less time for ion displacement to occur. For example, a 50 Hz ELF EMF cycles every 20 ms, with a change in field direction every 10 ms allowing for the EMF induced forces to cause displacement of ions. A 1 M Hz radiofrequency EMF cycles every 0.0010 ms, allowing only 0.0005 ms for the displacement of ions with every change in field direction. As a result, with increasing magnetic field frequency for the same ion displacement to occur that would overcome the threshold for a bioactive interaction, the strength of the applied field must be increased (Fig. 6). Through these kinds of mechanisms the lower the frequency of an alternating EMF, the less magnetic flux density is required to induce effects, and as a result extremely low frequency electromagnetic fields are highly likely candidates to cause biological excitability via this mechanism.

Whilst some examples have been given for potential biophysical mechanisms for interactions of electromagnetic fields with biological material, there is still very much more to learn about these the physical mechanisms that could give rise to biological effects. In many studies on the biological effects of EMFs the precise biophysical mechanisms which may give rise to the biological effects are often beyond the scope of the study. In such cases it is important to consider that EMFs will induce forces on charged particles in biological systems, which are fundamentally important in biological activities, and this may give rise to biological responses through the general pathway of biological responses to EMFs as described here.

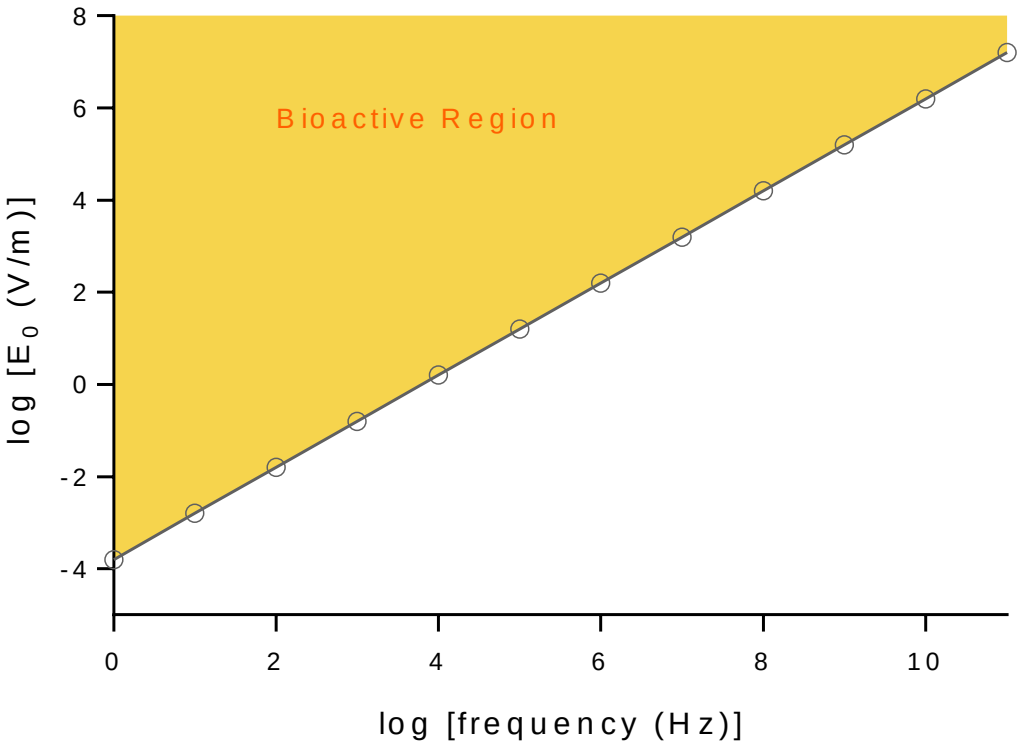

Figure 6. Relationship between the frequency of an applied EMF and the strength of the induced electric field. The area above the line indicates the 'bioactive region' where a field of the given frequency and strength will induce the opening of a voltage gated channel (adapted from Halgamuge et al., 2009).

1.4 Extremely low frequency electromagnetic fields

1.4.1 Study focus and rationale

The focus of this study is on the biological effects of extremely low frequency electromagnetic fields (ELF EMFs) which are pervasive in the environment, yet their biological effects (including impacts on insects and the environment) are poorly understood. ELF EMFs are classified on the electromagnetic spectrum in the 3 Hz - 3 k Hz frequency range. Environmental anthropogenic ELF EMF pollution has increased greatly, mainly at 50 Hz and 60 Hz frequencies, as these are the frequencies with which electrical power is transmitted. ELF EMF frequencies are close to the frequencies of important biological processes; for example, action potential durations in excitable tissue (nerves, muscles) are about 1-10 ms (100-1000 Hz) and turnover numbers of many enzymes (50 Hz) are similar to the frequencies of ELF EMFs (Blank, 1995a; 1995b). This makes ELF EMFs likely candidates to cause biological effects, that may lead to ecological and health impacts.

Understanding the biological effects of ELF EMFs emerged as an imperative research goal after a study by Wertheimer and Leeper (1979) associated ELF EMF exposure with increased childhood leukaemia, bringing about concern form the World Health Organisation (WHO) (WHO, 2007a) and leading to an enormous amount of associative epidemiological studies detailing potential human health effects of ELF EMFs. Current scientific opinion is divided, leaving the health effects of ELF EMFs on humans as a controversial topic, with the WHO concluding that ELF EMFs cause cell excitability and classifying them as ‘possibly carcinogenic’ (WHO, 2007a). This is very detrimental in terms of balancing the safe management of ELF EMF producing technologies with maximising their utility for human society, and it is imperative to elucidate whether ELF EMFs can cause biological effects that affect health, but also to extend the implications of such findings to potential impacts on the environment. In addition, while research has recently begun to identify potential ecological effects of other types of electromagnetic radiation e.g. particularly radiofrequency EMFs (Nicholls and Racey, 2007; 2009; Engels et al., 2014; Balmori, 2015; Lázaro, 2016) the ecological effects of ELF EMFs are extremely poorly understood (Gill, 2005; Saunders, 2005). There are many studies on the effects of ELF EMFs on animals (mainly mammalian) with which some potential ecological effects hypothesised, however this is limited as most of these studies have considered research questions from a human health (and not an ecological) perspective (Repacholi and Greenebaum, 1999; Saunders, 2005).

1.4.2 Sources and scale of ELF EMF emission

The most common source for ELF EMF pollution in the environment is transmission power lines. This is because AC electricity is transmitted at 50 or 60 Hz in most countries around the world, meaning that AC power transmission from power stations to homes/business where electricity is used generates 50 or 60 Hz (ELF) electromagnetic fields in proximity to these power systems. Around small to medium scale poles and pylons (11-40 kV), magnetic flux densities at ground level directly under the line can reach intensities of 10-40 μT, however larger (125-400 kV) high voltage transmission lines (HVTLs), can generate magnetic flux densities of 100 μT at ground level (WHO, 2007a). Whilst 100 μT is realistic at ground level directly below the conductor of a HVTL, the minimum ground clearance for HVTLs is 7.6 m, and much higher magnetic flux densities are experienced closer to the conductor and between the conductor and the ground (WHO, 2007a). From EMF modelling (Petrovic et al., 2013; Suleiman Sharkh personal communications), magnetic flux around HVTLs can reach 0.6 mT at 1 m from the conductor but as high as almost 14 mT at 1 cm from the conductor.

In the UK, there is 11,643 km of high-voltage transmission lines (HVTLs) at 400 kV, 11000 km of HVTLs at 132-275 kV as well as 279,427 km of distribution lines ranging from 132 kV to low voltage (ENA, 2011). Considering peak EMF exposure at ground level would occur in the 10 m area under the line, this gives an area of 116 km^2 of the UK (0.04% of UK land area) potentially affected by EMFs from 400 kV transmission lines, 111 km^2 of the UK (0.04%) affected by EMFs from 132-275 kV lines, and 2,794 km^2 of the UK (1.1% of the UK land area) potentially affected by EMFs from distribution lines which range from 136 kV to low voltage. This is just considering the area of land directly below power lines, without considering the movement range of animals that may come into contact with ELF EMFs produced by powerlines. With the full length of the transmission and distribution lines in the UK at over 302,070 km, forming an intricate network across the country bisecting habitats (Fig. 7) and producing ELF EMFs that may have an environmental impact there are large potential ecological effects of the emitted ELF EMFs. If, for example, ELF EMFs impede movement or distribution of organisms this could have large-scale ecological implications.

Figure 7. 132-275 kV high voltage transmission powerlines crossing a natural landscape in the United Kingdom

Many household electrical devices utilise electricity with conventional current and generate 50/60 Hz ELF EMFs. From an ecological perspective the impacts of these devices is almost certainly negligible, as the intensity of these electromagnetic fields deteriorates rapidly, however these devices do increase human ELF EMF exposure that may lead to biological effects. A few examples of household appliances producing ELF EMFs include vacuum cleaners (23-130 µT at 10 cm, 0.8 µT at 50 cm), hair dryers (6-2000 µT at 10 cm, 0.2 µT at 50 cm), microwave ovens (10-50 µT at 10 cm, 2 µT at 50 cm), and washing machines (1-20 µT at 10 cm, 1 µT at 50 cm) (Preece et al., 1997; Rifai et al., 2014). Other areas for potential human exposure to ELF EMFs are on transport networks, for example on the London underground passenger carriages can experience a 20 µT ELF EMF (100 Hz), and on mainline electric trains ELF EMFs reach 5-50 µT (50 Hz) in passenger coaches, 2,500 µT (100 Hz) in the equipment car and 15000 µT (100 Hz) above the inductor (Allen et al.,

1994; Chadwick and Lowes, 1998). This highlights a differential between general public exposure and occupational exposure that occurs in certain scenarios. For example, Hämäläinen et al. (1999) found that train passengers in Finland could be exposed to ELF EMFs up to 290 μT, whereas workers could be exposed to ELF EMFs up to 6000 μT. In certain occupations (particularly electrical manufacturing), workers are at risk of very high ELF EMF exposures, for example, working with resistance heaters (600-1,400 μT), arc welding (100-1000 μT), or electrogalvanizing rectification (200-460 μT) (Allen et al., 1994; NIOSH, 1996). Some of the largest occupational exposures occur for workers with induction furnaces which can commonly reach 10000 μT but can be as high as 60000 μT depending on distance (Lövsund et al. 1982).

1.4.3 Examples of ELF EMF effects

Studies to determine the biological effects of extremely low frequency electromagnetic fields are becoming more commonplace as the research field grows. It is important to consider the current evidence both in identifying knowledge gaps as well as in determining where likely effects may occur on a large scale, e.g. ecological effects.

1.4.3.1 Molecular examples

Indicators of biological or physiological stress, after exposure of biological tissues to ELF EMFs, can be valuable signals as to the biological effects of ELF EMFs. One of the most widely recognised molecular indicators of stress is a group of proteins called heat-shock proteins. The genes encoding heat-shock proteins are highly conserved and occur in every species in which they have been sought (Feder and Hofmann, 1999). Heat-shock proteins function as molecular chaperones; i.e. they interact with other proteins and as a result minimize the probability that these other proteins will interact inappropriately in biological processes. Heat-shock proteins recognize and bind to proteins that are in non-native conformations, whether due to protein-denaturing stress or because the peptides they comprise have not yet been fully synthesized, folded, assembled, or localized to an appropriate cellular compartment, and consequentially they are common molecular indicators of stress (Lindquist and Craig, 1988; Feder and Hofmann, 1999; Mayer and Bukau, 2005). Heat-shock proteins can be induced in response to a variety of stresses, including extremes of temperature, cellular energy depletion, and extreme concentrations of ions, osmolytes, gases, and various toxic substances (Feder and Hofmann, 1999; Mayer and Bukau, 2005). Activation of various intracellular signalling pathways results in expression

of heat-shock proteins. As a result, changes in heat-shock protein expression can be detected as a reliable indicator that stress on a molecular level has occurred, and a source to begin determining the mechanism of that stress. Methods for detection include transcriptomic techniques such as Western-blotting (Smith and Fisher, 1984) and real-time PCR (Nicot et al., 2005) technologies.

There is some evidence that ELF EMF exposure can increase stress-related protein expression in organisms, potentially indicating that ELF EMF exposure activates stress pathways and can lead to biological issues on a molecular level. For example, Tokalov and Gutzeti (2004) found that 50 Hz 10-140 μT EMF exposure increased Hsp70 expression in human myeloid leukaemia (HL-60) cells. Li et al. (2013) found in *Drosophila* that short and long term 3 mT 50 Hz EMF treatments caused different expression of *hsp22, hsp68, hsp70bc, hsc70-4, hsp60d*. As well as heat shock proteins, activities of other stress related proteins have been shown to increase after ELF EMF exposure. For example, Todorović et al. (2011) found that stick insects, *Baculum extradentatum*, exposed to a 50 Hz 6 mT EMF during embryonic development increased antioxidative defence activities of superoxidase dismutase (SOD) and catalase (CAT), both enzymes that prevent oxidative damage.

The mechanisms by which alternating EMFs may affect transcription and translation pathways and generate cellular stress responses have been investigated. Blank and Goodman (2004) suggest that electric and magnetic fields generate forces on electrons that destabilise H-bonds in DNA such that the forces holding the DNA strands together are weakened, which could lead to 'unwinding' of DNA strands and initiation of the complex process of transcription. The energy needed to induce transcription with a magnetic field is about one thousand times lower than the energy needed with an electric field (Blank, 1995a). DNA strand breaks are proposed by Blank and Goodman (2004) for explaining electromagnetic responsive elements (EMREs). An example of these occurs with prominent indicators of stress, heat-shock proteins. *Hsp70* gene expression by electromagnetic fields (in human HeLa cells) has been associated with nCTCTn binding sites in the *Hsp70* promoter that lie upstream of the transcription binding site. The sequences act as EMREs as the ability to induce stress proteins with an EMF gradually disappears as you mutate each of these sequences one by one (Lin et al., 2001). As the DNA structure will determine electron transferability, the makeup of these sequences could make them preferential for interactions with EMFs, like those described by Blank and Goodman (2004), that would make these more susceptible to initiate DNA transcription. However Lin et al. (2001) also admit that whilst they have shown that modifying these sequences affects EMF responsiveness the actual

mechanism for this could be more indirect. It is noted by Blank and Goodman (2004) that the nature of an oscillating field is much more likely to influence the hydrogen bonds in DNA in such a way that would decrease the stability of these bonds, and for this reason much lower level magnetic flux densities may be able to induce such a reaction where fluctuating magnetic fields are applied. No further developments in this field have been made since Blank's group in 2004, and so further detail on direct ELF EMF-induced gene expression has not been established. As mentioned previously, ELF EMF proliferation in the environment has only occurred in the last century, giving no clear historical environmental selection pressure that would be likely to evolve ELF EMF responsive elements in the promotor regions of genes that may protect against ELF EMF damage, especially in genes so highly conserved as heat shock proteins are. With this evidence weighed, ELF EMFs may affect gene expression through direct DNA interaction, but if this occurs it is likely to be a non-specific process where multiple genes are upregulated in the event that, by chance, their promoter regions contain sequences that are more responsive to ELF EMFs. Alternatively, for example with the upregulation of stress-related genes, upstream changes in molecular biology from ELF EMFs may lead to downstream changes in gene expression. Scientifc understanding of the mechanisms of molecular ELF EMF interactions must be advanced as this will improve knowledge of how specific or extensive molecular ELF EMF effects are.

1.4.3.2 Physiological effects

ELF EMFs may also cause effects on a physiological level. The nervous system functions via electrical signals, and as a result it is inherently susceptible to ELF EMFs (WHO, 2007a). ELF EMF exposure is known to induce electric fields within organisms which can excite neurons (Dimbylow, 1998; 2000; Jacobson et al., 2005). A variety of mechanisms have been proposed by which ELF EMFs can affect neuronal signalling, and most of these are related to fluctuations in important charged ions, for example via the forced vibration model. As a result, the WHO has set out as a high priority research 'to determine threshold response to ELF-induced multi cell systems, such as neural networks' (WHO, 2007b).

A variety of interactions with charged ions could affect physiological processes. For example, there is potential for the Na^+/K^+ ATPase pump to be affected by ELF EMFs as the frequency range of these electromagnetic fields are very close to the turnover rate of the enzyme (Yoda et al., 1984; Blank, 2005). There are many studies that report modified activity of the Na^+/K^+ ATPase when induced electric or electromagnetic fields are applied

(Serpersu and Tsong, 1983; 1984; Blank and Soo, 1989; 1990; 1996; Liu et al., 1990; Blank, 1992). One suggested mechanism for this effect is modified binding of ions to the enzyme activation sites (Blank, 1992), which is similar to the mechanosensitive effect Halgamuge (2009) suggests. This model was then improved by Blank (2005) with better understanding of the biochemical and biophysical mechanisms in the Na^+/K^+ ATPase. This model explains how the Na^+/K^+ ATPase, which pumps ions across their concentration gradients in biological membranes (Skou, 1957), is made up of an α and β polypeptide chain, of which the α-chain catalytic activity is directly related to ion concentrations in contact with the enzyme (Läuger, 1991). As a result the enzyme exists in two conformations, one with ATP and Na^+ bound intracellularly and one with K^+ bound extracellularly (Rephaeli et al., 1986), and it alternates between these two confirmations, interchanging charge between intracellular and extracellular solutions. It has been shown that the mechanism of conformational changes of the Na^+/K^+ ATPase is reliant on charge shifts that occur within the protein during ATP splitting (Bühler et al., 1991; Glynn, 1993). Blank (2005) suggested that flickering H-bonds allow for electrons and proton charges to exist transiently, and the effects of electromagnetic fields on these transient charges could initiate the conformational changes in the enzyme that result in disrupted ion binding to the enzyme activation sites, and ultimately the observed EMF-dependent effects on its function. While the mechanism of interaction is not completely understood, it is clear that ELF EMFs are able to affect the activity of the Na^+/K^+ ATPase, and through this route could critically alter the levels of important ion gradients and function of neurons.

Calcium ions are another important charged particle involved in physiological responses that may be susceptible to ELF EMFs. Ca^{2+} is involved in muscle contraction, and therefore the effects of ELF EMFs could occur directly in muscle tissue due to modified Ca^{2+} levels. Bone, and also muscle tissue, are piezoelectric due to the polar orientation of collagen, which means that mechanical stress in these tissues can lead to the accumulation of electric charge (Christensen and Durney, 1999). In humans, when quietly standing, vibrations are generated in muscles that induce mechanical stains and currents in the frequency range 5-30 Hz (Antonsson and Mann, 1985). This leads to an increase in intracellular calcium levels which aids the generation of muscle contractions, and aids strengthening of bone tissue (Christensen and Durney, 1999). Consequently there is potential for 50 Hz ELF EMFs to stimulate this piezoelectric tissue, and affect this interaction. Hemmersbach et al. (1997) found that mechanosensitive Ca^{2+} channels in ciliates are stimulated by a 2 mT ELF EMF causing an ion influx, which directly increases their velocity. In addition, it has been shown in many human cell types that ELF EMFs can increase Ca^{2+} levels (Carson et al., 1990; Lyle

et al., 1991; Lindström et al., 1993; 1995; Fitzsimmons and Baylink, 1994; Barbier et al., 1996; Pessina et al., 2001).

There are also examples of cellular and whole animal effects of ELF EMFs. Exposure to a 50 Hz EMF at 1 mT for 20-120 min can cause a change in the morphotype of coccoid *E. coli* cells, which is likely to be due to changes in cell division (Cellini et al., 2008). Electromagnetic fields at the same frequency and magnetic flux density but with whole organism exposure for 15-30 min has also been shown to affect cellular shape of immunocytes in the invertebrate mussel *M. galloprovincialis* with potential links to K^+ channel activation (Ottaviani et al., 2002). A variety of studies have found developmental effects of ELF EMFs in *Drosophila* For example, exposure to 250 µT 50 Hz EMFs for 48 hr increased developmental time (Patenković et al., 2015), however 500 µT 48 hr 50 Hz EMFs decreased developmental time (Dimitrijević et al., 2014; Zmejkoski et al., 2017) in post-embryonal stages of *D. subobscura*. Patenković et al. (2015) found that post-embryonal *D. subobscura* exposed to a 250 µT 50 Hz EMFs had abnormal adult morphology (e.g. enlarged wings), and similar findings have been found for *D. melanogaster* with a 80 µT 60 Hz EMF (Graham et al. 2000) and an 11000 µT 50 Hz EMF (Mirabolghasemi and Azarnia, 2002).

1.4.3.3 Behavioural effects

In terms of health and ecology, behaviour is an incredibly important attribute in determining measurable biological effects. In the cases of ELF EMFs, changes in behaviour can indicate that electromagnetic interactions with physiological and molecular processes are being translated into actual changes in the activities of an organism. Behavioural changes can be highly indicative of health (Weary et al., 2009) and are enormously important in ecological systems (Sih et al., 2004).

Our current understanding of the effects of ELF EMFs on behaviour are limited. A few studies have analysed the cognitive effects of ELF EMFs in humans. For example, Preece et al (1998) found that a 50 Hz 0.6 mT EMF reduced performance in numerical memory and work recognition tasks. Keetley et al. (2001) found reduced performance in a visual-motor memory task with exposure to a 50 Hz 28 µT EMF. Podd et al (2002) found reduced performance in a recognition memory test for 50 Hz 100 µT EMF exposure. Trimmel and Schweiger (1998) found that with a 1 mT 50 Hz EMF there were reductions in visual attention, perception and verbal memory. Several studies report a reduced task performance in mice and rats for ELF EMFs above 100 µT (Kavaliers et al., 1996; Lai, 1996; Lai et al.,

1998; Sienkiewicz et al., 1998a; 1998b). There appears to be no non-mammalian studies to determine the effects of ELF EMFs on cognitive behaviour, as research focus has been in mammalian models. In contrast, for locomotory behaviour, there are mammalian and non-mammalian examples. Janać et al. (2005) found that 7 day exposure to 50 Hz 500 µT ELF EMFs increased locomotory activity in rats, however Rostami et al (2016) found double half day exposures of rats to a 4 mT 60 Hz ELF EMF decreased locomotory activity. In one of the few non-mammalian studies Zmejkoski et al. (2017) found that exposure of 1-day old *Drosophila subobscura* to 50 Hz 500 µT EMFs for 48 hrs decreased locomotor activity.

1.4.3.4 Ecological effects of ELF EMF and applications

Although the variety of EMF-related biological effects are in many cases poorly understood, the potential for biological effects could lead to clear ecological impacts. There is a depth of evidence for the ability of many insect groups to be able to detect electric and magnetic fields. As a result an observed behavioural effect of electromagnetic fields on insects is often an attraction or repulsion to/from the electromagnetic field. These effects vary between insect groups. For example, low-frequency EMFs have been shown to diversely affect insect groups causing positive taxis for some groups, and negative taxis for others (Wijenberg et al., 2013). This simple behavioural effect provides a potential pest control method via the application of electromagnetic fields which may even be selective on insect groups based on different repulsive/attractive interactions. Electrified coils could act as bait traps as a non-toxic alternative to pesticides for pest control in an urban environment (Wijenberg et al., 2013).

The attractive or repulsive effects of electromagnetic fields are not limited to insects, and neither are the applications of these technologies. Sharks have electrosensory pores known as the ampullae of Lorenzini which allow them to detect weak electromagnetic fields, but powerful magnetic fields may overwhelm this sense, repelling sharks. Permanent magnetic deterrents have been shown to have high potential to reduce shark bycatch in fishing (Robbins et al., 2011). The Shark Shield Freedom 7TM is a device for surfers or divers that can strapped to the ankle with a control panel on the strap and 2 m of light tubing (which can trail behind the user in the water) which contains two 40 cm electrodes. The device generates a pulsed electromagnetic field and open water studies in South Africa and Australia with decoy seal baits provide promising evidence of the device as a successful deterrent of great white sharks (Huveneers et al., 2013).

Only a few studies have asked whether ELF EMF pollution can actually have ecological effects. Vertebrates have been found to be affected by weak magnetic fields (1.2 μT) around 735 kV power-lines, which cause kestrels to be more active during courtship, brood-rearing, and incubation periods (Fernie et al., 2000). In the 1950s the US Navy used extremely low frequency EMFs to communicate with submerged submarines with large antennae's that alternate between 72 or 80 Hz to produce a binary EMF signal (National Research Council, 1997). The ELF EMF produced by the antennae is very weak at 1.03 μT at 28 m distance and 0.55 μT at 58 m (National Research Council, 1997). A report summarising the studies conducted by the US navy was produced by the National Research Council (1997) but was very critical of the experimental design of the studies. In one example, the effects of ELF EMF exposure was tested on two species of leafcutter bees (Megachilidae), and for 5 of 8 parameters tested significant effects were found, including nest orientation and overwinter mortality. Despite this, however, the authors concluded ELF EMF effects were absent or at most minimal (National Research Council, 1997). The quality of these studies is very low, and the intensities of magnetic fields generated around communications antennae are much lower than those found around HVTLs. This indicates how poor the current understanding is of the ecological effects of ELF EMFs in a modern environment.

Many organisms use Earth's static geomagnetic field for orientation, or have been linked to magnetoreceptive capabilities including vertebrates such as birds (European robins, Wiltschko and Wiltschko, 1972; homing pigeons, Walcott and Green, 1974), reptiles (loggerhead sea turtles, Light et al., 1993), amphibians (red-spotted newts, Phillips and Borland, 1994), fish (sockeye salmon, Mann et al., 1988) and mammals (Zambian mole rats, Marhold et al., 1997; cattle and deer, Begall et al., 2008; Burda et al., 2009), as well as invertebrates such as insects (ants, Cammaerts et al., 2013; honey bees Collett and Baron, 1994; termites, Jacklyn and Munro, 2002) and crustaceans (spiny lobsters, Lohmann et al., 1995). There are two major mechanisms which are considered to potentially allow magnetoreception to occur, magnetite-based magnetoreception (Kirschvink et al., 2001), and cryptochrome-based magnetoreception via free radical pair interactions (Ritz et al., 2004).

There is some evidence regarding whether or not ELF EMFs may be able to affect magnetosensory capabilities. In several organisms magnetoreception is particularly disrupted at Larmor frequency, which as mentioned previously is dependent on Earth's geomagnetic field in a given location, and is believed to be the same frequency as radical-pair resonance in free-radical based magnetoreception (Ritz et al., 2004). Due to the strength

of Earth's geomagnetic field, Larmor frequency is often in the M Hz range (much higher than ELF EMF frequency). In Ritz et al. (2009), with the background magnetic field considered, Larmor frequency was 1.315 M Hz, and a 1.315 M Hz applied EMF > 5 nT was sufficient to disrupt magnetic orientation in birds. At higher (0.685 M Hz) and lower (2.630 M Hz) frequencies magnetic orientation was still disrupted, however a stronger EMF (> 150 nT) was required to affect magnetoreception. Similar effects have been shown in insects. For example, in Vácha et al. (2009) where Larmor frequency was 1.2 M Hz, with a threshold for magnetoreception disruption of 12-18 nT whereas at 2.4 M Hz the threshold for disruption was 18-44 nT. While ELF EMFs are far lower-frequency than Larmor frequency, the intensity in magnetic flux density of ELF EMFs generated around power lines from ground level to 1 m away from the conductor can be 84000-840000 times stronger than those required to disrupt magnetoreception in birds (Ritz et al., 2009) and insects (Vácha et al., 2009).

There may also be a secondary threshold for magnetic sensitivity at low frequencies as magnetic fields approach static properties. For example, in honeybees the threshold for static magnetic field discrimination is 26 nT (Walker and Bitterman, 1989). Kirschvink et al. (1992) found that honeybees could discriminate 2.2 mT alternating EMFs in a choice experiment, an ability which approached randomness when the applied frequency was greater than 60 Hz. In a follow-on experiment Kirschvink et al. (1997) found with honeybees that at 10 Hz, field strengths above 4 μT were required for EMF discrimination, whereas at 60 Hz, field strengths above 100 μT were required for EMF discrimination. These are the only experiments to date which directly tested the thresholds for magnetoreceptive discrimination of ELF EMFs. The only other study to date regarding ELF EMF effects on magnetosensory systems was that of Begall et al. (2008) who found that grazing ruminants align with the Earth's static geomagnetic field. The same research group, found that ELF EMFs from powerlines disrupted this alignment (Burda et al. 2008). Given that there are so few studies there is still a considerable need for a better understanding of the mechanisms of magnetoreception, and thresholds for different types of EMFs that can affect this sense, as this is a potential route for ecological effects of ELF EMFs.

1.4.3.5 Epidemiological studies

Whilst most epidemiological studies regarding ELF EMFs focus on their impacts from a human health perspective, they are still relevant in considering the bigger picture of improving our understanding of the biological effects of ELF EMF exposure, which in turn

may give rise to environmental effects. A real concern developed regarding the potential biological effects and health impacts of anthropogenic ELF EMFs in the late 1970's after a study by Wertheimer and Leeper (1979) associated ELF EMF exposure with childhood leukaemia. This led to decades of research into the field and multiple reports published by the WHO describing in detail the evidence of health and environmental effects of ELF EMFs (WHO, 2007a). From this the WHO have aligned their current knowledge of the effects of ELF EMF radiation with new research needs in their 2007 research agenda (WHO, 2007b). These two reports summarise that ELF EMFs are known to acutely induce electric fields and currents in biological tissues which can cause nerve and muscle excitation, however, it is urgent for research to address the uncertainty with regards to the thresholds for these effects, as well as clearer evidence for the direct effects of ELF EMFs on whole organisms and mechanisms for effects (WHO, 2007a; 2007b). This is because many epidemiological studies have been correlative, and estimate ELF EMF exposure crudely, with some particularly controversial associations of ELF EMF health effects. The number of ELF EMF studies that have monitored direct experimental exposure are in the minority, and of those most studies test the effects of a controlled ELF EMF on cell cultures and not whole organisms (most likely due to ethical barriers).

Epidemiological studies have linked ELF EMF exposure to a variety of stress responses (Silny, 1999) (including eczema), depression, mood and alertness (Dowson et al., 1988; Stevens and Rea, 2001), and as contributory effects to conditions such as Alzheimer's (Fernie and Reynolds, 2005; Garcia et al., 2008; Zhang et al., 2015), amyotrophic lateral sclerosis (ALS) and Parkinson disease (WHO, 2007a; Reale et al., 2014). There are also proposed mechanisms by which ELF EMFs could cause reproductive effects (Panagopoulos et al., 2004; Fernie and Reynolds, 2005; Li et al., 2013) and cardiovascular effects (Ptitsyna et al., 1996; Mohamed et al., 2011; Zhao et al., 2012).

As discussed previously, childhood leukaemia remains the strongest current potential health association with ELF EMF exposure (Wertheimer and Leeper, 1979; Ahlbom et al., 2000; Feychting et al., 2005). There are studies, however, that do not find this association consistently (Kheifets, 2001) or are inconclusive on the relationship between EMFs and leukaemia (Juutilainen et al., 2006). The WHO currently classifies ELF EMFs as 'possibly carcinogenic' (WHO, 2007a; 2007b) and ask for more studies to determine potential synergistic effects with other carcinogens. In addition, they state there is a range of inconclusive weaker evidence for other epidemiologies including depression, suicide, reproductive dysfunction, developmental disorders, immunological modifications,

neurological disease and cardiovascular disease; all of which needs further research to elucidate if any relationships exist.

Part of the issue in discerning whether ELF EMFs may have harmful biological effects, is in determining the mechanism by which ELF EMFs may affect biological tissues. Many of the studies that cause concern with regards to certain epidemiologies are correlative, or look at the frequency of certain epidemiologies in people who have higher than normal exposure to ELF EMFs, in comparison to people with lower exposures. Many of these studies fail to identify a clear cut mechanism for how ELF EMFs could lead to a particular disease, mainly due to the variety of biological mechanisms that could be affected by ELF EMFs.

1.4.4 ELF EMF safety, standards, and costs

The inconclusive state of opinion of the effects of ELFs on public health and the environment makes it difficult for policy makers as there are a range of potential measures that may, or may not, be taken (WHO, 2007a). The WHO (2007a) clarifies that the major objective of policy is to protect public health, however, with the implementation of any policy there are direct costs to society (i.e. the direct changes made by policy) and indirect costs (e.g. less than optimal technology usage due to EMF restrictions) that policy makers must consider. As a result this has led to a variety of ELF EMF exposure standards around the world being recommended, observed or adhered to. Exposure standards are recommended limits to EMF exposure under various technical specifications which can consider anything from intensity to frequency, exposure time, body part exposed, the status of the exposed individual (public or occupational) etc. A very important standard reference level is for power frequency, often either 50 or 60 Hz. The main consideration universally for determining ELF EMF exposure standards is public/occupational exposure risk (i.e. human health), and thus the extent to which the environment is protected from ELF EMF pollution depends on the exposure limits that are deemed safe to protect public health in different localities around the world.

There are various independent institutions that provide exposure standards including the International Commission on Non-ionizing Radiation Protection (ICNIRP) and the Institute of Electric and Electronics Engineers (IEEE), as well as recommendations made internationally by governments such as the European Union (EU). The ICNIRP recommends a maximum of 1 mT for occupational exposure and 200 μT for public exposure (which is recognised by the United Nations as safe exposure levels). The EU has EMF standards of

500 μT for occupational exposure and 100 μT for public exposure (Swanson, 2014). Neither of these recommendations are legally binding, however, and the European Union just recommends that EU member states implement measures to maintain levels below the standards. As a result there is variation in the EMF standards set by different countries and the legal force applied to these standards. At one end of the spectrum there are legally enforced, very low EMF limits e.g. Argentina 25 μT at a substation perimeter, Brazil 83.3 μT substation barrier, Italy 10 μT median 24 hr exposure for 50 Hz transmission line with 100 μT limit (Swanson, 2014). In the UK the government policy is to follow the EU recommendation of 100 μT for public exposure and 500 μT for occupational exposure, with no legal force, however due to the Health and Safety at Work Act (1974) there is some legal occupational protection (Swanson, 2014). At the other end of the spectrum there are some countries with no legal EMF coverage and complicated recommendations. For example, Australia recommends for up to 2 hours 5000 μT can be reached for occupational levels, and 25,000 μT provided the exposure is just in limbs, and the United States has an advisory occupational exposure standard of 1,200 μT for the whole body, 6,000 μT for arms and legs and 12,000 μT for hands and feet (Swanson, 2014). Bosnia, Canada, New Zealand, Peru, Spain and Turkey have no restrictions or recommendations for EMF exposure limits at power frequencies (Swanson, 2014).

There is clearly no agreement on what is a safe level of EMF exposure, which makes interpreting all of the standards around the world very difficult. Safe levels can even vary within an individual country. Within the United States there are no federal laws regarding EMF safety, however 6 states have exposure laws for power lines for 20 μT public exposure. The Netherlands Health Council 2,000 recommends a public exposure limit of 120 μT and an occupational exposure limit of 600 μT but due to the concerns about childhood leukaemia the Dutch ministry of housing made a recommendation in November 2005, that was confirmed policy in 2008, that local authorities should not give permission to build new homes in areas where annual average magnetic fields calculated from power lines is above 0.4 μT (Swanson, 2014). A study from Kelfkens et al. (2002) compared four different methods of reducing magnetic fields for homes in Holland to 0.4 μT: vector-sequence rearrangement, phase conductor splitting, line relocation and undergrounding which costed €18,000, €55,000, €128,000, and €655,000 per dwelling, respectively. These are large direct costs that are clearly incurred immediately for any government choosing to restrict EMF exposure to a certain level, and this is before considering the indirect costs such as inefficiencies of technology as a result of policy. However there is also a potentially great cost to public health for not implementing restrictions, if these ELF EMFs do actually cause

health effects. Furthermore, the impacts of ELF EMF pollution on the environment has been largely neglected as a research field, and effects here could add further costs to ELF EMF impacts. As a result it is absolutely critical for research in this field to resolve the uncertainties around the biological effects of ELF EMFs.

The scale of ELF EMF pollution in the environment is large. The lack of scientific consensus on the biological effects of large scale ELF EMF pollution has measureable economic effects. In modern urban environments transmission powerlines are often located in close proximity to urban developments. Sims and Dent (2005) found that in the UK the value of a detached property was reduced by 38% when within 100 m of a high-voltage overhead transmission line, and that reduction was still as high as 30% when within 300 m of a high voltage transmission line, when compared to similar properties in the same locality. Knowing that 22,643 km of high voltage overhead transmission lines (132 kV – 400 kV) exists in the UK amplifying this result to the whole UK would mean there is a zone of 600 m (300 m each side of a HVTL) around HVTLs, with a total UK area of ≈ 13,586 km^2 (5.6% total UK land area), where the value of a detached house would see a 30 % reduction due to the presence of an overhead transmission line. Most importantly, Sims and Dent (2005) found the highest factor affecting this value was the perceived health risk of powerlines, ahead of any visual issue. This represents a large potential economic loss, due to the general public's perceived biological effects of ELF EMFs that could be mitigated by better understanding of ELF EMF effects.

1.4.5 Research needs

Given the current situation regarding ELF EMF understanding, the WHO have devised a research agenda to drive research focus to high priority areas where understanding must be improved to tackle the problems cause by the ELF EMF knowledge gap. Some of the high priority research needs include a need for pooled analyses of brain cancer studies, neurodegenerative studies, and potential effects of EMFs on cognitive behaviour (WHO, 2007b). The WHO (2007b) state that animal studies have an advantage as they can be used when it is unethical or impractical to perform studies with humans, and the experimental conditions can be more rigorously controlled, especially at chronic levels. In addition, as such a large portion of ELF EMF studies have used cell cultures, it is essential for whole animal studies to be conducted to improve understanding of ELF EMF interactions with whole organisms. With this in mind it is a high priority research need for animal studies with EMFs to continue and due to evidence in animals suggesting that EMFs are not carcinogenic

to investigate the potential of EMFs to act as a co-carcinogens, or a multiple stressor that aids the stressing abilities of a different carcinogens (WHO, 2007b). The research needs in these areas are diverse, with an agenda for behavioural studies, studies of immature animals to indicate potential cognitive effects in children, possible pre/postnatal effects of EMF exposure (particularly with regards to nervous system and cognitive function), and further investigation of physiological effects of EMFs to determine mechanistic basis's for behavioural EMF effects. In addition to this a specific high priority research need is to determine threshold responses for ELF EMF effects in multicellular systems such as neural networks, and a general crucial research need is to clarify threshold levels and exposure parameters.

The research focus for the last 38 years has been directed towards the health effects of ELF EMFs and not the potential ecological effects. The Millennium Ecosystem Assessment (MEA, 2005) explains the essential requirement in a modern environment with increasing anthropogenic change to identifying anthropogenic activities that may lead to adverse ecological effects, as well as knowledge gaps, thresholds for effects and management solutions. As a major anthropogenic change that may give rise to biological effects, and consequentially ecological impacts, ELF EMFs represent a major potential environmental stressor that must be investigated further.

1.5 Studying the effects of ELF EMFs on insects

In this study it is a major aim to understand the potential effects of ELF EMFs on insects and the ecological processes they are involved in. There are clear human benefits to understanding how an anthropogenic factor affects the biology of pest insects and beneficial insects, as well as how these species may respond to modified environmental stimuli. In addition, improving the understanding of the biological effects of ELF EMFs on insects may lead to a better understanding of the biological effects ELF EMF in general. Given the current research needs for ELF EMFs and their biological effects, there are a variety of experimental techniques, and attributes of insect biology, that can be used to address some of these knowledge gaps.

1.5.1 Key attributes of insect research

Insects, and indeed many invertebrates, have relatively high reproductive rates, relatively short lifespans, many sequenced genomes, less ethical considerations, they are often far cheaper and easier to obtain (and to keep and handle due to smaller body size and general maintenance), and they are often experimentally easier to manipulate (e.g. dissections, but also many other biological benefits) (Burrows, 1996; Ballatori and Vollalobos, 2002). This means that insects in particular are often able to overcome ethical, practical and economic issues that arise with targets of a particular study, thus making them attractive experimental models. As a result insects have been used (or their use has been well justified) as experimental models for many different fields of biological sciences including ageing (social insects including ants and bees) (Keller and Jemielity, 2006), cognition (honeybees) (Menzel, 2012), epigenetic mechanisms (many insects, honey bees included) (Lyko and Maleszka, 2011), exercise physiology (flying insects) (Wegener, 1996), genetics and developmental biology (*Drosophila*) (Kohler, 1994), human pathogens (many insects) (Scully and Bidochka, 2006), neurobiology (locusts) (Burrows, 1996), wound healing and tissue regeneration (*Drosophila*) (Belacortu and Paricio, 2011). Each of these processes are well understood because of studies with model organisms, and processes like these could be good potential areas to test the effects of EMFs because the biological processes are so well understood.

Due to their economic importance honey bees have become a focus for ecological issues, but they also represent a species for analyses of cognitive behaviour. Bees exhibit a proboscis extension response (PER) which represents a Pavlovian conditioning assay for

appetitive learning (Matsumoto et al., 2012), and a sting extension response (SER) assay for aversive learning (Vergoz et al., 2007). PER and SER have different broad ecological implications, with SER being associated with the ability to learn and avoid threats in the environment (McNally and Westbrook, 2006) whereas PER is often associated with the ability to learn and assess quality of food sources in the environment (Ramirez-Romero et al., 2008). Learning and memory of bees is correlated with pollination success (Raine and Chittka, 2008), and the effects of environmental stressors, and potentially ELF EMFs, on these cognitive behaviours may have large scale ecological and economic impacts.

1.5.2 Potential for ecological effects of EMFs on insects

1.5.2.1 Potential exposure

Flying insects may be particularly susceptible to ecological effects of ELF EMFs. As mentioned, in the environment ELF EMFs at ground level under HVTLs can reach 100 μT, but the minimum clearance for HVTLs is 7.6 m, and with greater distances from the ground in closer proximity to the conductors of HVTLS much higher levels up to 14 mT can be experienced. This highlights a critical point that current safety limits for ELF EMF exposure are only made from a perspective of protecting public health, and not environmental impacts, both in terms of what intensity is considered an environmentally safe exposure level, but also where measurements are made to enforce ELF EMF exposure limitations (ground level). Flying animals are particularly likely to be exposed to higher levels of ELF EMFs in close proximity to a HVTL conductor (Fig. 8), including flying insects that do not always fly at ground level. For example, undulations, obstacles and terrain can cause bumblebees to fly > 7 m high when foraging (Osborne et al., 1999) and drone honeybees can often fly > 30 m high when congregating for mating (Gary, 1963; Loper et al., 1992).

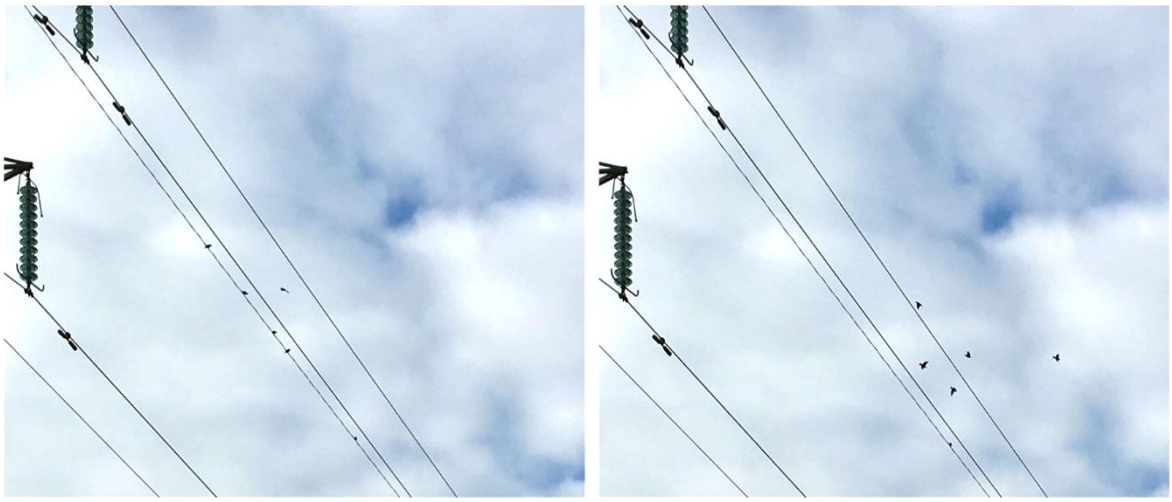

Figure 8. Birds perched on and flying in very close proximity to the conductors of a high voltage transmission line

Not only are ELF EMF levels potentially encountered by insects high, but the range of habitats where ELF EMFs may affect pollinators is very large. Power networks emit EMF pollution at these high levels and cross huge areas of habitat where insect pollinators may be exposed. As mentioned previously there are 22,643 km of HVTLs (132-400 kV) in the UK electricity transmission system (ENA, 2011). The foraging distance of bees varies markedly between species and also depends on body size (Greenleaf et el., 2007), however if it is conservatively estimated that bees forage regularly up to 1.5 km then a corridor of 3 km is present around a transmission line that could be within foraging distance of bees. With 22,643 km of HVTLs alone this gives a 67,929 km^2 area of land (equating to 28% of the land in the UK) within bee foraging range of HVTLs where high levels of EMF pollution can be experienced. For honey bees this range could be even larger as Beekman and Ratnieks (2000) found the mean distance of foraging flights was 5.5 km and median distance was 6.1 km, with only 10% of bees foraging within 0-5 km of the hive. This large land area is only considering the impacts of HVTLs on foraging bees, but local distribution companies own over 279000 km (ENA, 2011) of lower voltage overhead lines, which generate ELF EMFs near the cables up to 1 mT, and the total electricity network in the UK is over 1 million km long (ENA, 2015), which means the area of land within foraging distance of relatively high EMFs is vast. It is pertinent therefore to explore the potential impacts of increased ELF EMF exposure from HVTLs and other sources on flying insects in the environment.

1.5.2.2 Potential effects

As well as the evidence that flying insects may be highly likely to be exposed to ELF EMFs, there is also evidence that insects may be affected by electromagnetic forces of electric and magnetic fields, and this may make insects susceptible to ELF EMF exposure. Static electric fields can be detected by insects mechanically by the forces produced on different body parts such as antennae (Newland et al., 2008; Jackson et al., 2011) and wings (Newland et al., 2015). It has been shown that honey bees can detect static electric fields similar to those that naturally occur within the hive, that these signals are transduced via the antennae and that the detection of surface change of other bees may be a component of how information is communicated in the waggle dance (Greggers et al., 2013). Bumble bees can detect and learn floral electric fields (Clarke et al., 2013; 2017) through mechanosensory hairs (Sutton et al., 2016).

Static/low frequency EMFs cause differential locomotory effects on different insect taxa (Wijenberg et al., 2013), and there is evidence that the European honey bee can detect Earth's static geomagnetic field as a back-up for its sun compass mechanism (Collet and Baron, 1994; Dovey et al., 2013). Evidence for this is supported by the presence of magnetite particles in the European honey bee (Schiff and Canal, 1993; Keim et al., 2002; Hsu et al., 2007; Liang et al., 2016; Lambinet et al., 2017) which are strongly linked to magnetoreceptive abilities (Kirschvink et al., 2001; Wajnberg et al., 2010). Further to this, Walker and Bitterman (1985) showed that honey bees could be conditioned with a static EMF stimulus at 40 μT and 100 μT in semi-field conditions to feed at sucrose feeders. Static magnetic fields have been shown to cause misdirection in the waggle dance (Lindauer and Martin., 1972) and an impairment of magnetic field discrimination (Walker and Bitterman, 1989) in honeybees. Radiofrequency EMFs have been shown to generally affect Hymenoptera and pollinators, for example by reducing orientation success in ants (Cammaerts et al., 2013), and reducing abundance of beetles, wasps and hoverflies as well as composition of wild pollinators in natural habitats (Lázaro et al., 2016). Favre (2011) observed increased noise levels from honey bee piping behaviour after exposure to radiofrequency EMFs and Darney et al. (2015) found increased mortality in honeybees exposed to high frequency radiowave EMFs. There is consequentially varied evidence of the effects of different types of EMFs on honey bees, or abilities of honey bees to detect static EMFs, that may make bees susceptible to ELF EMFs, but it is important to focus on the properties of ELF EMFs that insects may be affected by.

There have been very few direct ELF EMF studies with insects. Bergh (1979) found that natural atmospheric low frequency electromagnetic radiation from storms stimulates take-off behaviour in the desert locust *Schistocerca gregaria*, which may provide an evolutionary advantage to initiate movements of locusts towards environmental conditions that are favourable for breeding purposes (recent storms). As discussed in more detail earlier (1.4.3), there are a variety of studies showing effects of short-term ELF EMF exposure on *Drosophila* development (Ramírez et al., 1983; Graham et al. 2000; Mirabolghasemi and Azarnia, 2002; Dimitrijević et al., 2014; Pantenković et al., 2015; Zmejkoski et al., 2017) locomotion (Zmejkoskia et al., 2017) and molecular biology (Li et al., 2013) as well as molecular biology of stick insects (Todorović et al., 2011).

There is little experimental evidence from the literature for potential ELF EMF effects on honey bees but there are some studies including experiments where hives have been placed under powerlines, and some studies which suggest honeybees may be able to detect and learn ELF EMFs. A variety of studies (Wellenstein, 1973, Rogers et al. 1982) have found that honey bee hives are not successful or stressed from induced electric fields under power transmission lines, with Morse and Hooper (1985) reporting that bee swarms hived under high voltage power lines will readily abscond. Greenberg et al (1981) found that bee hives exposed to HVTLs had increased motor activity, abnormal propolisation, reduced weight gain of hives, queen loss, impaired production of queen cells, decreased sealed brood and poor winter survival. As a precaution, the BPA, a federal American agency, recommends that bee hives not be placed on a 500 kV right of way, especially near mid-span between towers where the electric field is strongest (Lee, 1989).

With the evidence from literature considered regarding ELF EMFs and honeybees, there appears to be some suggestion that bees, as critical pollinators, can detect ELF EMFs in the range emitted by powerlines, and hives kept under powerlines have evidence of biological stress, that may or may not be linked to the EMF levels they have been exposed to. This is important as powerline strips (i.e. the strips of land under powerlines) have been suggested to be important refuges for insect pollinators as large areas of land with beneficial host plants for pollinators, leading to relatively good species richness including rare species of bees (Russell et al., 2005; Wojcik and Buchmann, 2012; Wagner et al., 2014; Hill and Bartomeus, 2016) as well as other important pollinators such as butterflies (Berg et al., 2016). As described earlier, in a modern environment pollinators are under strain from a variety of combined environmental stressors, including agrochemical use, land-use change, climate change, disease/parasites, and competition with invasive species. If important

pollinator species such as honey bees are affected by ELF EMFs, this could mean that ELF EMF pollution contributes to the complex system of interacting environmental stressors that affect pollinators (Fig. 9). Further to this, the evidence that ELF EMFs may affect insects may have wider ecological implications, including how pest species respond to these environmental stimuli.

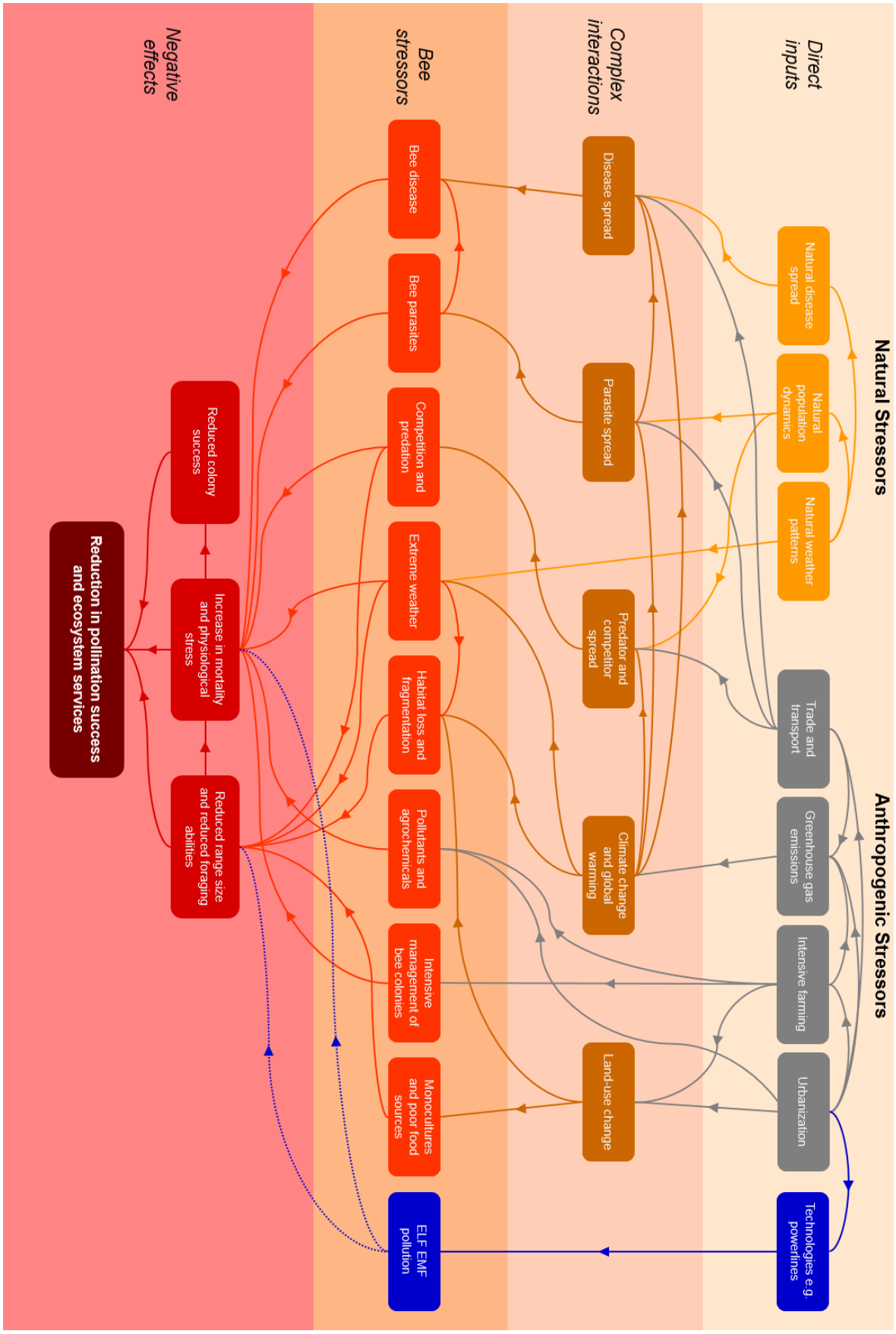

Figure 9. Interaction between ELF EMF pollution and other environmental stressors to affect pollination success of bees. Potential impacts are shown in dotted blue lines.

1.6 Aims and Objectives

Previous studies of the effects of ELF EMFs in insects have focussed primarily on the potential biological effects of EMFs, such as changes in development and locomotion. By contrast, little is known of the impacts of EMFs on insects on an ecological level, with field-realistic exposure levels. There is some evidence that radiofrequency EMFs may affect insect geomagnetic orientation. There is also evidence that power lines (which emit ELF EMFs) have detrimental effects on pollinators, however these studies have not directly determined whether EMFs *per se* are the cause of these effects. Studies focused on magnetoreception in honey bees have come the closest to exploring the relationships between pollinators and EMFs, however such studies have never asked whether the EMFs affect honey bee biology, and instead have examined magnetic fields (and mainly static fields) as an environmental stimulus that bees can detect. The extent to which ELF EMFs specifically affect bees, and other insect species, is not yet known in detail.

Aims:

Given the many gaps that exist in our current understanding of the effects of ELF EMFs on insects the broad overarching aims of this thesis are twofold:

1. Improve our understanding of the biological effects of ELF EMFs on insects.
2. Determine whether field realistic exposure levels of ELF EMFs have the potential to affect honey bees and the pollination services they provide.

This thesis will address these aims with objectives in each results chapter.

Objectives:

Chapter 2 – Effects of ELF EMFs on desert locusts:

The objective of this chapter is to use desert locusts as a well-known, tractable, model insect to understand the effects of exposure to high levels of ELF EMFs at the behavioural, physiological and molecular levels, as well as begin to explore some of the acute effects of ELF EMFs on insects. This chapter should lay foundation for whether ELF EMFs should be explored in an environmental context in other insect species, particularly in determinining whether potential locomotory effects of ELF EMFs occur consistently.

Chapter 3 – Effects of ELF EMFs on aversive learning, appetitive learning and stress protein levels in honey bees:

Honey bees are critically important pollinators word-wide and are far more likely to encounter ELF EMFs on their foraging flights than locusts. Bees are also well known for their learning abilities which underlie their foraging and pollination of crops. The objectives of this chapter are therefore twofold:

- First, to determine to what extent ELF EMFs can affect the cognitive behaviour of bees.
- Second, to determine some of the underlying molecular effects of EMF exposure in bees.

ELF EMF effects on cognitive behaviour, which is so critical for a variety of ecological processes, may indicate that ELF EMF effects should be explored in a more complex field scenario, e.g. foraging.

Chapter 4 – Effects of ELF EMFs on honey bee foraging and flight:

Understanding the effects of ELF EMFs on bees in the laboratory does not necessarily translate to similar effects on bees in the field. The objectives of this chapter are therefore twofold:

- To determine if ELF EMFs have the potential to affect honey bee locomotory behaviour at field-realistic exposure levels and exposure times, and on tethered and free flight.
- To determine the effects of ELF EMFs on feeding in honey bees; a complex ecological process that combines cognitive and locomotory behaviours, and which is critical to pollination behaviour.

Outcomes of this chapter should indicate whether ELF EMFs are likely to act as an environmental stressor in the field and should help determine whether ELF EMF effects should be studied in larger field studies, as well in combination with other environmental stressors.

Chapter 5 – The combined effects of ELF EMFs and the neonicotinoid insecticide clothianidin on flight and learning:

Globally, honey bee numbers are in decline which has the potential to cause significant effects on global agricultural production. Recent studies have revealed that the use of neonicotinoid insecticides has adverse effects on bee health, and in combination with other stressors significantly increases mortality.

The objective of this chapter is to determine the extent to which environmentally stressed bees that have been exposed to neonicotinoids are affected by acute field realistic ELF EMF exposure in terms of locomotory and cognitive behaviour.

Chapter 2 Effects of Extremely Low Frequency Electromagnetic Fields on Desert Locusts

2.1 Abstract

The effects of ELF EMFs on behaviour, physiology and molecular biology of insects are poorly understood. Here the desert locust was used to determine the short-term and acute effects of ELF EMFs. Short-term exposure at high levels > 4 mT reduced walking, caused increased spike latency and broadening of the spike from the fast extensor tibiae motor neuron, reduced hind leg force generated by stimulating the extensor tibiae muscle, and increased stress protein levels. Acute exposures of locusts to ELF EMFs during flight caused changes in wingbeat frequencies, with faster flying locusts showing decreased wingbeat frequency, and slower flying locusts showing increased wingbeat frequency. Further exploration of the frequency properties of the applied ELF EMF indicated that some synchrony of locust wingbeat frequency with the oscillating electromagnetic field may occur. These results indicate that ELF EMFs have the potential to cause molecular, physiological and behavioural changes in insects, as well impact acutely on key behaviours such as flight. This highlights a need to further understand the mechanisms and implications of these biological effects.

2.2 Introduction

2.2.1 Rationale for studying ELF EMFs

Electromagnetic fields (EMFs) are pervasive in the environment, especially at extremely low frequencies (ELF 30–300 Hz) where they are given off by electrical appliances and overhead power lines. There have been many studies on the effects of exposure to EMFs. In humans there has been considerable interest in the negative health effects caused by high exposure levels (WHO, 2007a), with the European Union suggesting an occupational exposure level of 1 mT (Directive 2013/35/EU; EU, 2013) to reduce the potential for damage. Insects, in common with many birds (Walcott et al., 1979; Walker et al., 1997), have a ‘magnetic’ sense including ants (de Oliveira et al., 2009), flies (Gegear et al., 2008), bees (Gould et al., 1978) and cockroaches (Vacha et al., 2009). These insects are able to detect very low levels of static magnetic fields and use them to drive orientated

movements. There is also evidence for different mechanisms of magnetoreception by organisms responsive to these low level static magnetic fields in the environment, including direct detection through either ferromagnetic crystal (Fe_3O_4) deposits (Kirschvink et al., 2001; Liang et al., 2016) or through cryptochrome molecules (Gegear et al., 2008; Bazalova et al., 2016).

Surprisingly few studies have asked whether higher magnetic field strengths have an effect on insect behaviour, yet for flying insects much higher levels of EMFs are experienced in close proximity to the high voltage power lines where exposure levels under 400 kV transmission lines can be 0.6 mT at 1m from the conductor but as high as almost 14 mT at 1 cm from the conductor, calculated from EMF modelling (Petrovic et al., 2013). The consequence of this is that there is real potential for these higher field strengths of 0.1 to 10 mT to impact on their behaviour and physiology. Moreover, few studies have analysed the effects of ELF EMFs on the responses of individual neurones and synapses. In the crayfish Ye et al. (2004) analysed the effects of very high levels of magnetic fields on the responses of an identified interneurone in crayfish, the lateral giant (LG) interneurone, which is responsible for coordinating an escape response. In response to electrical stimulation of tail afferents the spikes and synaptic potentials in LG increased in amplitude during exposure leading the authors to suggest that these changes could lead to an increase in the sensitivity of LG. They also suggest that these effects were caused by the action of the magnetic fields on Ca^{2+} channels as they are in mice where EMF exposure leads to an increase in Ca^{2+} channels (Sun et al., 2016).

At EMF levels less than 0.1 mT exposure in human cell lines (Mannerling et al., 2010), dipteran eggs (Carmody et al., 2000) and Planaria (Goodman et al., 2009) led to increased Hsp70 levels during a single exposure, while in chick embryos repeated exposure led to reduced Hsp70 and cryoprotection (Di Carlo et al., 2002) suggesting an effect of treatment duration on Hsp70 levels. More recent studies on ELF-EMF over 1mT have been restricted to *in vitro* cell systems but have shown marked effects, including an increase in Hsp70 transcription that leads to protection of chronic hypoxia (Wei et al., 2016), and Ca^{2+} channel expression in neuronal synapses (Sun et al., 2016) leading to effects on neuronal activity. In insects such as the locust, stress or heat shock leads to a marked increase in heat shock protein levels (Barclay and Robertson, 2000; Robertson, 2004) that play a key role in thermoprotection. Thermal stress-induced heat shock responses have been described in locusts leading to an upregulation of heat shock proteins (Whyard et al., 1986). Barclay and Robertson (2000) showed that these stress responses could stabilize neuromuscular

signalling during thermal stress, and that this could underlie thermoprotection of leg extensor muscle force output, possibly due to heat shock protein alteration of pre- or postsynaptic K^+ channels (Ramirez et al., 1999). In addition, in the locust flight system heat shock proteins reduce the thermosensitivity of synaptic delay and excitatory postsynaptic potential amplitude (Dawson-Scully and Robertson, 1998). Stressors, including EMFs, may activate a wide spectrum of interacting neuronal, molecular and neurochemical systems that underpin behavioural and physiological responses. Currently, however, nothing is known about the interaction between EMFs and heat shock protein expression in intact adult insects.

2.2.2 The desert locust

As a starting point to understanding how ELF EMFs may affect flying insects, the desert locust is studied here. There is some evidence that desert locusts may be responsive to low levels of ELF EMFs, which increase take off behaviour in the insects (Bergh, 1979). The thresholds for such a response however are not known, and no such studies further elaborating on or supporting potential EMF responses in desert locusts have been published since. If desert locusts are responsive to EMFs then understanding this response in detail is a priority because there are many features of the desert locust both scientifically and environmentally that make it an important study species.

2.2.2.1 Desert locust ecology

From an ecological perspective the desert locust is one of 12 other locust species that when living singly behave like the other members of the Acrididae family, grasshoppers, and assume a ‘solitary phase’; however when living in larger groups they undergo a number of behavioural and physiological changes and are known to be in the ‘gregarious phase’ (Uvarov, 1921; Showler, 2002; Alessi et al., 2014). In these aggregations locusts can form very large swarms which can be highly concentrated (up to 80 million/km^2) and incredibly mobile (they can fly up to 100 km/day or walk 1.5 km/day) (Steedman, 1988). This feature of their lifestyle can put great pressures on food security.

In 1988 in Algeria a swarm of estimated 20 billion locusts (up to 150 per m^3) consumed 35000 tonnes of vegetation in a day, which is enough food to equivalently feed 20000 humans for a year (Skaf et al., 1990). The potential scale of this problem becomes apparent when one considers that *Schistocerca gregaria* alone affects 20% of the land in the world containing 10% of the worlds’ population (Lecoq, 2001). Between the years 1986 and 1989 $400 million USD was spent attempting to control this issue in the Sahel region of Northern

Africa alone (Lecoq, 2001). The Food and Agriculture Organisation (FAO) of the United Nations monitors desert locust activity and updates a bulletin online recommending control procedures across North Africa, the Middle East and Indian subcontinent, which relies on data of moving hopper bands, and swarm take-offs and movement. Bergh (1979) suggested EMFs may increase take-off behaviour in locusts, and if desert locusts are responsive to electromagnetic fields, then potentially EMF data could improve swarm modelling. As well as this there may be a potential for EMF application from a biological control perspective, for example Wijenberg et al. (2013) found that some insect taxa exhibited negative locomotory responses to EMFs. Alternative control methods could be highly beneficial if implemented, or at least in an integrated pest management approach, as currently the main control methods for desert locust swarms are insecticide sprays (Story et al., 2005) which have associated negative environmental impacts from affecting non-target species.

2.2.2.2 Desert locust neurobiology

Another benefit to understanding ELF EMF effects in desert locusts is to improve neurobiological understanding of ELF EMFs. In an intensive research agenda (WHO, 2007b) to understand the effects of extremely low frequency electromagnetic fields on health, the World Health Organisation has identified a key priority to research the effects of ELF EMFs on intact neural networks to determine the mechanisms and thresholds for effects, as current understanding is 'limited'. In the same research agenda the WHO also calls for more animal based studies and these usually are able to explore the mechanisms of EMF effects in much more detail.

In desert locusts the components of the neural circuits controlling limb movements are known in detail (Burrows, 1996). For example, in kicking and jumping the fast extensor tibiae motor neuron (FETi) spikes causing contractions in the extensor tibiae muscle (ETi) which is critical for extension of the tibiae (Burrows, 1995). When jumping, the co-contraction of ETi and flexor tibiae muscles, caused by spikes in FETi and flexor tibiae motor neurons, causes power to build-up in the ETi muscle in preparation for the jump, as the contraction of the flexor tibiae muscle prevents the force in ETi from being released (Burrows, 1995). To trigger the jump, excitation of flexor tibiae muscle is stopped and inhibited, allowing the power built up in ETi muscle by FETi to be released, producing a powerful extension of the tibiae and pushing the locust from the surface in which it is in contact (Heitler and Burrows, 1977; Burrows, 1995, 1996). This circuit is further mediated in a positive feedback loop with two mechanosensory campaniform sensilla, on the proximal

tibia, which spike when the leg is load bearing or meets a resistance (Burrows and Pflüger, 1988). These spikes produce a wave of excitatory postsynaptic potentials (EPSPs) in FETi, following FETi spikes, which can be sufficient to elicit more spikes in FETi, and produce more force in ETi muscle against the mechanical resistance (Burrows and Pflüger, 1988; Norman, 1996). This positive feedback loop can continue until the mechanical restraint is pushed away or removed (Burrows, 1996).

There are well established methods in locusts to record from identified neurones (Alessi et al., 2014) and monitor muscle output (Wilson et al., 2012) such as the FETi/campaniform sensilla feedback loop with ETi muscle mentioned above. As well as this, stress responses have been clearly identified in locusts (Robertson, 2004). In the locust there is also the opportunity to study the effects of high levels of ELF EMFs in an animal where cognitive, behavioural, physiological and molecular effects can be analysed in detail to begin to understand how exposure to high levels of ELF EMF could impact behaviour. Investigating the effects of ELF EMFs in the desert locust may be able to address high priority research needs with regards to the potential health effects of EMFs, as well as investigate potential applied ecological benefits in increasing understanding of EMF effects on a globally important pest.

2.2.3 Aims and Objectives

While previous studies have considered the effects of ELF EMFs on *Drosophila*, the extent to which ELF EMFs affect other insect species is unknown. Locusts are a very useful species for understanding neurobiology, and are also an economically important species. The aim of this chapter is to utilise the tractability of the locust nervous system to understand the effects of ELF EMF on walking, flight and physiology, to improve understanding of the biological effects of ELF EMFs on insects. The extent to which ELF EMFs may cause acute effects in insect behaviour is completely unknown, and assays for locust flight allow this behaviour to be monitored in a controlled lab environment where any clear direct acute effects of ELF EMFs on insect behaviour can be observed. The overarching aim of this chapter is to determine the biological effects of ELF EMFs on insects at multiple levels.

This chapter addresses the following questions:

Do ELF EMFs affect locomotory behaviour in locusts?

Do ELF EMFs affect locust physiology that underpins locomotory behaviour?

Do ELF EMFs affect stress protein levels in locusts?

Does acute ELF EMF exposure affect locust flight?

2.3 Methods

2.3.1 Locusts

Experiments were performed on adult desert locusts, *Schistocerca gregaria* (Forskål), aged from 4 to approximately 9 days post-moult and of both sexes, taken from a crowded colony at the University of Southampton. Locusts were fed on seedling wheat and oats and housed under a 12:12 light/dark cycle at 32 °C.

2.3.2 Short-term exposure

2.3.2.1 Magnetotherapy applicator coil

Overnight EMF exposures were applied with a 20 cm diameter magnetotherapy applicator coil (Elektronika i Elektromedycyna Sp. J.; Poland) composed of 282 turns of insulated copper wire (Fig. 10A). The coil and Variac power supply produced homogeneous, sine-wave alternating electromagnetic fields at 50 Hz and with intensities ranging from 0.1 to 8 mT (Fig. 10, B). Field polarization was vertical so that field lines were perpendicular to the bottom plane of the chamber. The distribution of field strength within the coil varied along the length of the coil). Measurements of the fields for calibration were made using a Gaussmeter (Model GM2, AlphaLab, Inc, USA).

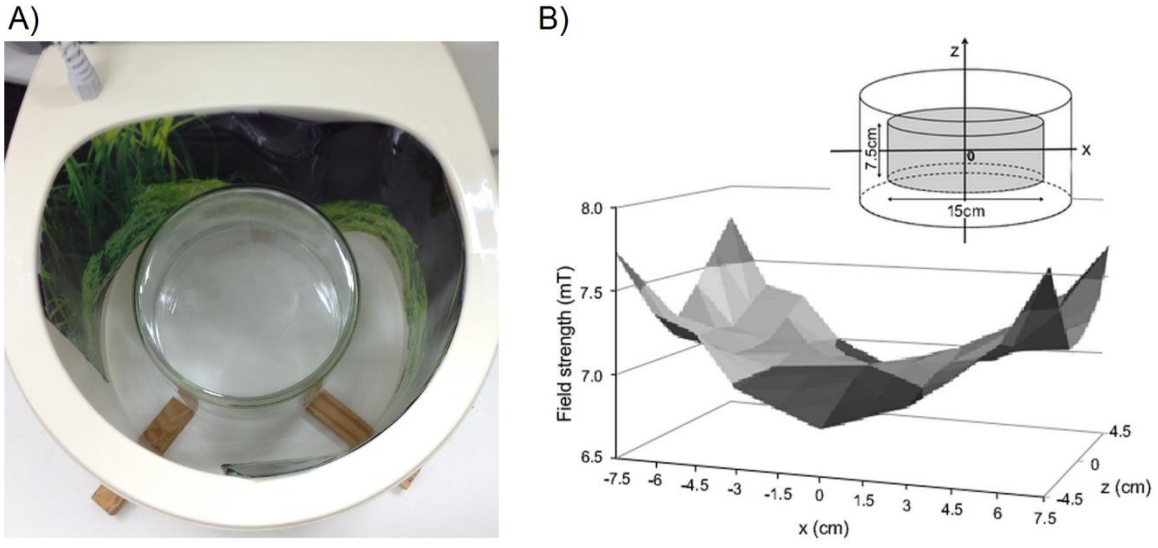

Figure 10. Experimental set up for applying ELF EMFs for short-term exposures (A) Glass treatment container in the magnetotherapy applicator/coil. (B) The average field strength (magnetic flux density, mT) distribution inside the solenoid along the Z and X axes. Inset shows the coordinate system (from Wyszkowska et al., 2016).

2.3.2.2 Treatment parameters

Animals were exposed for 24 hr to control conditions or EMFs whilst kept in a glass chamber (15 cm diameter × 7.5 cm deep) located within the coil (Fig 10, A). 6 locusts were exposed together to ensure they remained in the gregarious phase (Burrows, 1996). Exposure to 1, 4 and 7 mT EMFs were analysed. 24 hr exposure to 4 and 7mT EMFs led to heat within the coil. To control for this heat, temperature-matched controls were made using a hot plate (Dishwarmer 2; Photax®, UK) under the coil to ensure the same temperature in the glass chamber for each respective EMF treatment. At 7 mT the temperature at the centre of the glass chamber increased to 29.3 °C ± 1 °C, while at 4 mT it was 24.5 ± 1 °C. At 1 mT there was no heating in the coil and control EMF treatments were compared at room temperature (22.2 ± 1°C). Temperature during experiments was monitored using thermocouples mounted under each chamber to ensure that experimental conditions were similar, except for the presence of the ELF EMF.

2.3.2.3 Walking behaviour

Walking behaviour was analysed in an open-top tunnel (30 cm × 10 cm × 8 cm) placed within a glass tank (30 cm × 40 cm × 60 cm) an environmentally controlled room maintained at 25ºC. At one end of the tunnel a stimulus group of 5 gregarious locusts was placed in a mesh pot along with 7 g of seedling wheat and illuminated with a light source to act as an attractant. Individual test locusts were placed at the other end of the tunnel with their hind leg tarsi touching a line 5 cm into the tunnel. Walking was initiated by a single brushstroke with a fine tipped brush to tactile hairs on the dorsal tibia of one hind leg. Video recording was made from above with a HDR-CX115 Sony Handycam camera (Sony, Japan) and videos subsequently analysed offline. Locusts were given 5 min to walk the length of the tunnel, and for those that covered the entire length the trial was deemed to be complete, while for those that failed the trial was considered incomplete. The number of incomplete and complete trials were counted to calculate tunnel completion, while video analysis allowed measurements of cumulative distance travelled and completion time. In total 36 locusts were treated for each EMF level and control, and any reductions from 36 were due to losses over the 24 hr treatment period.

2.3.2.4 Muscle force

To determine the effect of chronic ELF EMF exposure on muscle function the twitch force produced by the hind leg extensor tibia muscle (ETi) was analysed using previously

established methods (Norman, 1995; Wilson et al., 2010; 2012). The ETi muscle was stimulated at a range of frequencies (10 pulses at 0.1 Hz, 50 pulses at 1, 5, 10, 20, 50, 100, 125, 150, 175, 200, 225, 250 Hz, with a 30 s rest period between each train) to generate a full range of forces in the muscle. For a 1 mT exposure, 24 locusts were tested (6 female and 6 male for both control and EMF groups), for 4 mT 48 locusts were tested (12 female and 12 male for both control and EMF groups), and for 7 mT 50 locusts were tested (13 control locusts and 12 EMF treated locusts for each gender). The mean of individual pulses at 0.1 Hz were analysed to determine twitch force, whereas 1-250 Hz, were analysed to determine the effect of ELF EMF on the tetanic properties of the muscle.

2.3.2.5 Physiological recordings

In collaboration with J. Wyszkowska, intracellular recordings were made in the metathoracic ganglion from the cell bodies of the hind leg fast extensor tibiae motor neuron (FETi), which innervates the extensor tibiae muscle (ETi) of the left hind leg, using methods previously described (Alessi et al., 2014; Newland and Kondoh, 1997). A pair of insulated 50 μm copper wires, exposed only at their tips, was implanted in the tibial extensor muscle of the hindleg and used to stimulate the ETi to produce antidromic spikes in FETi. The properties of the FETi spike and compound EPSP, resulting from synaptic input from campaniform sensilla (Burrows and Pflüger, 1988), were compared between temperature-matched control and EMF exposed groups using Spike7 software (Cambridge Electronic Design, UK). Measurements made included the amplitude, latency and duration of the FETi spike and compound EPSP (Fig. 11). Amplitude was taken as the difference in membrane potential from baseline resting potential to spike peak, latency was time between stimulus and initiation of membrane depolarization phase, and duration was the time between the membrane depolarization phase and the membrane repolarization phase as measured from the midpoint of the spike. 8 control locusts, and 11 7 mT EMF-exposed locusts were tested. J. Wyszkowska made intracellular recordings, and S. Shepherd prepared EMF treatments, and analysed data of physiological recordings.

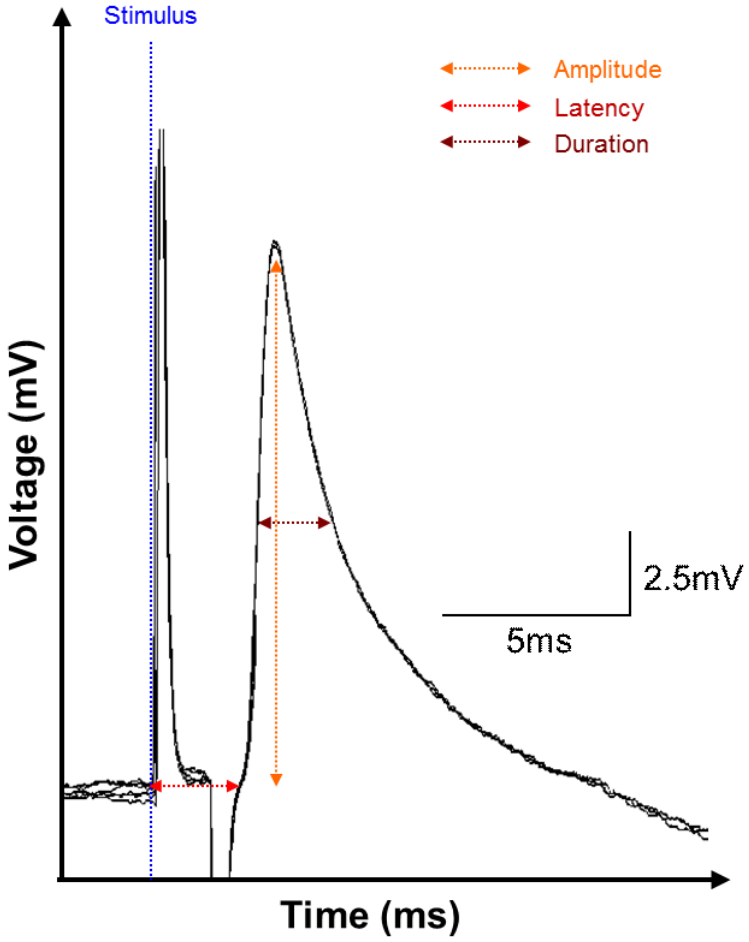

Figure 11. How measurements were made of amplitude, latency and duration (from the midpoint of spike amplitude) from physiological recordings. Labels are made on superimposed sweeps of intracellular recordings of FETi spikes evoked by antidromic stimulation of ETi muscle in control exposed locusts.

2.3.2.6 Stress protein levels

2.3.2.6.1 Sample preperation

Metathoracic ganglia (containing the somata of FETi) were isolated from male locusts and the levels of Hsp70 detected via Western-Blotting. 7 mT EMF and respective temperature matched control 24 hr treatments were compared, a heat shock treatment (45ºC for 3 hrs and 1 hr rest at room temperature), and heat shock negative control (25ºC for 3 hrs and 1 hr rest at room temperature) method for locusts (Wu et al., 2001). Following treatment each locust was snap frozen in liquid nitrogen and the metathoracic ganglion removed. Ganglia from three locusts were grouped in each sample to ensure adequate protein levels for blotting.

2.3.2.6.2 Western blotting

Samples were lysed in 2% sodium dodecyl sulfate (SDS) containing 1 × Halt™ protease and phosphatase inhibitor cocktail (Thermo-Fisher Scientific, UK) , of which 45 µl was added to each Eppendorf. Samples were pestled and then placed on a heat block at 95ºC for 5 min. The samples were then centrifuged at 14000 rpm for 10 min. The supernatant was removed into new Eppendorfs and the pellet discarded.

A Bradford assay was then carried out using a microplate reader to determine protein levels in each sample using a Pierce™ BCA Protein Assay Kit (23225; Thermo-Fisher Scientific, UK) containing BCA reagents (A and B) and albumin standard. Known protein standards were then used in a linear regression analysis on Graph Pad Prism 6 (Ver 6.00, Graph Pad Software Inc.) to interpolate unknown protein levels from a standard curve using the quantified colorimetric values from the protein assay. Using the now known protein concentrations of each sample, original samples were adjusted to 2.5 µg/ µl using 2% SDS 1xHalt, to equalise the protein concentration in every sample, and then to 2.0 µg/µl with 5 × Loading Buffer (0.25% bromophenol blue, 0.5M DTT, 50% Glycerol, 10% SDS).

A 7.5% separation gel (2.5 ml 30% Acrylamide (Sigma-Aldrich, UK), 1.25 ml 3M TRIS (pH8.8), 50 µl APS, 10% SDS, 10 µl TEMED (Sigma-Aldrich, UK), topped up to 10 mls with dH_2O) and stacker solution (15 ml 30% Acrylamide, 37.5 ml, 0.25TRIS HCL (pH6.8), 1 ml 10% SDS, up to 100 ml in dH_2O) were prepared for SDS PAGE. 30 µg of protein (15 µl of each sample) was loaded into each well, and 5 µl of protein ladder (both Precision Plus Protein™ Dual Colour (Biorad, UK) and Pageruler Prestained (Thermo-Fisher Scientific, UK) protein ladders were used. The gel was loaded in a tetra tank with running buffer (Tris-Glycine/SDS pH 8.3 - 25 mM Tris, 190 mM glycine, 0.1 % SDS) and run at 125 V for approximately 1 hour 20 minutes to resolve samples by SDS-polyacrylamide gel electrophoresis (PAGE). Gel sandwiches were then prepared for transfer on to a nitrocellulose membrane (Bio-Rad Inc.) in transfer buffer (running buffer with 20% Methanol). Protein transfer onto nitrocellulose was done overnight (approximately 17 hrs) at 30 V in a cold room at 4ºC.

After the transfer process nitrocellulose membranes were cut in half such that β-actin loading control antibody (ab8224; host – mouse; monoclonal; immunogen – synthetic peptide derived from residues 1-100 of Human beta-actin; Abcam®, UK) could be applied independently of Hsp70 primary antibody (ab2787; host – mouse; monoclonal; immunogen – recombinant fragment within Human Hsp70 122-264; Abcam®, UK). Membranes were

blocked in 50 ml blocking solution (5% non-fat dried skimmed milk (Marvel; Premier Foods, UK) in tris buffered saline (TBS) (Thermo-Fisher Scientific, UK)) for 1 hr, and then washed for 15 min in TBS 0.1%Tween-20 (Sigma-Aldrich, UK). Membranes were then incubated separately overnight in primary antibodies at a 1:1000 dilution in 5ml of TBS 0.1%Tween-20 in 50 ml Cell-Star sterile tubes (Greiner Bio-One, UK) overnight on a shaker in a cold room at 4ºC. Primary antibody was then washed off with TBS 0.1% Tween-20 and the membranes were incubated in secondary antibody (IRDye® 800CW Goat anti-Mouse polyclonal antibody 926–32210; Li-cor Biosciences, UK) at a 1:10000 dilution in probing solution (5% non-fat dried milk in TBS 0.1% Tween-20) for 1 hr at room-temperature (22ºC). Secondary antibody was then washed off with TBS 0.1% Tween-20.

Finally, membranes were imaged on a Li-cor Odyssey scanner using Image Studio version 4.0 (Li-cor Biosciences, UK). Detection strips were drawn in Image Studio around Hsp70 and beta-actin bands to determine Hsp70 and beta-actin signals, with a median up/down background detection removal setting. The Hsp70 signals were then normalised with respect to beta-actin loading controls, to control for unequal loading between wells. Hsp70 signals were then analysed to determine the effects of ELF EMF exposures on stress protein levels.

2.3.3 Acute exposure

For primary experiments determining the effect of acute ELF EMF exposure at differing field strengths on locust flight, the same exposure system as short-term experiments with the magnetotherapy applicator coil was used (Fig. 10). In secondary experiments where frequency was modulated, this exposure system was no-longer available, and a Helmholtz coil apparatus was used for acute ELF EMF exposure.

2.3.3.1 Helmholtz coil

Electromagnetic fields were generated with a custom-made Helmholtz coil (paired wire for 350 turns; 700 turns total) consisting of two 25 cm (inner diameter) copper wire solenoid electromagnets (Faculty of Engineering and the Environment, University of Southampton). Electromagnets were paired together on the same axis, on an adjustable custom stand (Fig. 12, A) 14 cm apart. Coils were powered with 240 V 50 Hz AC electricity through RS Pro 1 Phase 1.92 kVA 1 Output 240 V Variacs (RS Components, UK) to generate homogenous 50 Hz sinusoidal AC electromagnetic fields with a total range of field strength from ~10 μT-10000 μT at the centre of the Helmholtz coil (Fig. 12, B-E). Field strength (magnetic flux

density) was measured with a Model GM2 Magnetometer (Alphalab Inc., USA). For control exposures no current was passed through the coil system.

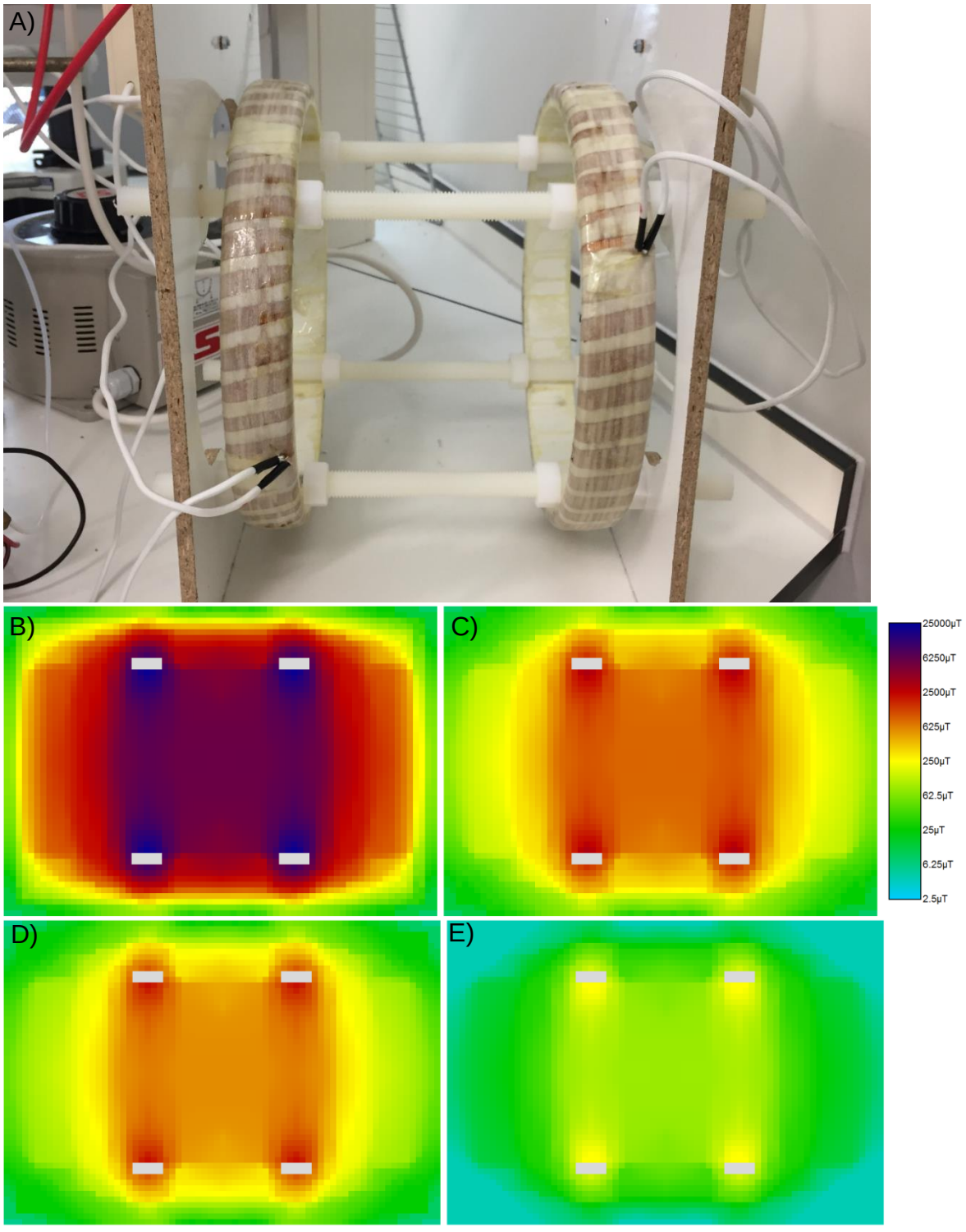

Figure 12. (A) Custom-made Helmholtz coil on stand with solenoid electromagnets for generating homogenous 50 Hz sinusoidal AC electromagnetic field placed 14 cm apart. (B-E) Magnetic field mapping for recorded field strength in lateral cross-section of the Helmholtz coil for various EMF exposures. (B) 7000 µT. (C) 1000 µT. (D) 700 µT. (E) 100 µT

2.3.3.2 Frequency modulation

To determine the effect ELF EMF frequency on flight the frequency of the sinusoidal ELF EMF was modulated by passing a 50 Hz 240 V supply through a TPA power amplifier (HH Electronics, UK) and a function generator, allowing the frequency to be modulated from 10-50 Hz. A field strength of 700 µT was consistently generated across all frequencies (Fig. 13) and was therefore adopted for these experiments.

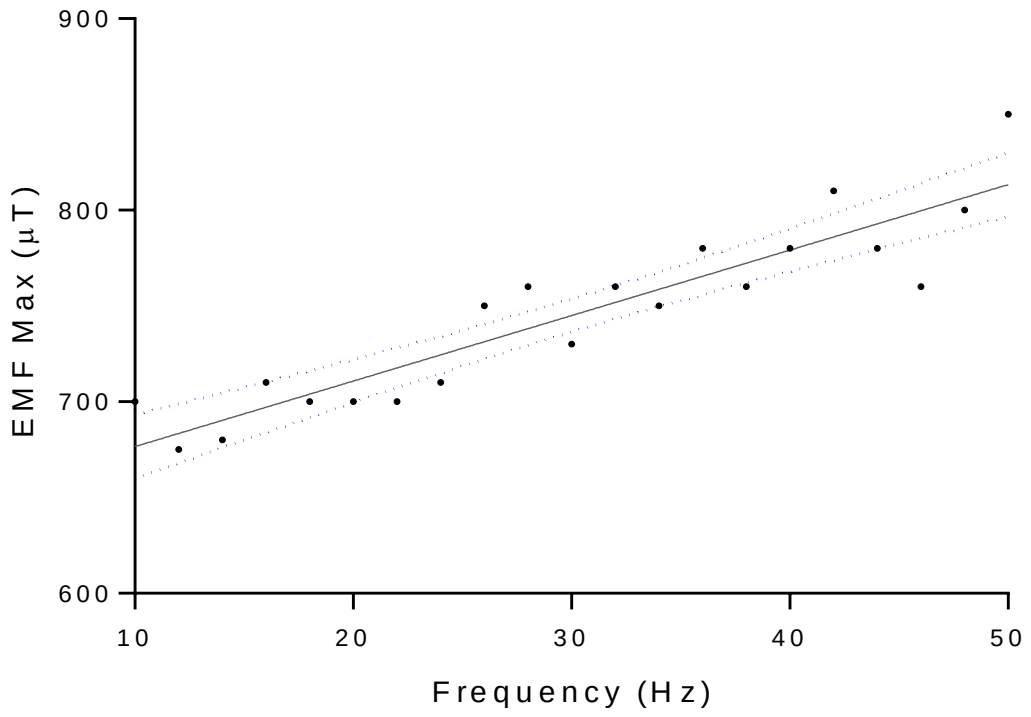

Figure 13. Maximum magnetic flux density for each tested power frequency using power amplifier and function generator to power Helmholtz coil.

2.3.3.3 Flight responses

Locust flight ability was assessed to determine if ELF EMFs cause acute effects on locomotory behaviour in the desert locust. Male locusts were individually removed from the colony and fixed to a custom made tether using resin-wax, made from a 50:50 w/w mixture of natural resin (Sigma-Aldrich, UK) and beeswax (Sigma-Aldrich, UK). Once harnessed, locusts were suspended and given a small paper ball (3 cm^3) to hold at rest. Tethered locusts were suspended in the centre of the electromagnet such that an EMF could be applied to the locust during flight.

Flight was initiated by applying a constant warm airflow over the locust, stimulating hair sensilla on the head of the locust (Arbas, 1986), and removal of the ball (Wilson, 1961). The airflow source (D5015 hairdryer; Remington®, UK) was placed 112 cm in front of the Helmholtz coil. Locust flight was recorded using a high-speed video camera (MotionScope 1000S, Redlake Imaging, CA, USA) at 125 fps. After 15 s of consistent flight, EMF treatment was activated and high-speed video triggered (Fig. 14) to store 5 s before EMF initiation (pre-treatment) and 15 s after EMF initiation (treatment).

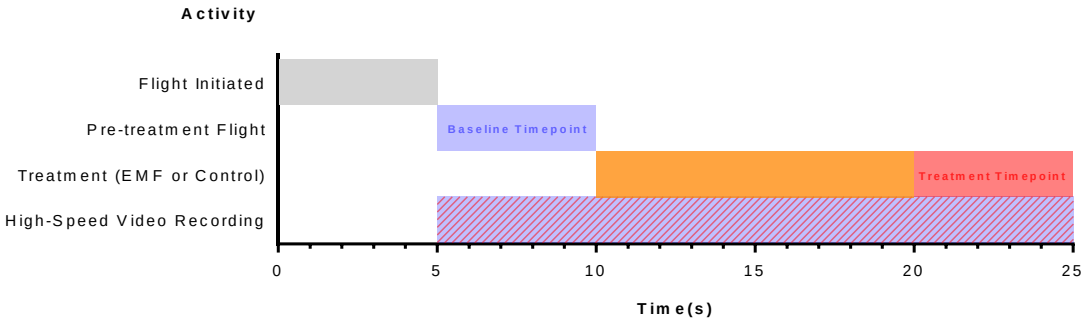

Figure 14. Experimental timetable for analysis of ELF EMF effect on locust wingbeat frequency. 5 seconds were given for consistent flight and 5 seconds of pre-EMF flight to determine baseline wingbeat frequency levels. Then the EMF was switched on (or control treatment initiated) for 15s. In analysis the wingbeat frequency over the time-period 10-15 s after initiation was used. High-speed video was triggered to record the 20 s of relevant flight data.

High-speed video was analysed to determine wingbeat frequencies of locusts in the 0-5 s before the initiation of EMF (pre-treatment) and the wingbeat frequencies of locusts in the 0-5 s, 5-10 s, and 10-15 s time periods after the treatment began (treatment). The effect of acute EMF exposure on flight was determined by calculating the change in wingbeat frequency from the pre-treatment time period to final treatment time period (10-15s after treatment began). To determine the effects of 50 Hz ELF EMFs on locust flight 100 µT, 1000 µT and 7000 µT field strengths were used. Absolute change in wingbeat frequency (i.e. overall change irrespective of increase or decrease) was calculated 10-15 s after flight onset. To determine the effects of ELF EMF frequency on flight 17, 20 and 22 Hz EMFs were applied at 700 µT. To determine whether locust wingbeat frequency phase-locked to the frequency of the EMF the difference in locust wingbeat frequency from the applied magnetic field frequency was calculated and compared to pre-treatment frequencies. In all acute exposure flight dynamics experiments 162 locusts were used.

2.3.4 Statistical analysis

Data were analysed using SPSS v. 24 (IBM SPSS Inc., USA) and Graphpad Prism (v.7, Graph Pad Software Inc.). Where appropriate, homogeneity of variance and normality assumptions were tested. If required, data transformations were made, or alternative statistical tests were chosen.

To determine the effect of EMFs on tunnel completion, the number of locusts that had complete and incomplete trials were totalled for EMF exposures and their respective controls. A χ^2 test was used to compare data in a 2×2 contingency table for both 4 mT and 7 mT exposures and high and low controls. To determine the effect of EMF exposure on completion time, a students' unpaired t-test using Welch's correction for uneven variances was used to compare the mean completion time between EMF exposed locusts and control animals. To determine the effect of EMF exposure on the cumulative distance travelled by locusts in the tunnel assay a two-way repeated measures ANOVA was used to compare the effects of 'treatment' (EMF exposure or respective control) and 'time' on the cumulative distance travelled along the tunnel.

To assess whether exposure to EMF had an effect on ETi muscle force output a Students' unpaired t test was used to compare mean muscle force output for a single stimulus in locusts exposed to EMF with untreated control locusts. To satisfy assumptions for parametric analysis, for this experiment analysis was conducted on square root transformed data. As well as this the effect of ELF EMF exposure on the tetanic properties of the ETi muscle were analysed with a two-way repeated measures ANOVA, with 'stimulus frequency' as a repeated measure, and testing for interaction effects between 'EMF *vs.* Control exposure' and 'stimulus frequency'. To determine whether exposure to EMF resulted in a change in the FETi spike properties, a students' unpaired test was used to compare spike width and latency in EMF exposed animals with control animals. Finally, to determine the effects of EMF on Hsp70 levels a students' paired t test was used to compare EMF exposed groups with control animals. In every case two-tailed Students t tests were used.

The acute effects of ELF EMF exposure on locust flight were analysed using a one-way ANOVA to determine the effect of different treatments (Control, 100 μT, 1000 μT and 7000 μT) on the absolute change in locust wingbeat frequency from pre-treatment levels. A pattern emerged in the data suggesting that EMF exposure was differential, dependent on the initial wingbeat frequency of the locust, with locusts synchronizing to 20 Hz. To

determine whether locusts synchronized to 20 Hz the difference in locust wingbeat frequency from 20 Hz before and after treatment was calculated and analysed in a two-way repeated measures ANOVA, with time points as a repeated measure and EMF treatments as a main factor. To determine whether locusts synchronized wing beat frequency to different EMF frequencies a two-way repeated measures ANOVA was used to compare the difference in locust wingbeat frequency from the applied EMF frequency between pre-treatment at 700 μT treatment, for each of the different EMF frequencies tested, with time point as a repeated measure.

2.4 Results

2.4.1 Short-term exposure

2.4.1.1 Behaviour

To determine the effect of short-term 24 hr exposure to ELF EMFs on walking behaviour a walking tunnel assay was used. 4 mT and 7 mT treatments were compared to their respective controls. Distance travelled in the tunnel assay depended on EMF exposure. When EMF treatments the mean distance travelled by locusts over the 5 min time period was lower than control exposed locusts for both a 7 mT EMF exposure (Fig. 15, A), and a 4 mT EMF exposure (Fig. 15, B). The effects of EMF on cumulative distance travelled was greater over time compared to controls, with exposed locusts covering less distance by the final time point (5 min) (7 mT exposure Interaction factor, $F_{12,804} = 10.88$, $P < 0.0001$ and 4 mT exposure Interaction factor, $F_{12,840} = 9.78$, $P < 0.0001$). In the final time point 7 mT exposed locusts had travelled 15.0 ± 1.7 cm in average in comparison to 23.2 ± 0.7 cm travelled by temperature-matched control locusts, and 4 mT exposed locusts travelled 16.2 ± 1.3 cm in comparison to 22.2 ± 1.0 cm travelled by temperature-matched control locusts.

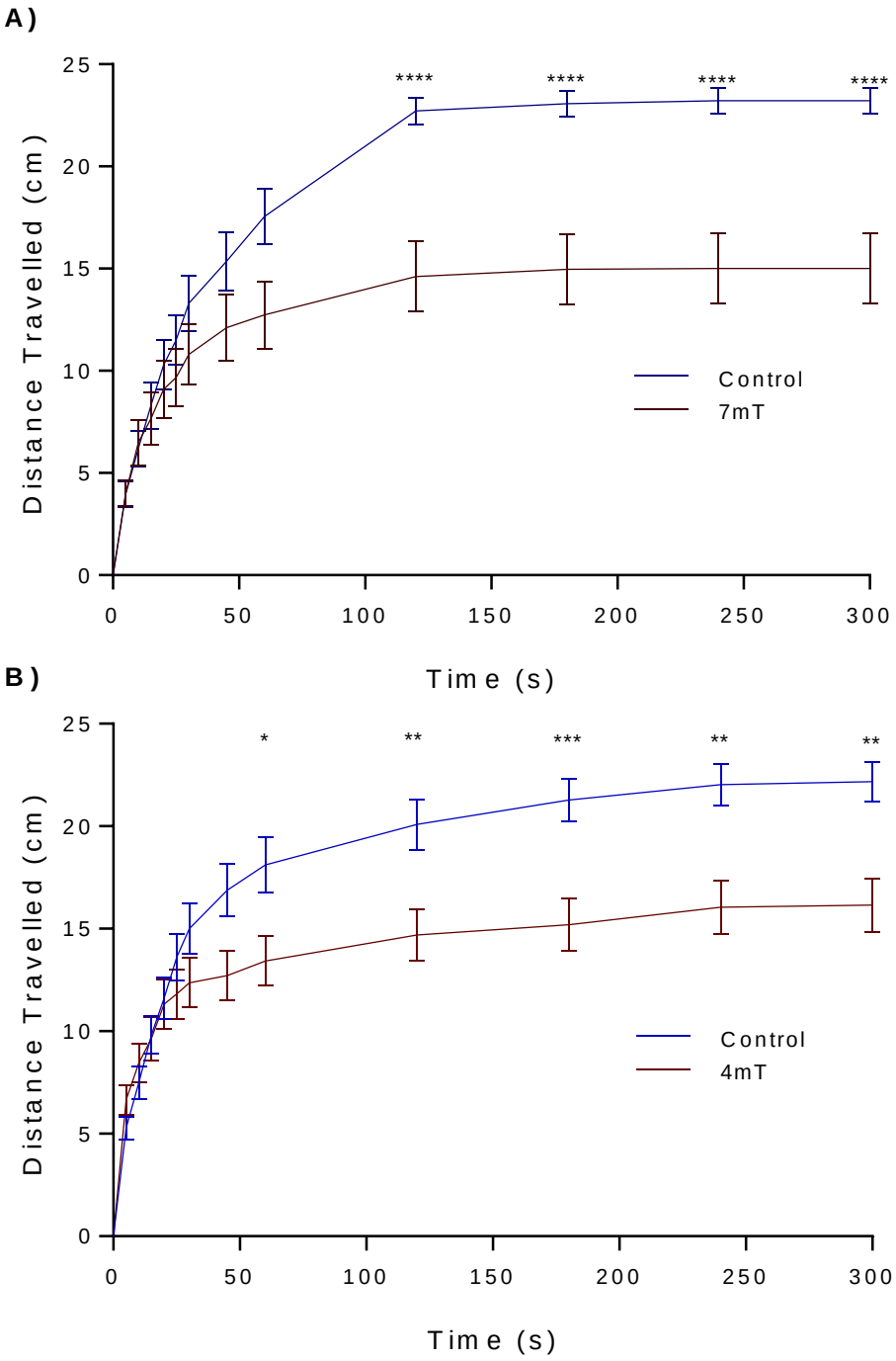

Figure 15. The effect of 24 hr 50 Hz ELF EMF exposure on the distance travelled by locusts in the walking tunnel assay. A) 7 mT EMF treatment and respective control. B) 4 mT EMF treatment and respective control. Mean and SEM are plotted. N=36 for each treatment.

To determine the effect of exposure to EMF on walking behaviour the numbers of locusts completing the tunnel assay were compared between those exposed to EMF and their respective temperature matched controls. For both 7 mT (Fig. 16, A) and 4 mT (Fig. 16, B)

EMF treatments the number of complete trials were less following exposure to EMF compared to controls. Following a 7 mT exposure 15 locusts completed the tunnel while 19 did not, whereas for matched temperature controls 28 locusts completed the tunnel while 7 did not ($\chi^2 = 9.46$, d.f. = 1, P = 0.0021). For a 4 mT exposure 12 locusts completed the tunnel while 24 did not, whereas for temperature-matched control 28 completed the tunnel while 8 did not ($\chi^2 = 14.40$, d.f. = 1, P = 0.0001).

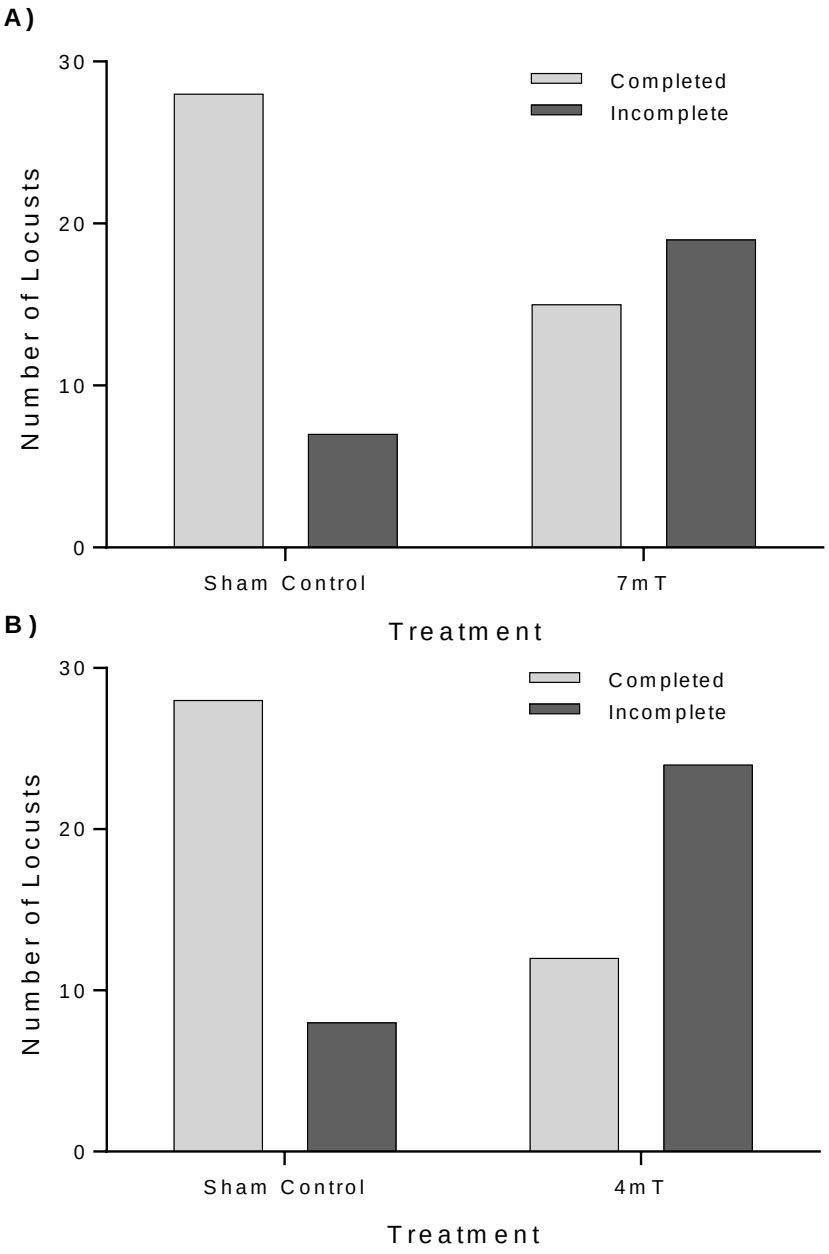

Figure 16. Effects of 24 hr EMF exposures on tunnel completion, measured by the number of locusts that did or did not complete the tunnel. (A) 7mT EMF exposure and respective control. (B) 4mT EMF exposure and respective control

In total 83 locusts completed the tunnel ($Control_{4mT}$ = 28, EMF_{4mT} = 12, $Control_{7mT}$ = 28, EMF_{7mT} = 15). From this stage the attributes of tunnel completion of only the 83 locusts that completed the tunnel were analysed. Of these locusts, following a 7 mT exposure (Fig. 17, A), completion time (49.3 ± 8.6 s) was reduced compared to controls (78.0 ± 10.7 s) such that EMF exposure increased the speed at which locusts completed the tunnel (Welch corrected students' t test, t = 2.08, d.f. = 40.54, P = 0.044). With a 4 mT exposure (Fig. 17, B) there was no significant difference in the time taken for individuals to complete the tunnel (Welch corrected Students' t test, t = 0.64, d.f. = 15.67, P = 0.53), with a mean completion time of approximately 90 s.

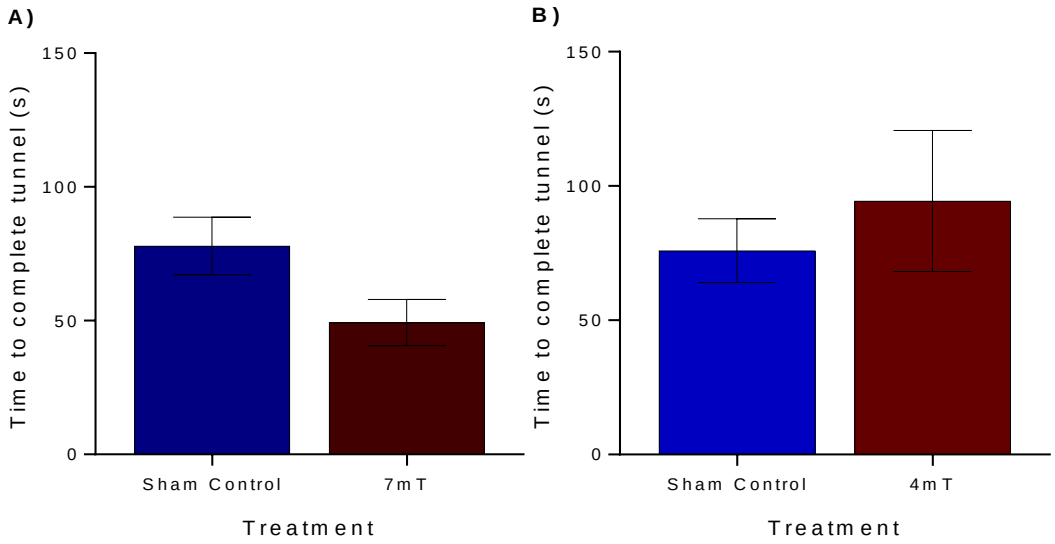

Figure 17. The effect of EMF on time to complete the walking assay. A) 7 mT EMF exposure and respective control. B) 4mT EMF treatment and respective control. Analysis for 4 mT was done on Log_{10} transformed data to satisfy parametric analysis. Data shown is reverse Log_{10} transformed.

2.4.1.2 Muscle force

To determine the effect of 24 hr ELF EMF exposure on muscle force, the hind leg ETi muscle was stimulated and the force of the extended tibia was measured. Temperature matched controls showed that with decreasing control temperature (from 29.3 °C ± 1 °C for 7 mT exposure to 21 ± 1 °C for 1 mT exposure) there was a decrease in ETi force (Fig. 18, A-C). With 1 mT and 4 mT EMF exposures there were no significant differences in force compared to respective temperature controls. For example at 1 mT the mean force generated

by ETi of exposed locusts was 58.7 ± 9.7 mN compared to 38.7 ± 5.4 mN for temperature matched controls (Students t test, $t = 1.838$, $P = 0.08$, d.f. = 22). At 4 mT the mean muscle force of EMF treated animals was 60.1 ± 9.0 mN compared to 79.5 ± 10.1 mN for temperature matched controls (students t test, $t = 1.548$, $P = 0.129$, d.f. = 46). Exposure to 7 mT for 24 hr, however, led to a significant decrease in force generated by ETi compared to the temperature-matched controls (Fig. 18, A). The mean muscle force of 7 mT exposed animals was 68.4 ± 6.7 mN compared to 124.2 ± 19.9 mN for controls (students t test, $t = 2.114$, $P = 0.040$, d.f. = 48). For 1 mT 12 locusts were used for EMF and respective control exposure, for 4 mT 24 locusts were used for EMF and respective control exposure, and for 7mT 24 locusts were used for EMF and 26 were used for control exposures.

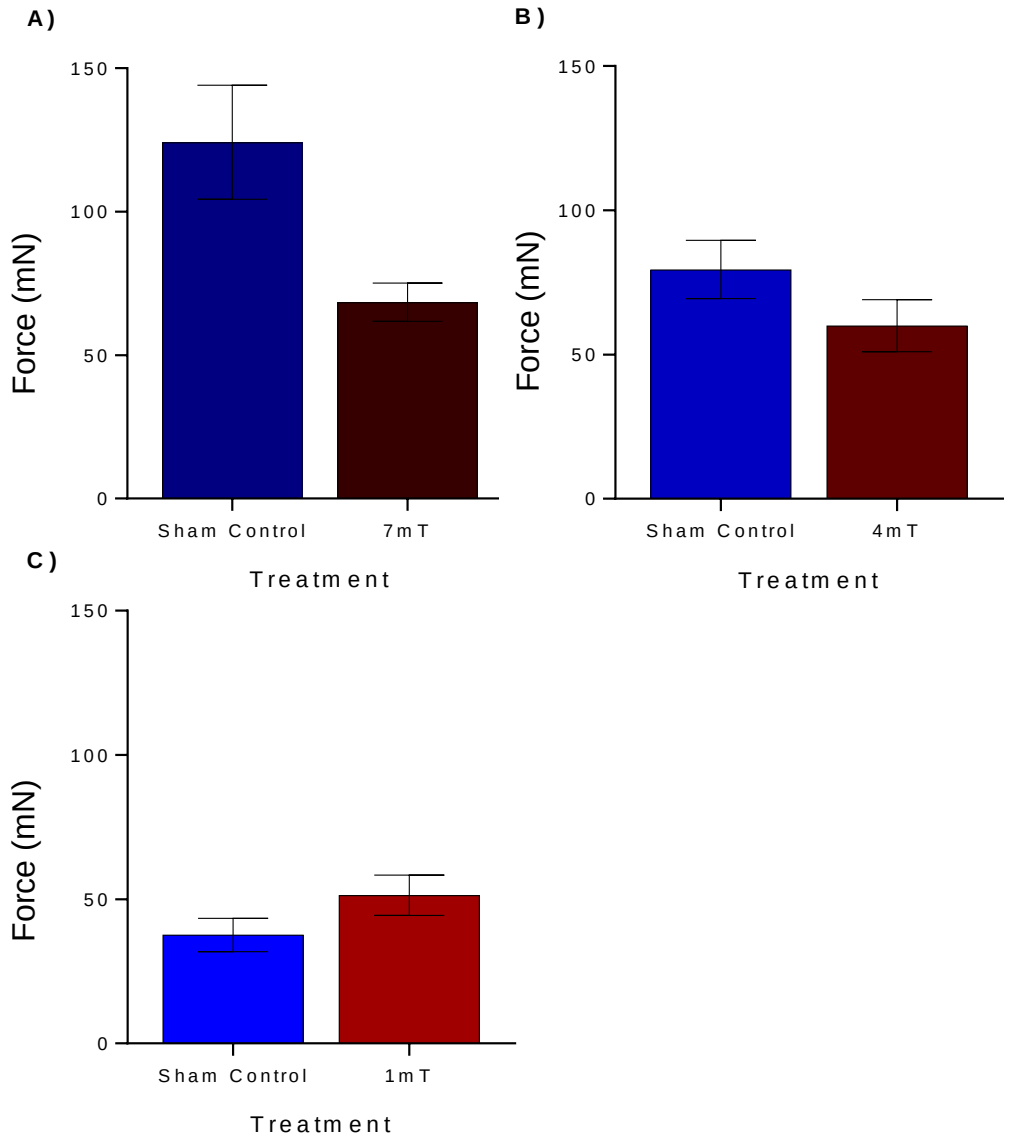

Figure 18. The effect of 24 hr ELF EMF exposure on the force generated by ETi muscle. A) 7 mT treatment and respective temperature control. B) 4 mT Treatment and respective temperature control. C) 1 mT Treatment and respective temperature control. Analysis of force was carried out on square-root transformed data to satisfy parametric analysis. Data shown is reverse square-root transformed, showing mean and SEM.

The effects of short-term chronic ELF EMF exposure on the tetanic properties of the ETi muscle were also analysed. Stimulation frequencies of 1, 5, 10, 20, 50, 100, 125, 150, 175, 200, 225, 250 Hz for ETi muscle were used. For all treatments tetanic summation of ETi force occurred (Fig. 19, A-C), peaking at 50-100 Hz, and gradually decreasing with increasing stimulation frequencies (RM-ANOVA, 'Stimulation Frequency' factor for: 7 mT/control $F_{11,407} = 59.81$, $P < 0.001$, 4 mT/control $F_{11,506} = 178.7$, $P < 0.001$, 1 mT/control

$F_{11,242}$ = 40.3, P < 0.001). There was no significant effect of EMF treatment on the force generated at different tetanic stimulation frequencies for a 7mT EMF (RM-ANOVA, $F_{1,37}$ = 0.26, P = 0.61) (Fig. 19, A), a 4mT EMF (RM-ANOVA, $F_{1,46}$ = 0.74, P = 0.39) (Fig. 19, B), or a 1mT EMF (RM-ANOVA, $F_{1,22}$ = 0.02, P = 0.89) (Fig. 19, C). There was no EMF*Stimulation Frequency interaction for any of the tested EMF exposures (RM-ANOVA: 7mT $F_{11,407}$ = 0.49, P = 0.91, 4mT $F_{11,506}$ = 0.68, P = 0.76, 1mT $F_{11,242}$ = 0.87, P = 0.57)

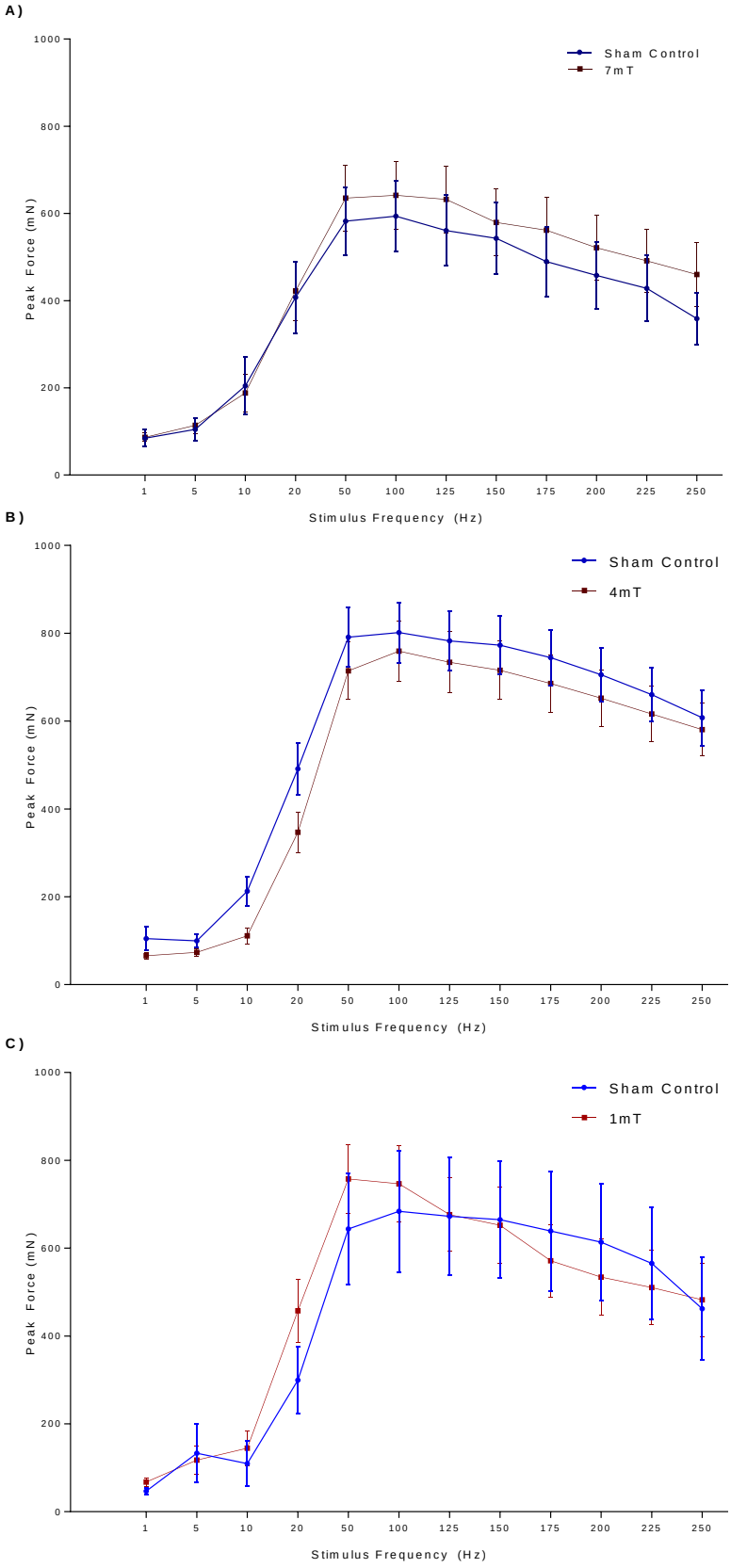

Figure 19. The effect of 24 hr EMF exposure on force generated by different frequencies of stimulation to cause summation of signal in ETi muscle. A) 7 mT EMF and control. B) 4 mT EMF and control. C) 1 mT EMF and control. Analysis of force was carried out on square-root transformed data to satisfy parametric statistics.

2.4.1.3 Electrophysiology

To determine the effects of short-term EMF exposure on the nervous system of the locust, the spike and EPSP properties of the fast extensor tibiae motor neurone in the metathoracic ganglion were analysed. 8 locusts were treated at control levels, and 11 locusts treated with a 7 mT EMF. The amplitude of the FETi action potential and resultant excitatory postsynaptic potential (EPSP) were both measured. The mean amplitude of the FETi action potential was 15.2 ± 2 mV for control and 17.6 ± 2 mV for EMF treatment, and the mean EPSP amplitude was 3.2 ± 0.5 mV for control and 3.8 ± 0.5 mV for treatment. There was no significant effect of EMF exposure on action potential (Fig. 20, A) and compound EPSP (Fig. 20, B) amplitude in FETi (FETi spike: $P = 0.33$, $t = 1.014$, d.f. = 17; EPSP: $P = 0.39$, $t = 0.88$, d.f. = 17).

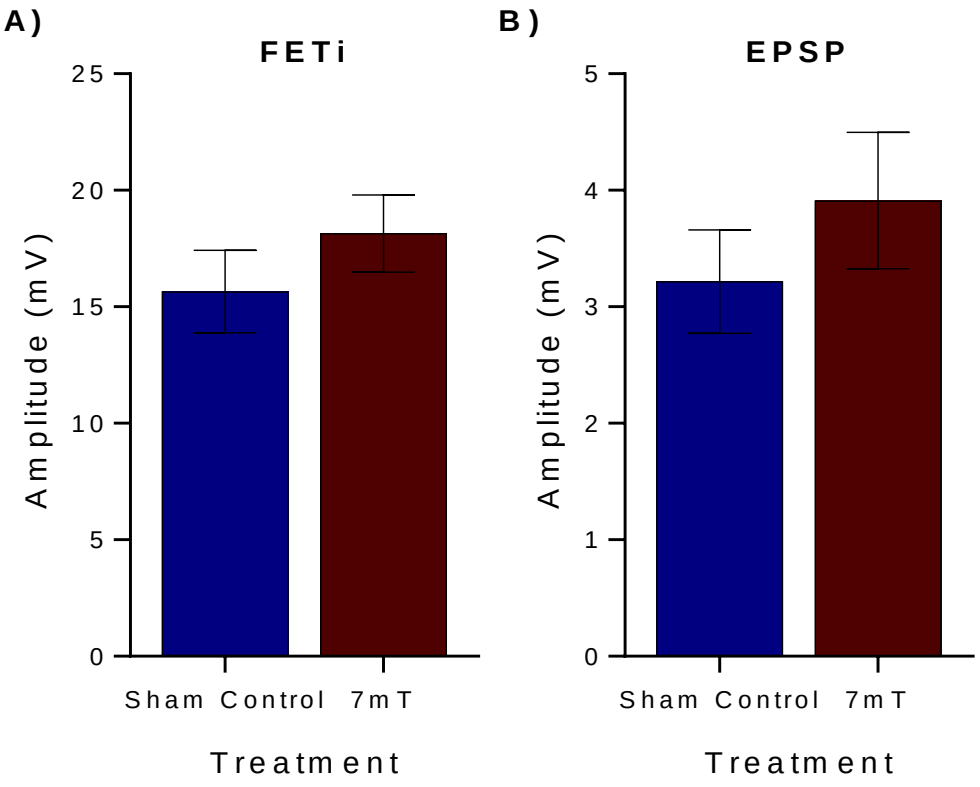

Figure 20. The effect of 7 mT 24 hr EMF exposure on the amplitude of A) FETi motor neurone action potential B) Excitatory post-synaptic potential from campaniform sensilla. Mean and SEM are plotted.

The latency of the FETi action potential from the stimulus increased from 4.98 ± 0.2 ms to 6.50 ± 0.5 ms after EMF 7 mT treatment ($P = 0.03$, $t = 2.44$, d.f. = 17) (Fig. 21, A).

By contrast there was no evidence of an effect of EMF treatment on EPSP latency (P = 0.87, t = 0.16, d.f. = 17) (Fig. 21, B).

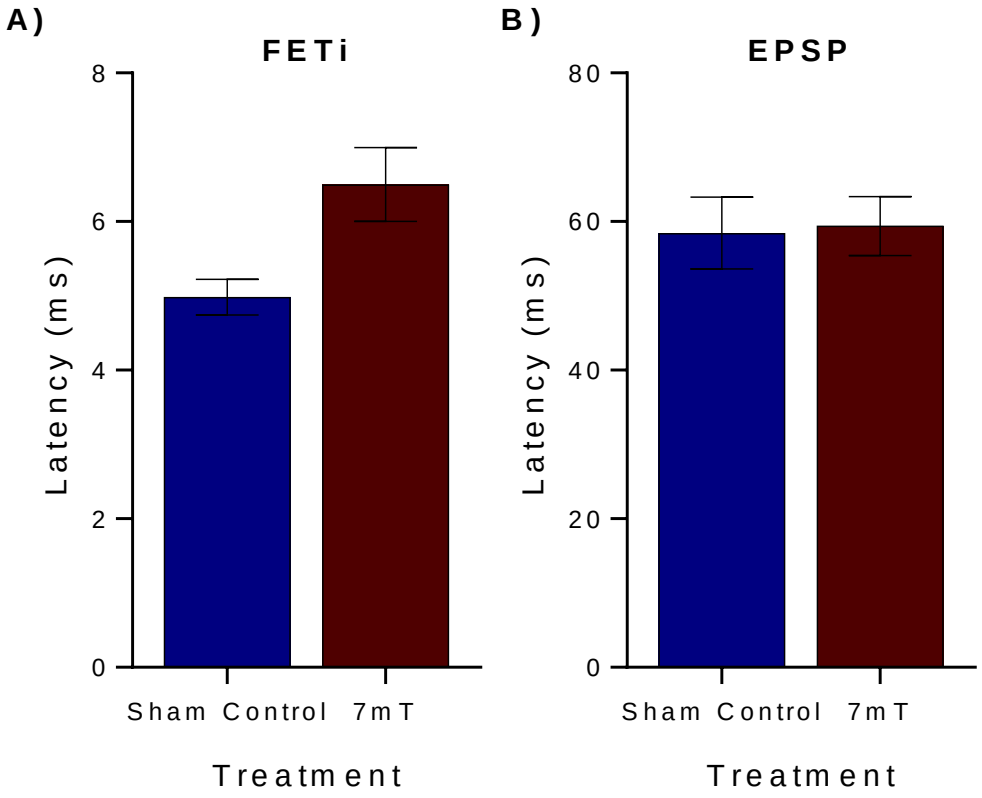

Figure 21. The effect of 24 hr EMF exposure on the latency of A) FETi motor neuron action potential. B) Excitatory post-synaptic potential from campaniform sensilla. Mean and SEM are plotted.

The effect of ELF EMFs on FETi action potential spike and EPSP duration was also analysed. There was a significant increase in the duration of FETi action potentials in 7 mT EMF treated animals, increasing from a mean of 4.4 ± 0.5 ms to 6.5 ± 0.6 ms ($P = 0.03$, $t = 2.43$, d.f. = 17) (Fig. 22, A). There was no evidence of an effect of EMF exposure on EPSP duration ($P = 0.51$, $t = 0.67$, d.f. = 17) (Fig. 22, B).

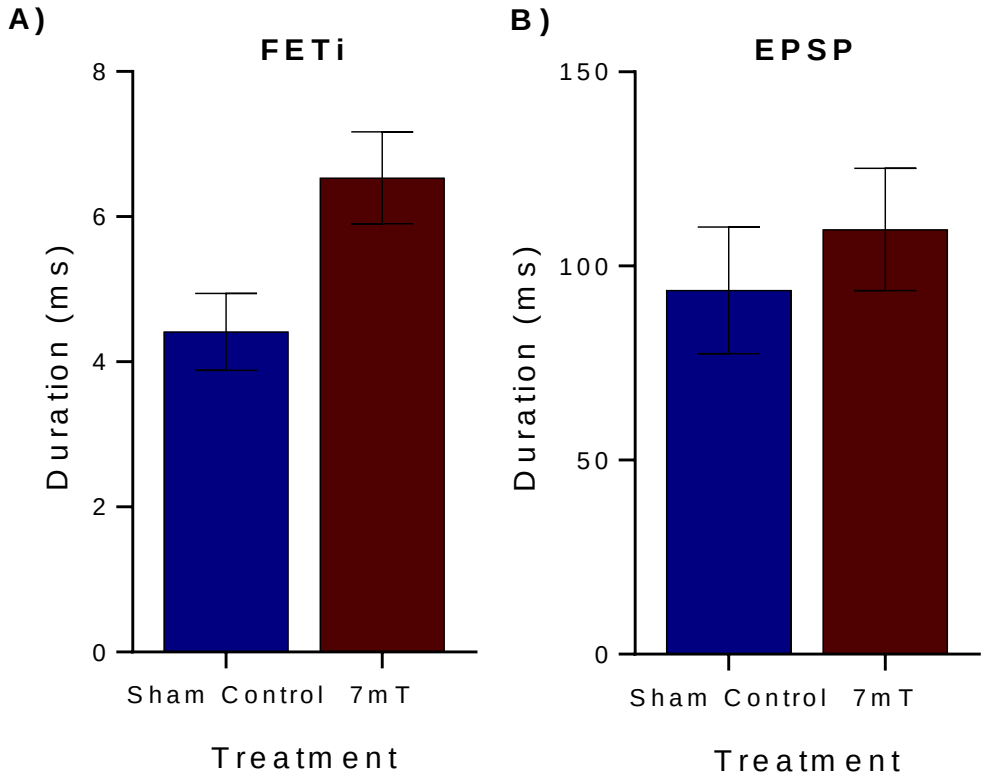

Figure 22. Duration of action potentials and EPSPs in the FETi motor neuron. A) FETi action potential duration B) EPSP duration. Mean and SEM are plotted.

2.4.1.4 Stress Proteins

The effect of EMF exposure on stress proteins was studied by measuring changes in the expression levels of the stress protein Hsp70 via Western-blotting (Fig. 23, A-B). As a positive control, heat-shock treatment led to an increase in protein levels by a factor of 1.4 ± 0.2 compared to the negative heat-shock control treatment (Fig. 23, B). The two treatments tested (control and EMF 7 mT) were normalised to values relative to the negative heat-shock control. Control (i.e. sham EMF) Hsp70 levels were lower relative to negative heat-shock control levels by a factor of 0.7 ± 0.1 whereas EMF 7 mT treatment levels resulted in Hsp70 levels higher than heat-shock control levels by a factor of 1.5 ± 0.3, showing a marked increase in Hsp70 levels after ELF EMF treatment in comparison to controls ($P = 0.02$, $t = 2.62$, d.f. = 12).

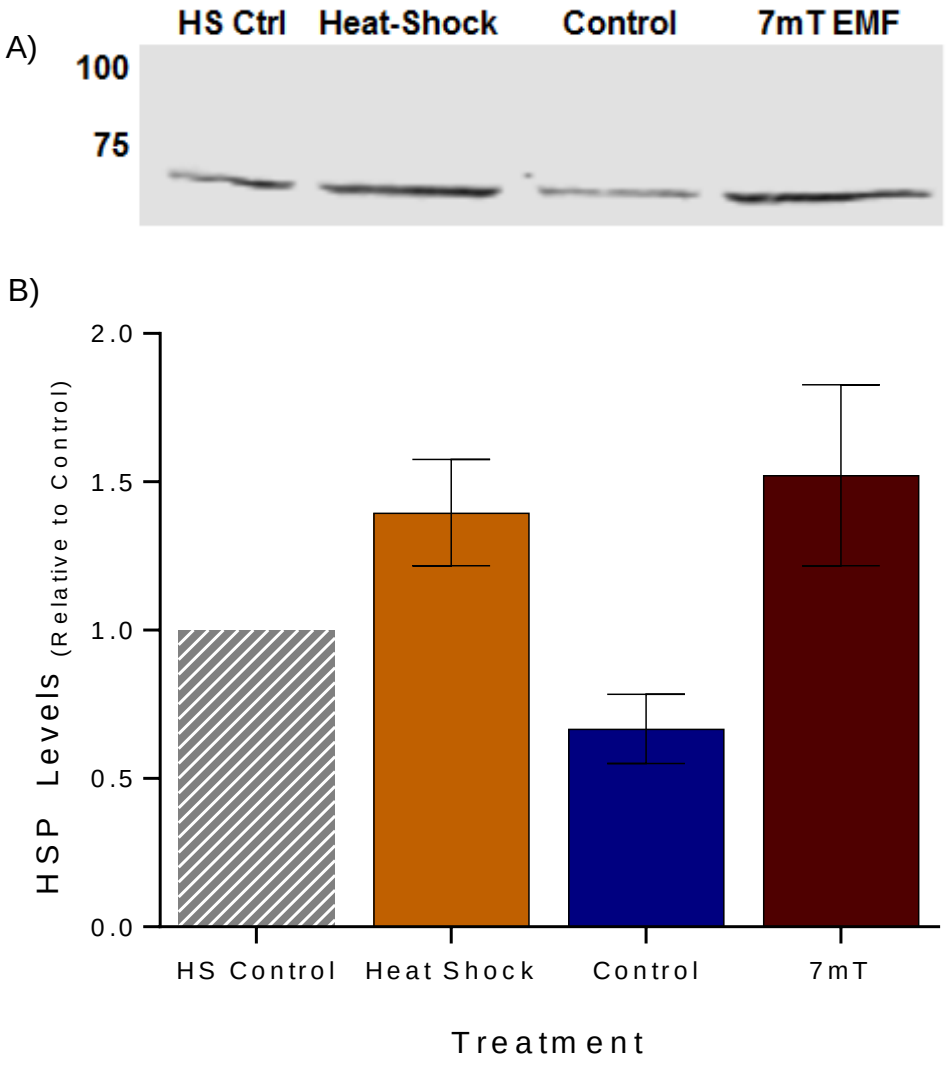

Figure 23. The effect of 7 mT 24 hr EMF on Hsp70 expression in the metathoracic ganglia of the locust. A 3 hr 45ºC heat shock treatment and heat shock control (3 hr 25ºC) are also shown for points of comparison. A) Example western blot with lanes labelled accordingly. B) Means and SEM Hsp70 signals. All values shown are relative to the heat shock control treatment.

2.4.2 Acute exposure

2.4.2.1 Effects of magnetic flux density on wingbeat frequency

The initial wingbeat frequencies of all locusts were measured and wingbeat frequencies ranged from 11-26 Hz. The mean wingbeat frequency of locusts in all experiments was 18.92 ± 0.27 Hz (Fig. 24).

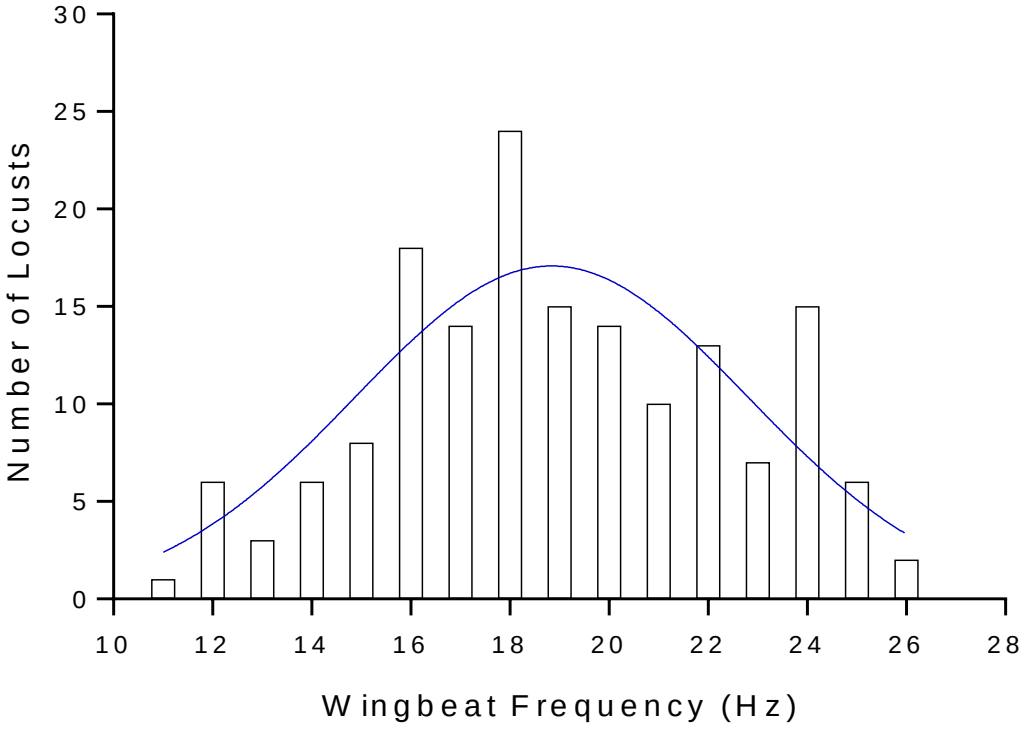

Figure 24. Number of locusts flying at each wingbeat. Bars show numbers of locusts, and the blue line shows curve of Gaussian distribution with the same mean and standard deviation as data.

The acute effects of ELF EMFs on locust flight were determined for control, 100, 1000, and 7000 μT exposures. When the exact change in wingbeat frequency was plotted, little effect of ELF EMF on wingbeat frequency was observed (Fig. 25), as there was not a consistent directional change (increase or decrease) in wingbeat frequency caused by ELF EMF exposure.

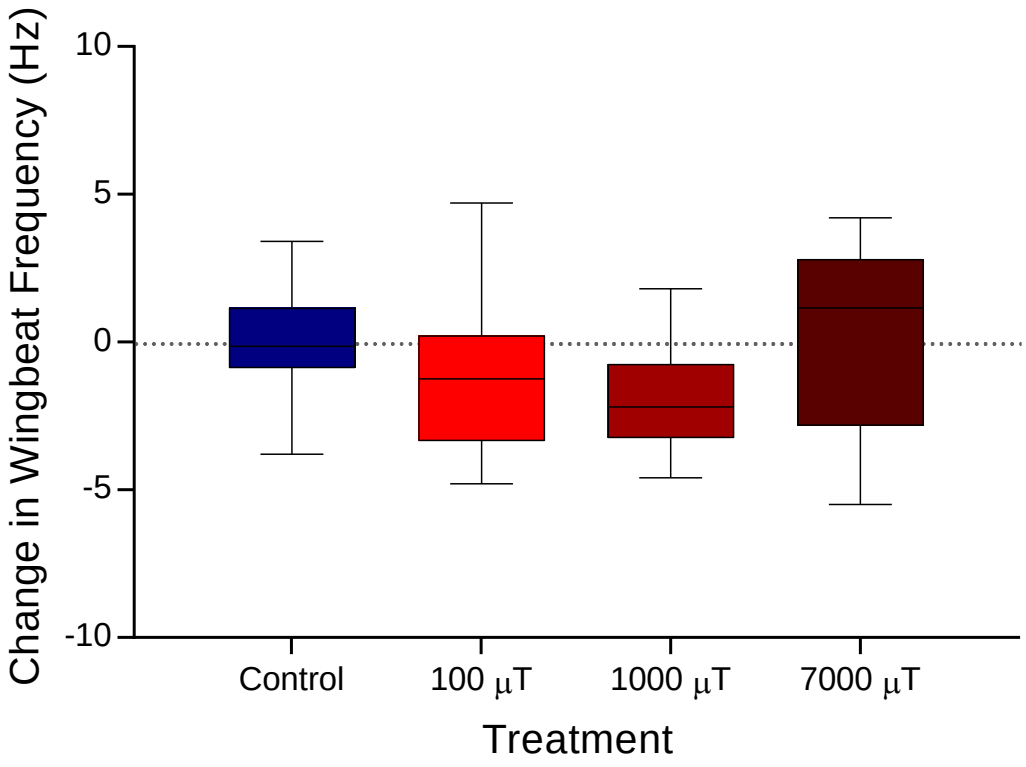

Figure 25. Change in wingbeat frequency after 10-15 seconds of ELF EMF exposure. With this analysis method, as some locusts increased wingbeat frequency and some decreased, very little change was measured overall.

To improve clarity of data the absolute change in wingbeat frequency from pre-treatment levels was measured (i.e. the magnitude of the change was considered but not the sign, +/-, of the change). For all exposures there was variation in wingbeat frequency 15 s after treatment from pre-treatment frequencies (Fig. 26). For control locusts the wingbeat frequency varied by 1.3 ± 0.3 Hz from pre-treatment levels. The absolute change in wingbeat frequency was higher for all ELF EMF intensities (100 μT: 2.3 ± 0.4 Hz, 1000 μT: 2.3 ± 0.3 Hz, 7000 μT: 2.7 ± 0.3 Hz) (Fig. 26). A one-way ANOVA showed that EMFs significantly increased the absolute change in wingbeat frequency (ANOVA, $F_{3,76}$=3.857, P=0.013), and a Bonferroni *post-hoc* analysis revealed 7000 μT exposure caused a significantly greater absolute change in wingbeat frequency from pre-treatment than control levels (Bonferroni adjusted P = 0.01).

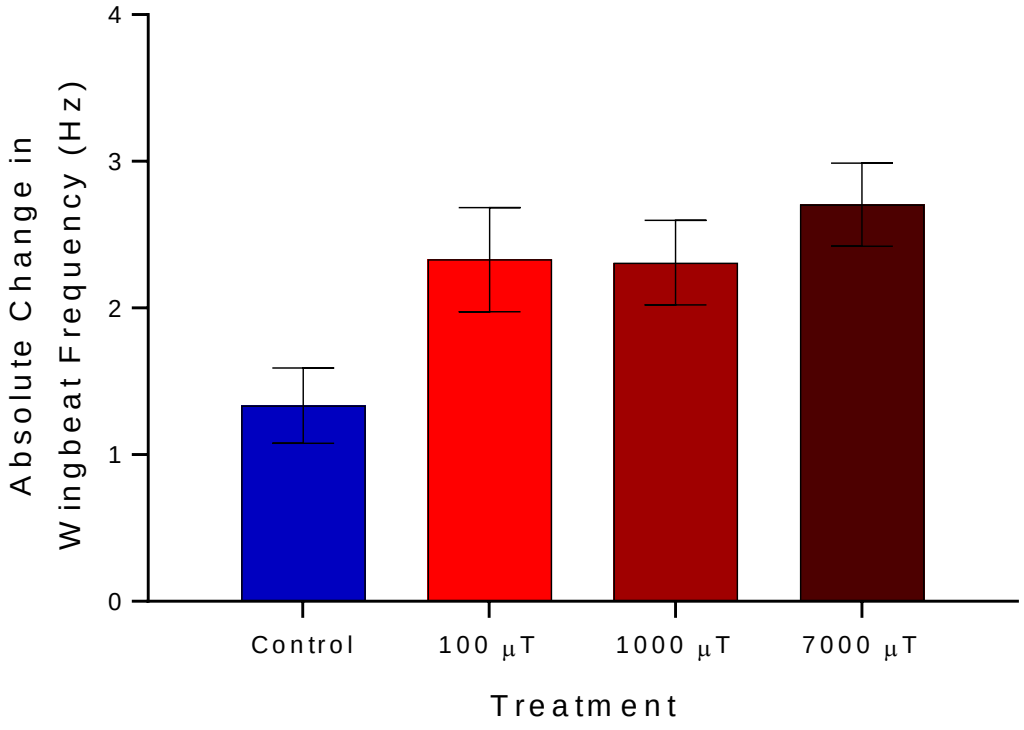

Figure 26. Effects of acute ELF EMF exposure on the absolute change in wingbeat frequency from pre-treatment levels. Mean and SEM change in wingbeat frequency is plotted.

In determining why for all EMF exposures, but not controls, some locusts increased wingbeat frequency and some decreased, the wingbeat frequencies of individual locusts were plotted. For control locusts mean wingbeat frequency remained the same at 19.3 Hz (SEM remained the same at 18.5-20.1 Hz), however for 100 µT exposed locusts mean wingbeat frequency changed from 22.1 Hz to 20.9 Hz (SEM from 21.3-22.9 Hz to 20.4-21.4 Hz), for 1000 µT exposed locusts mean wingbeat frequency changed from 21.8 Hz to 19.9 Hz (SEM from 21.1-22.5 Hz to 19.4-20.4 Hz), and for 7000 µT exposed locusts mean wingbeat frequency changed from 19.1 Hz to 19.3 Hz (SEM from 18.5-19.7 Hz to 18.9-19.7 Hz). The data indicates that, with 50 Hz EMF exposure, wingbeat frequency tends towards (potentially synchronizing to) approximately 20 Hz (Fig. 27).

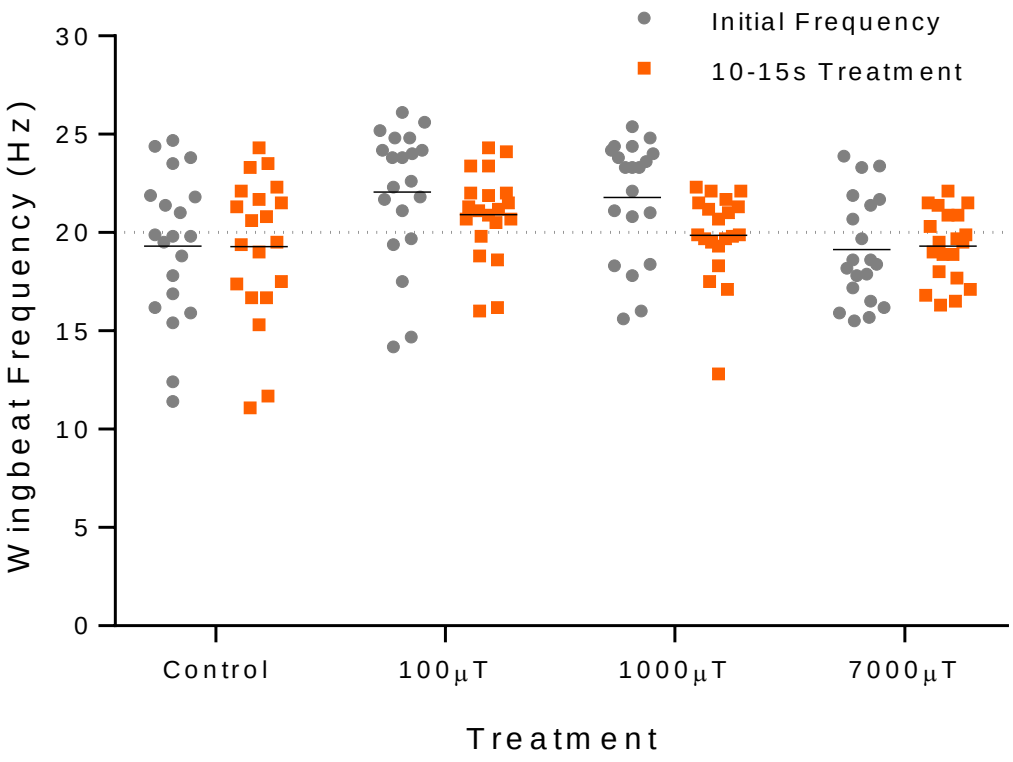

Figure 27. Individual wingbeat frequencies of locusts before treatment and over the 10-15s time point after treatment for control and EMF treatments. Black lines show the mean for each treatment. For all EMF treatments locust wingbeat frequency appears to tend towards 20 Hz.

To further test if wingbeat frequency synchronized to 20 Hz when a 50 Hz EMF was applied, the difference in wingbeat frequency from 20 Hz was calculated (e.g. a locust flying at 17 Hz or 23 Hz would have a difference of 3 Hz from 20). If wingbeat frequency synchronizes to 20 Hz, locusts flying faster than 20 Hz would decrease, and locusts flying slower would increase. Therefore the difference in wingbeat frequency from 20 Hz would get smaller. For all EMF exposures locust wingbeat frequency synchronized to 20 Hz (Fig. 28) as the difference in wingbeat frequency from 20 Hz decreased (100 µT: from 3.5 ± 0.4 Hz to 2.0 ± 0.3 Hz; 1000 µT: from 3.2 ± 0.3 Hz to 1.5 ± 0.4 Hz; 7000 µT: from 2.5 ± 0.3 Hz to 1.5 ± 0.3 Hz). There was a significant interaction between treatment (EMF or control) and time (before or after exposure) on wingbeat frequency (Two-way RM ANOVA: $F_{3,76} = 4.97$, $P = 0.003$) as all EMF exposures caused a significant synchronization of wingbeat frequency towards 20 Hz (Bonferroni adjusted P: 100 µT, $P < 0.0001$; 1000 µT, $P < 0.0001$; 7000 µT, $P = 0.015$), whereas control did not (Bonferroni adjusted $P > 0.9999$).

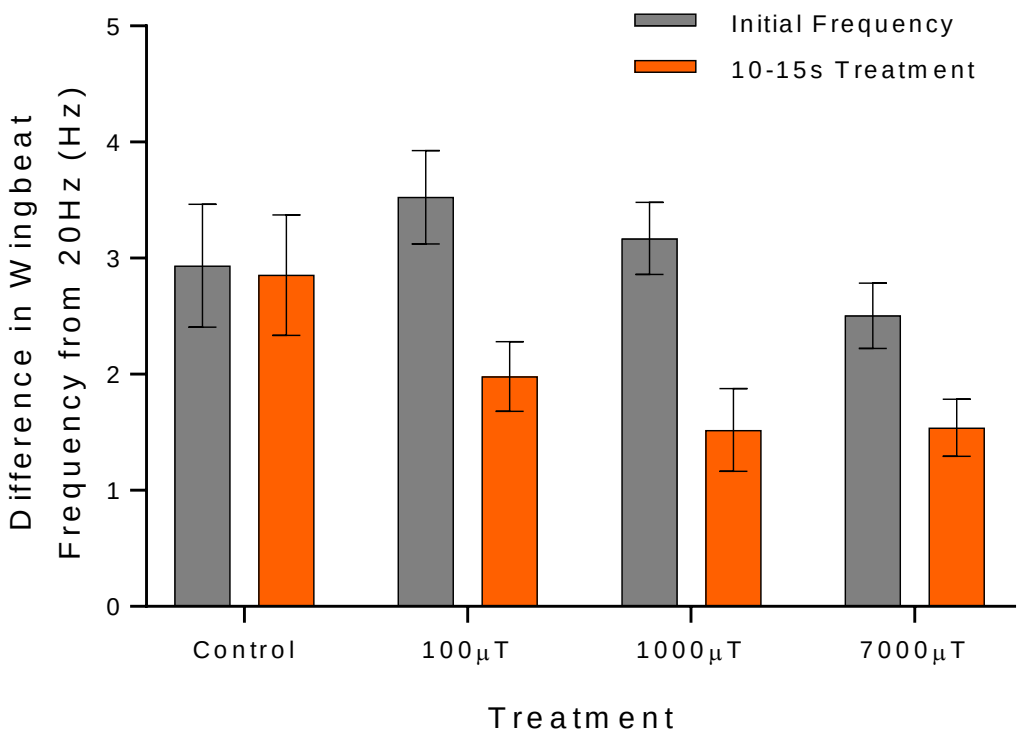

Figure 28. Difference in wingbeat frequency of locusts from 20 Hz for all treatments. Mean and SEM are plotted

To further visualise that ELF EMFs effects were dependent on initial wingbeat frequency and caused synchronization to towards 20 Hz, locusts were grouped based on their initial wingbeat frequencies. Locusts that were initially 'fast-flying' (flying above 20 Hz before treatment) would tend to decrease wingbeat frequency when the 50 Hz ELF EMF was applied, and locusts that were initially 'slow-flying' (flying above 20 Hz before treatment), would tend to increase wingbeat frequency when the 50 Hz ELF EMF was applied (Fig. 29, B-D). In comparison this pattern does not occur for control exposed locusts (Fig. 29, A).

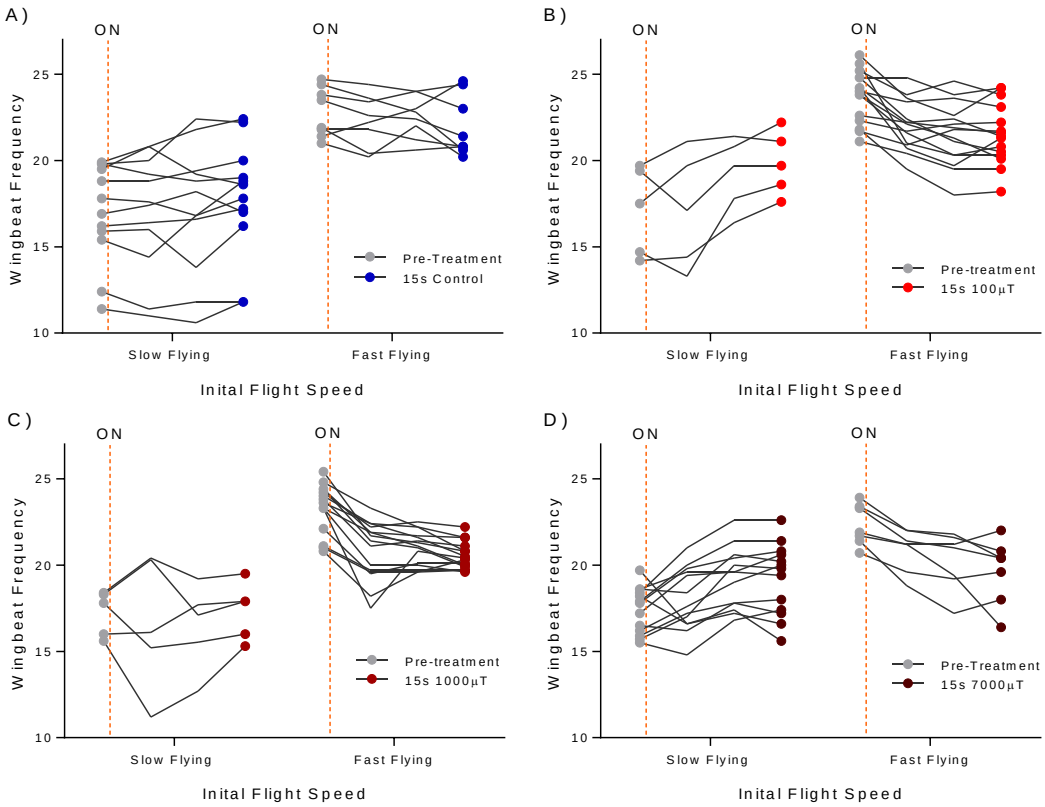

Figure 29. Effects of 50 Hz EMF on the wingbeat frequencies of individual 'slow-flying' (flying <20 Hz before treatment) and 'fast-flying (flying >20 Hz before treatment) locusts. Pre-treatment (grey) and treatment wingbeat frequencies (coloured) are shown. (A) Control, (B) 100 µT, (C) 1000 µT, (D) 7000 µT.

2.4.2.2 Effects of magnetic field frequency on wingbeat frequency

The effect of EMFs on flight were further analysed by exposing locusts to different ELF EMF frequencies. EMF strength was kept constant at 700 µT and ELF EMFs were applied at 17 Hz, 20 Hz and 22 Hz (Fig. 30).

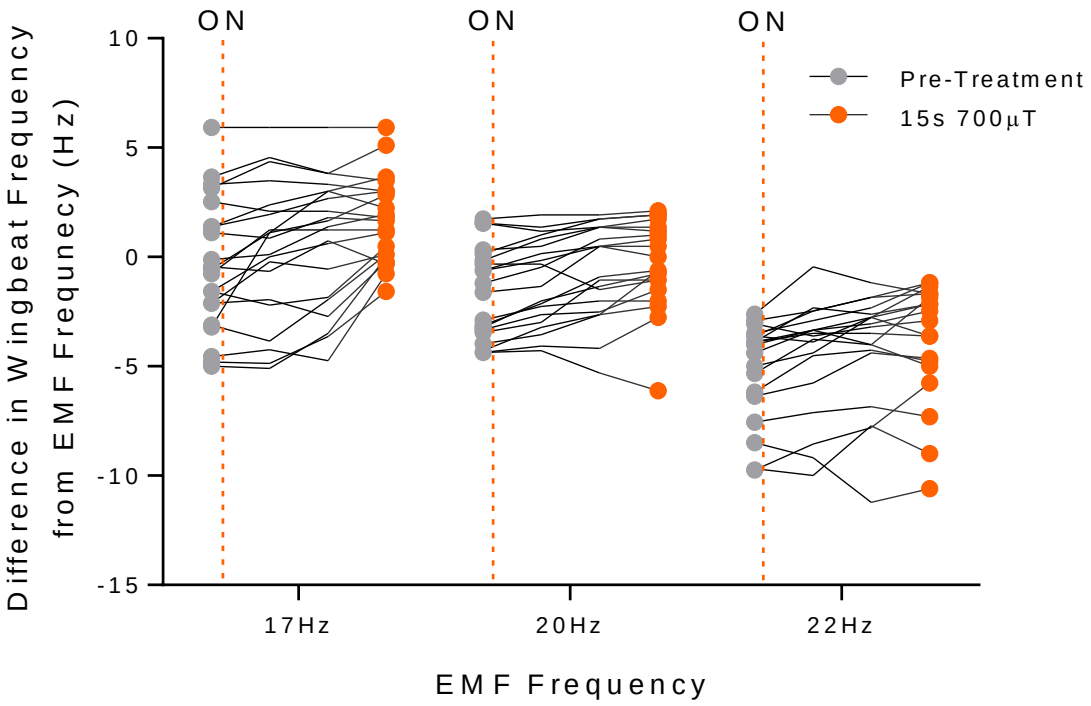

Figure 30. Effects of 700 µT EMFs at varying frequencies (17, 20, and 22 Hz) on the difference in wingbeat frequencies from the applied frequency of the ELF EMF. Pre-treatment (grey) and treatment (coloured) wingbeat frequencies are shown.

For all tested EMF frequencies the difference between locust wingbeat frequency and applied EMF frequency was lower during exposure compared to pre-treatment levels (Fig. 31) i.e. locusts synchronized to all applied EMF frequencies. Locusts flew 2.5 ± 0.3 Hz out of synchrony of a 17 Hz EMF before exposure and 1.9 ± 0.4 Hz out of synchrony during exposure. At 20 Hz locusts flew 2.1 ± 0.3 Hz out of synchrony before exposure, and 1.6 ± 0.2 Hz out of synchrony during exposure, while for 22 Hz locusts flew 5.1 ± 0.5 Hz out of synchrony before exposure, and 3.8 ± 0.8 Hz out of synchrony after EMF exposure. A two-way RM-ANOVA showed that application of a 700 µT EMF significantly increased synchronization of locusts towards the frequency of the applied EMF (Two-way RM ANOVA, $F_{1,51}$=14.69, P=0.0003). As the mean wingbeat frequency of locusts was approximately 19 Hz the difference in locust wingbeat frequency from the frequency of the applied EMF varied significantly (Two-way RM ANOVA, $F_{2,57}$=13.58, P<0.0001). There was no significant interaction effect between the differing test frequencies, and the applied EMF (i.e. synchronization caused by EMF application) (Two-way RM ANOVA, $F_{2,57}$=1.334, P=0.272).

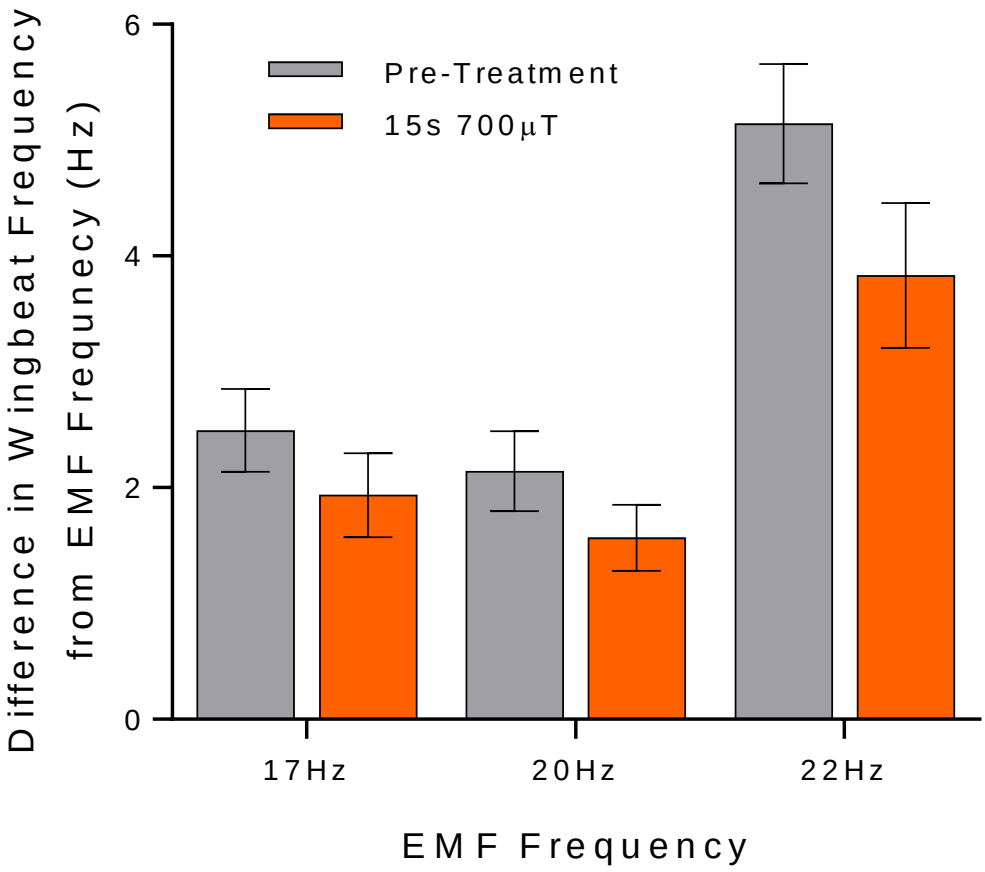

Figure 31. Effects of 700 µT EMFs at varying 17, 20, and 22 Hz on the mean difference in wingbeat frequencies of locusts from the applied frequency of the ELF EMF. Mean and SEM are plotted.

2.5 Discussion

2.5.1 Overview

Short-term exposure to relatively high levels of ELF EMFs, equivalent to that present around overhead power transmission lines, affect the behaviour, neuronal and muscular responses and levels of heat shock protein in the locust. Exposure to ELF EMF reduced walking in freely moving locusts. Force generated by ETi muscle was reduced during EMF exposure while the latency to the FETi spike increased and spike duration lengthened during exposure, and the levels of Hsp70 increased during exposure. Acute exposure to ELF EMFs caused changes in locust wingbeat frequency that were more pronounced at higher field strengths, and caused synchronization of locust wingbeat frequency to the applied ELF EMF frequency.

2.5.2 Short-term effects

2.5.2.1 Behaviour

ELF EMFs significantly alter the normal walking behaviour of locusts, with distance travelled being reduced following exposure to EMFs. The short-term effects of ELF EMFs have little ecological relevance to the locust specifically, however may have environmental impacts for other species that encounter ELF EMFs more regularly on a short-term basis. Walking/locomotory behaviour is critical for dispersal in insect species as a fundamental process that affects metapopulation dynamics (Vinatier et al., 2010). Successful behavioural responses to sensory cues are critical in terms of insect ecology, and the reduced success of gregarious locusts here to locomote towards the olfactory and visual cues of other gregarious locusts is certainly a deviation from normal behaviour (Despland, 2001). EMF exposed locusts still partially completed the tunnel assay towards attractive stimuli, and this finding alongside the observed effects of ELF EMFs on ETi muscle force and FETi signalling appear to indicate that a major cause of poorer performance in this assay is due to locomotory ELF EMF effects. That said, ELF EMF impacts on the abilities of locusts to respond effectively to sensory cues may also contribute to the findings here.

This behavioural finding now gives impetus to investigate the effects of ELF EMFs in other species, to determine how broad behavioural impacts may be, as well as investigating the thresholds for ELF EMF induced behavioural changes in insects, both in terms of intensity and exposure time. Previous studies have suggested responses of insects to ELF

EMFs at very low levels (Bergh, 1979; Wijenberg 2013). In other organisms short-term exposure to ELF EMFs has been shown to affect locomotory behaviour, for example in mice ELF EMF exposure reduces water maze performance, as well as causing oxidative stress (Cui et al., 2012). Some insects are known to have magneto-sensory abilities to Earth's static geomagnetic field (30-75 μT) (Wajnberg et al., 2010), which is much weaker than the fields tested here. Two studies have found that short-term ELF EMF exposure can cause changes in locomotor activity in *Drosophila* (Dimitrijević et al., 2016; Zmejkoskia et al., 2017), however little is known of the physiological and molecular changes that may occur to cause these behavioural deviations.

As discussed in detail in Chapter 1, ELF EMFs may cause a range of effects within biological tissues that may then underpin behaviour. Magnetic field effects on hydrogen bonds have been associated with destabilising DNA (Repacholi and Greenebaum, 1999) which may explain observed impacts of ELF EMFs on gene expression found in various studies (Blank and Goodman, 2004; Tokalov and Gutzeti, 2004; Li et al., 2013) and lead to changes in behaviour. ELF EMFs have been associated with modified function of critical proteins for neuronal signalling, such as a sodium potassium pump (Serpersu and Tsong, 1983; 1984; Blank and Soo, 1989; 1990; 1996; Liu et al., 1990; Blank, 1992), as well as being associated with excitatory effects on neurones (Dimbylow, 1998; 2000; Jacobson et al., 2005) which could lead to behavioural effects, including modified locmotory function. Changes in gene expression, neuronal function and excitation are a few examples that may explain how behavioural changes from ELF EMF exposure occur. Given the range of physiological effects found here, it is important to consider what physiological interactions occurred that may give rise to the behavioural effects that have been observed here.

2.5.2.2 Physiology

ELF EMF exposure reduced muscle force output in the locust, increased the latency to spike in FETi and broadened the FETi spike; effects most pronounced at high levels of exposure. The effects of EMF at multiple levels in an organism is not surprising given that the nervous system functions via electrical signals and, as a result, is inherently susceptible to ELF EMFs (WHO, 2007a). ELF EMF exposure is known to induce electric fields and currents within organisms that can potentially excite neurons (Dimbylow, 1998; Jacobson, 2005), and it is possible that under a 24 hr exposure, subthreshold repeated excitation of the locust neuro-muscular system could lead to poorer walking performance. Fatigue and

adaptation can occur in response to repetitive stimulation in insect muscle in general (Stevenson and Josephson, 1990) and in the force output of the extensor muscle stimulated using similar methods described here (Norman, 1995). Taniguchi and Tani (1999) also found EMF exposure increased the latency of motor-evoked action potentials in human erector spinae muscles while Bigland-Ritchie and Woods (1984) showed impaired action potential properties (including duration and delay, such as was found here in the locust) due to overstimulation of motor neurones, leading to a reduction in muscular force. These results provide striking similarities to those found in the locust and may explain why locusts do not complete the tunnel efficiently.

With this in mind the link between modified FETi action potential properties (e.g. FETi spike broadening that was found here) and reduced ETi muscle force seems possible. For example, Spencer et al. (1989) showed in *Polyorchis*, that spike broadening (i.e. increased action potential duration) was negatively associated with 'excitatory junction potential' amplitude. Cohen et al. (1978) found in *Aplysia* that the degree of contraction of the buccal muscle, and in turn muscle force, is dependent on depolarizing 'excitatory junction potential' amplitude, and so, if spike broadening leads to reduced 'excitatory junction potential' (Spencer et al., 1989) then spike broadening may reduce muscle force. Whilst these underlying mechanisms have been identified in other species it remains to be determined exactly how spike broadening (from ELF EMF exposure) may lead to reduced force in the locust extensor muscle.

Changed FETi action potential properties and reduced metathoracic ETi force may have implications for both defensive kicks and jumping. This was the only neuronal circuit tested in this study, and these physiological impacts are likely to be representative of other motor neuron and muscular effects throughout the locust. The behavioural findings provide some suggestion that this may be the case, as during free-walking (as in the behavioural experiments in this study) the metathoracic FETi motor neurons do not spike (Burrows and Pflügler, 1988), and therefore ELF EMF effects on prothoracic and mesothoracic motor neurons as well as (and including) the slow extensor tibiae (SETi) motor neurons may give rise to this behavioural effect, as opposed to effects on the metathoracic FETi motor neuron. There is a potential for physiological effects found here to be representative of ELF EMF effects on a range of other key behavioural motor programmes, including flight.

The effects of EMFs on the FETi action potential could be caused by any changes in Na^+ and K^+ levels, as these ions are critical for action potential generation. While few studies have explored the effects of ELF EMFs on Na^+ and K^+ levels, however, Na^+/K^+ ATPase is

known to be affected by ELF EMFs as the frequency of the electromagnetic fields used in this study (50 Hz) is very close to the turnover rate of the enzyme (Yoda et al., 1984; Blank, 2005). There are many studies that show modified Na^+/K^+ ATPase activity when EMFs were applied (Serpersu and Tsong, 1983; Liu et al., 1990; Blank, 1992). Given that ELF EMFs are able to cause disruption to the process by which ion gradients are maintained then it is not surprising that FETi action potential are affected. Moreover, Barbier *et al.* (1996) showed that there was a significant change in Ca^{2+} influx following 50 Hz EMF exposure. Given the key role of Ca^{2+} in synaptic transmission and muscle contraction any changes caused by EMF would be likely to lead to changes in muscular forces as found in the locust. In other invertebrates, such as the crayfish, high levels of EMFs affect the amplitude of action potentials in the lateral giant interneuron (Ye et al., 2004) and intracellular Ca^{2+} levels. Given that high Ca^{2+} influx can be intrinsically linked to K^+ efflux (Crest and Gola, 1993) EMF related calcium changes could lead to disrupted Na^+/K^+ gradients, and an indirect ELF EMF effect on action potentials.

As a limitation here, ELF EMFs could only be applied at separate temperature levels when the magnetotherapy applicator coil was used for short-term treatment. This gave little comparability to the ELF EMF treatments at this stage, as desert locust's optimal temperature is 25-35ºC (Weis-Fogh, 1956; Burrows, 1996), and temperatures for 1 mT and 4 mT treatments with the magnetotherapy applicator coil fell outside of this range. As the temperatures of these treatments were below optimum, it is likely that this contributed to the lower muscle force for respective controls. Increased temperatures, such as those for the 7mT EMF and control treatment, are more optimal for desert locusts, and increased temperature (within the range of 20-40ºC) is associated with increased the maximal muscle force output in insects (Stevenson and Josephson, 1990). The lower temperature of the other treatments therefore may have reduced the maximal force that may have been gneeated by ETi muscle, making it difficult to determine the effects of these ELF EMF exposures on ETi muscle force. To avoid this issue in future a large environmentally controlled room, with minimal metal to avoid magnetization from EMF generation, and an EMF generator with more airspace and air circulation (e.g. a Helmholtz apparatus with a fan) would be required to maintain a consistent temperature (within locust optimal conditions) for a single control treatment and all EMF exposures that occur over a short-term period.

2.5.2.3 Stress Proteins

ELF EMFs caused an increase in Hsp70 levels, which suggests that at the molecular level, stress processes are affected by short term exposure to high levels of ELF EMFs, and these changes may underpin the other effects observed in this study. There is evidence that heat shock proteins can be upregulated from EMF treatments. For example, Li *et al.* (2013) found in *Drosophila* that short and long term 3 mT 50 Hz EMF exposure caused different expression of *hsp22, hsp68, hsp70bc, hsp70, hsp60d*. There are multiple pathways underlying induction of heat shock proteins, the first being a direct increase in expression of Hsp70 through interaction with the promoter region DNA (Goodman and Blank, 2002). Whilst this pathway is likely, with the other results found in this study, the potential for ELF EMF exposure to induce Hsp70 expression through stress pathways is also likely. Heat shock proteins in insects (and other animals) are induced by a variety of molecular and/or physiological stresses (King and Macrae, 2015). As there are many pathways that could be affected to upregulate Hsp70 expression from stress it is difficult to determine if a specific pathway may be affected. There is however evidence that muscle fatigue increases Hsp70 expression in rats, after muscle tissue is electrically over stimulated (Jammes et al., 2012). This could potentially link with the results from other parts of this study as a molecular signal of the physiological effects that could lead to modified behaviour. There is also evidence from studies on rats (Laramee et al., 2014) that extremely high levels of static EMF can also lead to an increased Hsp70 expression, however these levels of static EMF are many orders of magnitude greater than geomagnetic fields normally encountered in the environment and which many insects can detect (Gould et al., 1978; Gegear et al., 2008; de Oliveira et al., 2009).

The protective properties of heat-shock proteins from heat stress on neuronal function has been investigated in detail, including Barclay and Robertson (2000) who found reduced neuromuscular thermosensitivity in heat-shocked locusts proceeding heat shock treatments. The pathways by which environmental stress can lead to neuromuscular protection via upregulation of heat-shock proteins are not completely described, but Barclay and Robertson (2001) suggest that stressful environmental conditions affecting ionic diffusion and flux as well as protein shape/function are likely to be detrimental to neuromuscular systems when encountered repeatedly, and potential triggers for stress responses. There are mixed findings with regards to the direct effects of heat-shock proteins on the specific properties neuromuscular systems, for example for excitatory junction potentials some studies have found no effect of heat-shock on latency (Karunanithi et al., 1999; Barclay and Robertson,

2000), decreased latency (Barclay and Robertson, 2001) and increased latency (Dawson-Scully and Robertson, 1998). Barclay and Robertson (2001) state that there may be multiple heat-shock pathways for alteration of synaptic performance, or a single heat-shock mechanism acting on different cellular targets, which may explain the variation in these findings. Part of the difficulty in determining the direct impacts of heat-shock proteins on neuromuscular systems may be in distinguishing what properties were affected by the initial environmental stress, and what were affected by increased heat-shock protein expression.

Relevent to the findings of this work, Barclay and Robertson (2001) found that pre-exposure to stressful environmental conditions enhanced synaptic facilitation, indicating an increase in presynaptic calcium levels, which could be achieved by axonal spike broadening, and Wu et al. (2001) found that prior heat-shock increased the duration of action potentials recorded from neuropil segments of depressor motorneurons in the locust flight system. There may therefore be a link between the changes in heat-shock proteins found in ELF EMF exposed locusts and some of the observed changes in neuromuscular properties of treated locusts, e.g. increased FETi action potential duration. It is also possible that through upregulation of heat-shock proteins that the neuromuscular systems of the exposed locusts have some buffer against potential future exposure to ELF EMF environmental stress. This could be explored in future studies of the impacts of repeat ELF EMF exposure on neuromuscular systems.

2.5.3 Acute effects

2.5.3.1 Effects of ELF EMFs on flight

Acute exposure to ELF EMFs caused a change in flight activity of locusts. Bergh (1979) suggested that very-low frequency EMFs caused by storms increased the take-off rate of locusts that initiates flight. Moreover, other studies (Ramchandra Rao, 1942; Clark, 1969) have associated locust flight activity with storms, which are known to generate ELF EMFs (Reiter, 1960; WHO, 2007a). These are the only indications so far in literature, before the findings of this study, that locusts may show acute behavioural responses to ELF EMFs. The distinct biological mechanism by which insects may respond to environmental ELF EMFs is not known. The most widely accepted potential mechanisms for magnetoreception are through exploitation of the quantum effect in cryptochrome flavoproteins (Dodson et al., 2013), or through forces on tiny crystals of magnetite (Fe_3O_4) within cells (Kirschvink et al.,

2001). There is no current non-behavioural evidence of locusts having the machinery for these processes, but advances in molecular biology, such as the sequencing of the 6,500,000,000 bp migratory locust genome (Wang et al., 2014), are increasing the likelihood of developments in this area. If magnetosensory abilities are present in locusts there is still not enough known about the thresholds for magnetic fields interacting with these sensory systems, however there is some evidence that ELF EMFs may be able to have impacts upon magnetosensory systems (general introduction section 1.4.3.4), and there is certainly not substantial evidence to rule this out as a possible cause for acute behavioural effects seen in this study.

ELF EMF exposure was also shown to cause a synchronization of locust wingbeat frequency to the frequency of the applied ELF EMF. Locust wingbeat frequency is controlled by a central pattern generator (Reye and Pearson, 1988; Marder and Bucher, 2001) meaning a central flight oscillator generates the wingbeat frequency pattern independently of peripheral receptors. A cooperative system of central neuronal, sensory (proprioceptive) and mechanical inputs (Horsmann et al., 1983) however, can modulate the activity of the pattern generator by increasing or decreasing the rhythmic activity of elevator/depressor motor neurons (Waldron, 1968; Pearson, 1985). From the evidence described above of ELF EMFs effects on motor neuron signalling in this study, as well as neuronal excitability in the literature, it is therefore possible that central neuronal processes as well as sensory and mechanical inputs, could be affected by ELF EMFs which could cause observed changes in wingbeat frequency. The acute effects here could be caused by the same physiological interactions of ELF EMFs that may give rise to the short-term effects seen with longer exposures.

The environmental stimuli that can affect locust wingbeat frequency are varied, as locusts are known to phase lock wingbeat frequency to a variety of oscillatory stimuli including strobe lights (Waldron, 1968; Hennigsson, 2015), modulated airflow (Horsmann et al., 1983), and even neighbouring locusts in flight (i.e. locust flying in the wake of another locust) (Kutsch et al., 1994). Mathematical analysis of phase locking in locusts has described the phenomenon as a function of the amplitude of the sinusoidal stimulus and the relative frequencies of the oscillator and the sinusoidal stimulus (Glass and Mackey, 1979). This makes it likely that with increasing EMF intensity, phase-locking with the EMF may be more likely. Phase-locking to oscillatory stimuli is more likely if the frequency of the stimuli are not too different to the initial locust wingbeat frequency (Waldron, 1968) or close to the natural wingbeat frequency of locusts, 20 Hz, or a factor of this (Hennigsson, 2015).

Wingbeat frequency is a critical parameter of locust flight as a principal determinant of aerodynamic power output (Taylor, 2001). Increases in wingbeat frequency are normally associated with increased force production (Taylor, 2001). Therefore when synchronizing with applied oscillatory stimuli insects may be unable to decrease wingbeat frequency by too great a magnitude, as they must maintain enough force to remain airborne, and they may not increase wingbeat frequency by too large a magnitude as energy efficiency and physiological limitations come into play. For example Horsmann et al. (1983) found that with wind modulation phase-locking of locusts occurred in a range of around 3 Hz of their initial wingbeat frequencies, however when the applied oscillatory stimulus was outside of this range this flight was found to be only relatively coordinated with the oscillatory stimuli.

In this study synchronization to the exact frequency of the applied EMF was tested (Fig. 32). If however the applied ELF EMF frequency was largely different from the initial wingbeat frequency of flying locusts, which as stated is a major determinant for synchrony with other oscillatory stimuli (Waldron, 1968; Horsmann et al., 1983; Hennigsson, 2015), there are harmonics, or factors (subharmonics), of the applied EMF frequency with which synchrony may have occurred (e.g. Fig. 32, B-D). For example when the 22 Hz ELF EMF was applied, synchrony may have occurred in 1 instance to the 11 Hz subharmonic i.e. a 1:2 factor of the applied EMF (Fig. 32, D), and when the 17 Hz ELF EMF was applied in 2 instances synchrony may have occurred to the 25.5 Hz harmonic which is the 3:2 harmonic of the applied ELF EMF (Fig. 32, C).

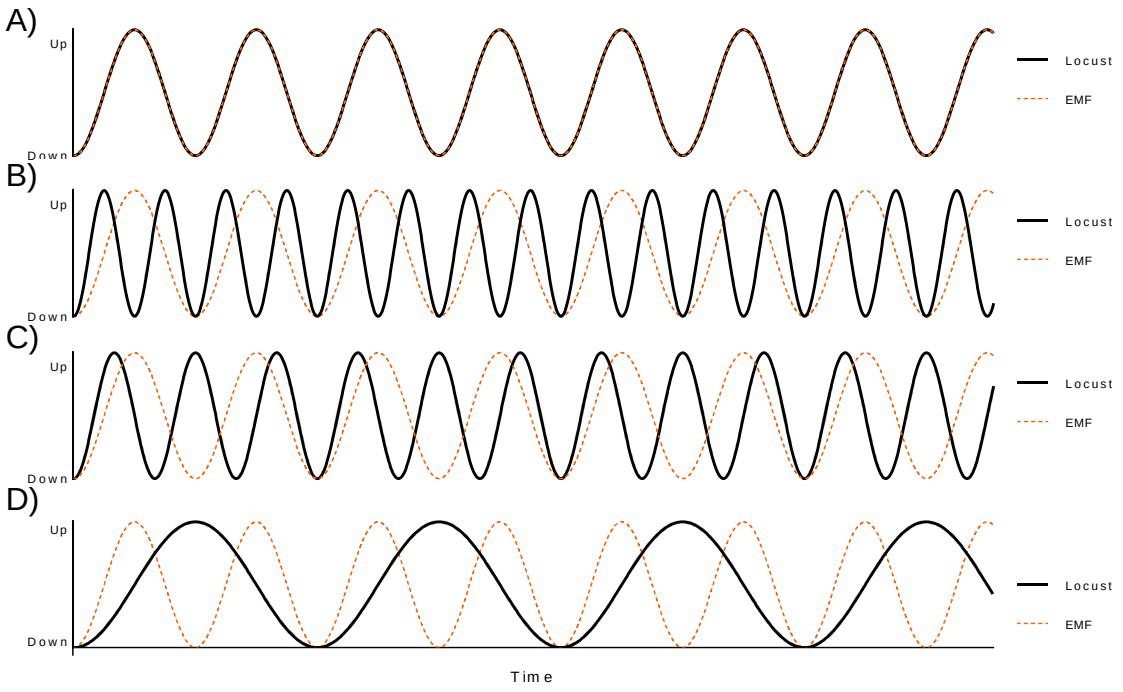

Figure 32. Varied forms of synchronization of locust wingbeat strokes (up/down) with an oscillatory EMF stimulus. A) 1:1 synchronization at the exact frequency of the applied EMF frequency. B) 4:2 synchronization, double the frequency of the applied EMF frequency. C) 3:2 synchronization, with 3 wingbeats to every 2 oscillations of the EMF. D) 1:2 synchronization, at one-half the frequency of the applied EMF frequency

In the primary experiment synchrony to a subharmonic of the applied 50 Hz EMF may be occurring, potentially explaining the 20 Hz synchrony in the primary experiment. 50 Hz is well beyond the initial wingbeat frequency of any locusts in these experiments e.g. the fastest locust flight recorded in this study was 26 Hz. Therefore, with a 50 Hz oscillatory EMF the forces produced by the applied EMF would have to elicit at least a doubling of locust wingbeat frequency for exact synchrony to occur. Synchrony with 1:2 (25.0 Hz), 1:3 (16.6 Hz), and 1:4 (12.5 Hz) subharmonics of the applied 50 Hz field may also be unlikely as these frequencies are not close to the initial wingbeat frequencies of many of the locusts in this study. 20 Hz however is a 2:5 subharmonic of the applied 50 Hz ELF EMF (Fig. 33) which is much closer to the mean wingbeat frequency of locusts in this study, and this may explain why synchrony to 20 Hz occurred during the primary experiment.

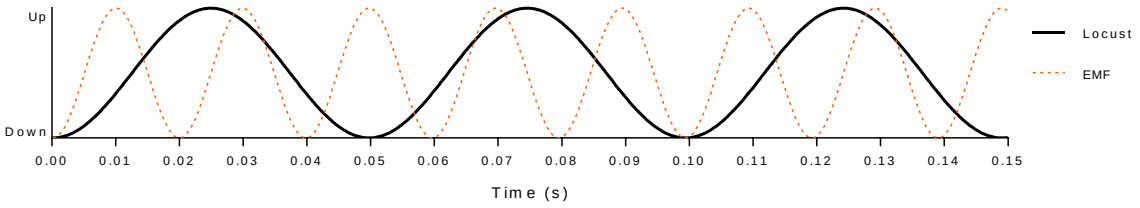

Figure 33. Synchronization of locust wingbeat frequency with an oscillatory EMF at a 2:5 factor, where for every 2 full wingbeats the EMF oscillates 5 times. This figure depicts a locust flying at 20 Hz in a 50 Hz ELF EMF that is generated by 50 Hz conventional mains electricity.

2.5.4 Summary and conclusion

What is clear from these findings is that ELF EMFs are a form of oscillatory environmental stimuli that affect locust flight, and flight pattern generation. Short-term exposures to ELF EMFs cause physiological changes that underpin locomotory behaviour, and increase molecular indicators of stress in these systems. The consequences of this are that ELF EMFs may affect a range of neuromuscular systems in insects potentially giving rise to diverse acute and short-term behavioural effects.

In future studies the applied ecological implications of ELF EMFs on insect flight should be considered as well as the mechanisms by which these environmental stimuli acutely bring about these effects. As most ELF EMFs in the environment occur around 50 or 60 Hz, it is likely that flight pattern will be affected differentially dependent on insect species. For example, locusts often fly with a 20 Hz wingbeat frequency (Hennigsson, 2015), however hemipterans often fly at 40-45 Hz, *Ochlodes* butterflies often fly at 50 Hz, and Bumble bee wingbeat frequencies can often be in a range of 100-115 Hz (San Ha et al., 2013). Considering the acute effects of ELF EMFs found here in combination with short-term effects there is evidence that ELF EMFs can induce stress responses in insects, and can cause physiological changes leading to behavioural changes, as well as acute behavioural responses to ELF EMFs. There is further impetus to study ELF EMFs from a physiological health perspective, e.g. whether such effects occur in other organisms and whether or not these effects are transient, as well as to determine the varied potential environmental impacts of ELF EMFs.

Chapter 3 Effects of ELF EMFs on aversive learning, appetitive learning, and stress levels in honey bees

3.1 Abstract

Learning performance is an imperative cognitive function in pollinating insects such as bees, both in terms of appetitive learning of profitable odours, and aversive learning of threats and negative stimuli. Here the impacts of ELF EMFs on both aversive and appetitive learning were explored, as well as the effects of ELF EMFs on stress proteins as potential indicators of molecular stress and modified gene expression that may underpin changes learning performance. Short-term 17 hr exposure to high ELF EMF levels of 7000 µT increased stress protein levels, but this effect did not occur at lower level exposures. In contrast, 17 hr exposure at both 1000 µT and 100 µT, the latter of which can be encountered at ground level under power lines, reduced performance in aversive learning assays. In appetitive learning assays short-term treatments slightly reduce cognitive function when applied as a secondary treatment after 5 conditioning trials. An acute exposure paradigm of 1-min exposure in-between each conditioning trial in the appetitive assay, to simulate ELF EMFs as may be encountered in a field scenario, largely reduced olfactory learning performance at 1000, 100, and 20 µT exposures.

These findings indicated that short-term exposure to ELF EMFs at levels which could be encountered for bee hives placed under power lines may reduce aversive learning performance, which could have wider ecological implications, however at levels which occur at ground level under powerlines there is no indication that short-term ELF EMF exposure will cause increased indication of molecular stress in bees. Acute ELF EMF exposure reduced honey bee appetitive learning performance at low levels which can be encountered regularly at ground level under power lines. This work lays the foundation to further explore the mechanisms by which ELF EMFs may affect learning processes, as well as the wider ecological implactions in field scenarios.

3.2 Introduction

3.2.1 Honey bee declines and potential for ELF EMF impacts

The European honey bee, *Apis mellifera*, is a species of particular economic and ecological importance (Potts et al., 2010a). As has been discussed in detail, honey bees, along with many other flying insects/pollinators, are currently in decline (Hayes et al., 2008; Potts et al., 2010b Hallmann et al. 2017). The current scientific consensus is that a variety of anthropogenic stresses have a major contribution to these declines (Goulson et al., 2015). As mentioned previously, pollution of the environment with ELF EMFs has increased dramatically in the last century, with a major environmental source for pollution being HVTLs. There is a variety of evidence (Wellenstein, 1973; Greenberg et al., 1981; Rogers et al., 1982; Morse and Hooper, 1985; Lee, 1989) that honey bees may be responsive to or affected by EMFs from HVTLs (1.5.2.2). If ELF EMFs can cause stress to honey bees, they could be contributory factors to honey bee declines and cause reductions in pollination ecosystem services.

3.2.1 Importance of learning and memory in bees

Learning is a critical cognitive behaviour for pollinating insects, including bees. Honey bees must make appropriate behavioural decisions in response to a variety of sensory cues, including visual, olfactory, tactile and auditory, whilst navigating over distances of several kilometres (Menzel and Giurfa, 2001; Smith and Raine, 2014). Honey bees learn spatial information, such as celestial cues and landmarks, which aid navigation (Dyer, 1996). Spatial information such as the distance and direction of potential food sources, water sources, or nesting sites, is communicated through the ‘waggle dance’ to other bees, which in turn learn these instructions (von Frisch, 1967).

Menzel and Giurfa (2001) define cognition as the sum of an organism’s ability to acquire perceived sensory information from the environment (learning) and an organism’s ability to retain acquired information and to use if for appropriate future behavioural responses to sensory cues (memory). The process of learning acquisition and memory formation has been studied in detail in honey bees by associating sensory cues with a positive or negative reinforcement in a learning ‘conditioning trial’, to an extent that different distinct phases of memory formation have been identified in honey bee cognition: short-term memory, medium-term memory and long-term memory. A single conditioning trial will

induce a sensitive short-term memory through neural activity in the seconds to minutes range that is independent of protein synthesis (Grünbaum and Müller, 1998; Menzel, 2001). Protein kinase A activity is required for middle-term memory in the hours range (Grünbaum and Müller, 1998; Müller, 2000), and repeated conditioning trials are required to generate a stable long-term memory in the days range which requires protein synthesis and activation of protein kinase C and NO pathways (Davis and Squire, 1984; DeZazzo and Tully, 1995; Müller, 1996; Menzel and Giurfa, 2001). The full suite of neurophysiological and structural changes that occur to acquire sensory information and consolidate this into a stable memory are critical to the abilities of bees, and many other organisms, in enacting suitable behavioural responses to stimuli in ecological processes.

3.2.2 Appetitive Learning

Foraging bees encounter hundreds of flowers in quick succession during flight, responding to floral cues, such as the smell, colour, shape and texture of the flower (Menzel et al., 1993). Floral cues are associated with the quality of food resource, which is learnt by bees, and memory of these cues is crucial in efficiently locating valuable food resources in further foraging activities (Menzel and Muller, 1996), with the ability to distinguish between the complex volatile emissions of flowers in olfactory learning being particularly important (Sandoz, 2011). Learning is associated with natural foraging success. For example, Raine and Chittka (2008) showed that bumble bee colonies which took less time to learn controlled floral cues had greater nectar foraging rates. As a result, factors that may impact upon learning, including electromagnetic fields, can negatively affect the pollination success of bees.

3.2.3 Aversive learning

In the environment bees are exposed to a variety of threats and negative environmental stimuli, whether these are deleterious conditions such as weather and toxins (Wright et al., 2010), or biotic threats such as colony diseases and parasites (Cappa et al., 2016; Goulson et al., 2017), invader bees from other colonies (Cappa et al., 2016) or predators (Nouvian et al., 2016; Tan et al., 2016; Goulson et al., 2017). How colonies respond to these environmental stresses is critical to long term colony fitness. In the environment bees must detect these negative stimuli (Cappa et al., 2016), learn that they are associated with a negative effect (Wright et al., 2010), respond in an appropriate behavioural manner (Nouvian, 2016), and even communicate this information to other individuals (Tan et al., 2016). For example,

many guard bees when confronted with a threat (e.g. predator or intruder) enter the hive to release alarm pheromone by extruding their sting, raising their abdomen and fanning their wings (Maschwitz, 1964; Collins et al., 1980; Nouvian et al., 2016).

For bees that approach a threat, an all-out response to sting the threat is not necessarily the most effective option. When stinging a threat the barbed lancets of the bee sting apparatus are very large, and these help the sting penetrate the target and deliver a greater dose of venom (Herman, 1971). However the barbs are too large to withdraw the sting from the target, and thus stinging a threat often results in sting autonomy, with an extreme rupture of the abdomen that causes the eventual (but not immediate) death of the bee (Herman, 1971; Cunard and Breed, 1998). As a result, before resorting to an all-out sting response, many bees will aggressively buzz and fly at a threat to ward it away (Collins et al., 1980; Cunard and Breed, 1998). This highlights the critical decision making processes of bees that occurs when processing environmental stimuli (Nouvian, 2015), which, if negative, must be recognised to enact the appropriate aversive response to avoid serious reductions in fitness for the colony (Wright et al., 2010; Nouvian et al., 2016).

3.2.4 Measuring appetitive learning with the proboscis extension response assay

Pioneering attempts to examine olfactory appetitive learning in bees were made by von Frisch (1919) who showed that free-flying bees could learn to visit artificial feeders based on olfactory discrimination of essential oils, and the association of these olfactory stimuli with sucrose rewards. Classical conditioning experimental assays were then developed as a method to examine and study learning in different organisms. Ivan Pavlov's classic experiments with dogs formed the foundation of classical conditioning (Pavlov, 1927) where a 'conditioned stimulus' (CS) that does not elicit a response in a subject, is associated with an 'unconditioned stimulus' (US) that usually does elicit a response, resulting in the learning of the CS, which subsequently elicits a conditioned response when presented. The first attempts to translate Pavlovian classical conditioning to bees with proboscis extension reflexes were made by Kuwabara (1957) with visual stimuli, and then Takeda (1961) with olfactory stimuli. The proboscis extension response (PER) assay was optimized to classical conditioning with honey bees by Bitterman et al. (1983).

In the PER assay a CS, which is usually an olfactory cue such as linalool, is paired with an US reward, such as sucrose. As the bee learns that the CS is associated with the US the bee extends its proboscis in response to the CS, a 'conditioned response'. In this way,

the PER assay simulates natural interactions of bees with plants, where a bee will reflexively extend its proboscis when its gustatory receptors come into contact with plant nectar, and the bee memorises the floral odours that it concurrently detects during this process (Desneux et al., 2007). Since Bitterman et al. (1983) first published details of their PER assay hundreds of studies have been published using PER to assess learning through olfactory conditioning, and PER has even expanded to other species such as bumble bees (Riveros and Gronenburg, 2009).

3.2.5 Biology of olfactory conditioning

The PER assay has allowed the biology of olfactory conditioning to be studied in detail, and improve our understanding of the neurobiological pathways that underlie olfactory learning in bees. Over 60,000 chemosensory olfactory receptor neurons (ORNs) transduce olfactory chemical signals (Esslen and Kaissling., 1976) and project along the antennal nerve to the primary olfactory centre of the brain (Fig. 34), the antennal lobe (AL). Here each ORN projects to one of 165 glomeruli (Flanagan and Mercer, 1989; Sandoz, 2011; Menzel 2012). In the AL approximately 4,000 local neurons (Galizia et al., 1999) mediate lateral interactions between glomeruli while excitatory projection neurons (PNs) represent the outputs from the glomeruli and transmit olfactory information to the mushroom bodies (MBs) and lateral horn (LH) which are higher-level centres of olfactory processing (Menzel and Giurfa, 2001; Sandoz, 2011; Menzel, 2012). The MB contains over 150,000 intrinsic neurons called Kenyon cells, and extrinsic neurons (ENs) from the MB project to multiple sites in the brain, including loops back to the MB, projections to the LH, and to the contralateral side of the brain (Homber and Erber, 1979; Mobbs, 1982; Grünewald, 1999). Descending interneurons (DN) transmit premotor instructions from the LH to the suboesophageal ganglion (SOG) where motor neurons drive the movement of mouthparts in the conditioned PER (Menzel, 2012).

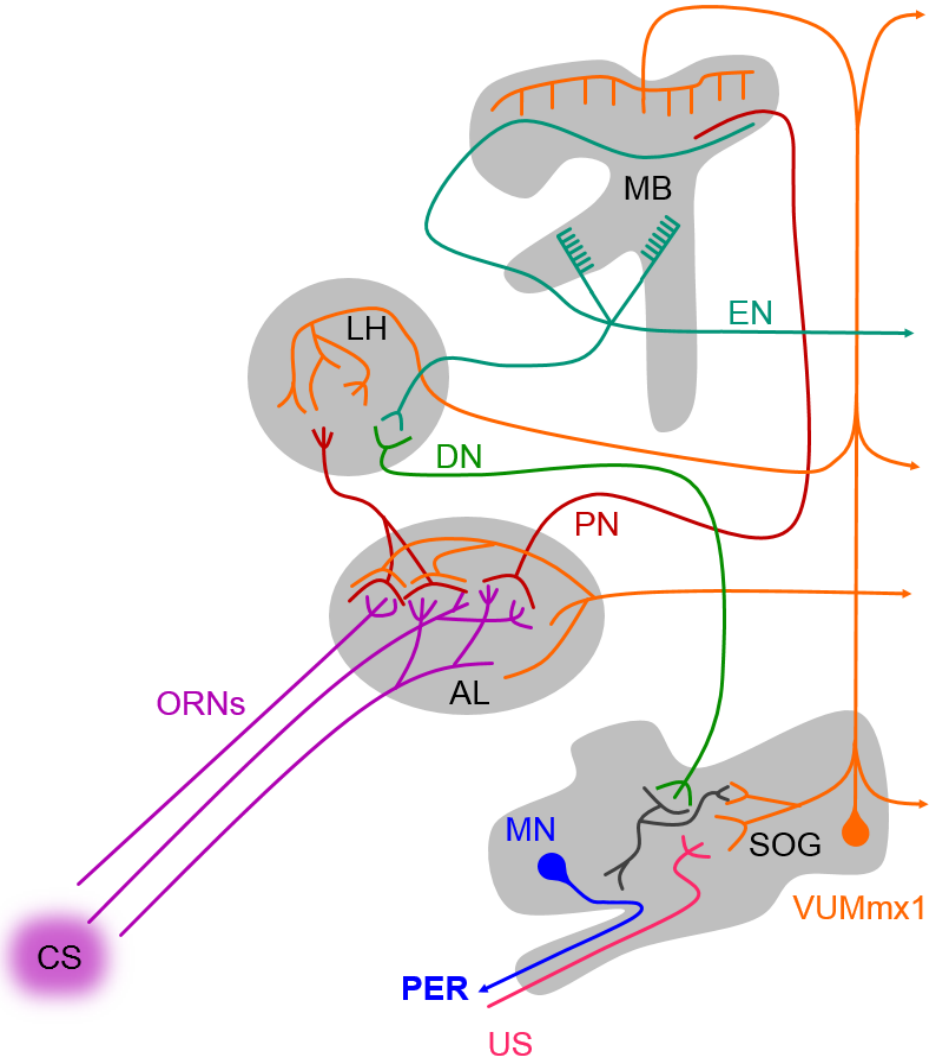

Figure 34. The neural pathways underlying appetitive olfactory associative learning in the honey bee, showing one side of the brain and SOG. Neurons that project to the contralateral side of the brain are shown with arrows. Adapted from Menzel (2012)

A single neuron, the ventral unpaired median neuron 1 of the maxillary neuromere (VUMmx1), has a critical role in value processing in olfactory learning. The cell body of VUMmx1 is located in the SOG and it projects to contact the olfactory circuit in the three main regions, the ALs, MBs and the LH, on both sides of the brain (Menzel and Giurfa, 2001; Menzel 2012). VUMmx1 responds to sucrose stimulation, and its direct activation through electrical stimulation can be used to replace sucrose as the US in associative learning. Moreover, it responds to a CS if the CS has previously been paired with an US (Hammer, 1993). Furthermore, VUMmx1 contains the biogenic amine octopamine that has a critical role in olfactory reinforcement in appetitive learning in honeybees (Hammer and Menzel, 1998; Farooqui et al., 2003). There is still much to learn regarding the neural

machinery that controls learning in bees, however these systems with critical roles in bee learning are candidates for the impacts of environmental stressors on cognitive behaviour.

3.2.6 Measuring aversive learning with the sting extension response assay

While the proboscis extension response of bees has been studied in detail, less is known of aversive learning in which the sting is extended when a bee is aggravated (Vergoz et al., 2007). In the sting extension response (SER) assay a conditioned stimulus (CS) (often olfactory) is applied and associated with an unconditioned stimulus (US) of a weak electric shock. Over repeated conditioning trials the bee learns to associate the US with the CS. In this scenario a confirmed learned response to aversive stimuli is recorded when a bee extends its sting after having been exposed to the CS, but before the electric shock (US). In SER studies a typical acquisition level is approximately 35-45% of the sample population (Vergoz et al., 2007), in comparison with an acquisition level of 70-80% in the sample population from proboscis extension response studies (Bitterman et al., 1983). SER is indicative of a bee's ability to perceive, learn and avoid threats in the environment (McNally and Westbrook, 2006). The SER assay therefore provides valuable information in a controlled experimental environment of how potential stressors such as ELF EMFs can affect a critical behavioural paradigm with regards to detecting, learning and responding to aversive stimuli.

3.2.7 Applications of learning assays

Learning ability is highly associated with natural foraging success in bees (Raine and Chitka, 2008). As a result, the learning assays can provide a low-cost method to investigate factors that may impact upon learning, as an indication of how specific cues may affect more complex ecological scenarios where cognitive behaviour is important, such as foraging. Whilst it cannot be assumed that a specific cue that causes lower performance in in a learning assay will have negative effects upon learning and foraging success of bees in the field, the assay can provide a valuable indication of whether factors should be explored in more complex field experiments. For example, initial studies on the impacts of neonicotinoid insecticides on learning through the PER assay (Lambin et al., 2001; Guez et al., 2001; Decourtye et al., 2003) led to an array of more complex field studies that confirmed the impact of these environmental stressors on foraging behaviour (Yang et al., 2008; Mommaerts et al., 2010; Henry et al., 2012; Schneider et al., 2012).

3.2.8 Potential impacts of ELF EMFs on learning

ELF EMFs may affect learning in honey bees. Exposure levels can be acute, as bees often fly above ground level (Gary, 1963; Loper et al., 1992; Osborne et al., 1999) where higher EMF levels of 100 μT – 14,000 μT can be experienced by flying organisms near the overhead powerlines themselves. Exposures could also be short/long-term, for hives placed under powerlines that show poor success (Wellenstein, 1973, Greenberg et al., 1981; Rogers et al. 1982; Morse and Hooper, 1985), or for bees that use habitat refuges under powerlines as nesting sites (Russell et al., 2005; Wojcik and Buchmann, 2012; Wagner et al., 2014; Hill and Bartomeus, 2016). Here the impacts of ELF EMF exposures, both short-term and acute, are assessed via olfactory PER conditioning in the honey bee, *Apis mellifera*.

3.2.9 Molecular indicators of stress

Heat-shock proteins are a robust indicator of biological stress on the molecular level. Heat-shock proteins are stress-related proteins, the genes for which are expressed in response to various environmental stressors such as: extremes of temperature, cellular energy depletion, and extreme concentrations of ions, other osmolytes, gases, and various toxic substances (Feder and Hofmann, 1999). They are molecular chaperones that recognise damaged/improper proteins, bind to these proteins and help minimize the probability that they will interact inappropriately with one another, or with other parts of an organism. Short-term exposure has already been found here to increase Hsp70 levels in locusts (Chapter 2) as well as *Drosophila* (Li et al., 2013). There is some evidence that EMFs are able to initiate heat-shock expression by interacting with EMREs in the promoter region of the Hsp70 gene (Lin et al., 2001). Here, Hsp70 levels will be measured in EMF exposed bees to determine if there are molecular indicators of stress caused by short-term exposure EMFs. Changes in Hsp70 levels with EMF exposure could indicate damage at the molecular level or direct upregulation of gene expression from EMF interactions with DNA, such as weakened H-bonds in DNA from ELF EMF interactions as suggested by Repacholi and Greenbaum (1999).

3.2.10 Aims and Objectives

In Chapter 2 acute ELF EMF exposure caused changes to locust flight pattern and short-term ELF EMF exposure affected locomotory behaviour in locusts, as well as the neuromuscular physiology that underpins locomotory behaviour, and stress protein levels. As discussed in the general introduction, there is some further evidence that short/long-term

exposure to ELF EMFs may affect insects, especially *Drosophila*, however the extent to which different insect taxa may be affected by ELF EMFs is unknown. The potential for important ecological species (such as pollinators) to be affected by ELF EMFs is also unknown, and attempts to explore environmental effects of ELF EMFs on insects from a behavioural ecology perspective have not been made. No studies have considered the effects of ELF EMFs on insect cognitive behaviour. Honey bees as crucial global pollinators may have a susceptibility to ELF EMFs, and also are a well-studied species in terms of cognitive behaviour. Whilst some studies suggest that bee colonies may be stressed or unsuccessful when kept in proximity of powerlines, no studies have directly tested how controlled ELF EMFs may affect key honey bee behaviours, such as learning, as well as other parameters such as molecular stress, which may indicate how ELF EMFs could lead to lower colony success.

The aim of this chapter is to expand our understanding of the effects of ELF EMFs on honey bees, which represent a key pollinator species, to determine whether EMFs affect insect cognitive behaviour, through measuring key honey bee behaviours that are critical to colony success. As no such effects of ELF EMFs have been explored before, this chapter will use a range of exposure parameters in determining ELF EMF effects, including single short-term EMF exposures, repeat short-term exposures, and acute exposure. As well as this a range of ELF EMF strengths will be used here to both indicate to some level the thresholds for potential effects and/or cover the range of possible magnetic field intensities in proximity to a powerline.

This chapter addresses the following questions

First, do ELF EMFs affect levels of stress proteins in honey bees?

Second, do ELF EMFs affect learning performance in honey bees?

3.3 Methods

3.3.1 Effects of short-term ELF EMF exposure on aversive learning, appetitive learning and stress levels

3.3.1.1 Magnetic Fields

For the assessment of the effects of short-term exposure to 50Hz EMFs on honey bee stress protein levels and olfactory appetitive learning (PER) the magnetotherapy applicator coil (2.3.2.1) was used, and for the assessment short-term ELF EMF effects on aversive learning (short-term effects of ELF EMFs on SER) 50 Hz EMFs were applied using the Helmholtz coil apparatus (2.3.3.1).

For quantitative stress protein experiments, 50 Hz EMFs were applied at 4 different intensities (7000 µT, 1000 µT, 100 µT, and 20 µT). For the 7000 µT exposure a high temperature matched control at 29.3 ± 1 ºC was used (as described in section 2.3.2.2). For other treatments (20 µT, 100 µT and 1000 µT) no heat was generated at the centre of the coil and therefore one low temperature matched control was used at RT 21ºC (2.3.2.2).

For SER experiments two EMF treatments, 100 µT and 1000 µT were used along with a control exposures. No multiple temperature settings were required for controls.

For olfactory appeititve learning (short-term effects of ELF EMFs on PER) two EMF exposures were used (100 µT and 7000 µT), each with temperature matched controls. For 7000 µT exposure a high temperature matched control at 29.3±1ºC was used (2.3.2.2). For 100 µT no heat was generated at the centre of the coil and temperature matched control at room temperature 21ºC was used (2.3.2.2).

3.3.1.2 Bee Collection and Harnessing

Honey bees were kept on the University of Southampton campus in an apiary (50° 56' 10"N, 1° 23' 39"W). Returning forager bees were collected for all short-term exposure experiments. Foragers were identified by the pollen in their corbiculae (pollen baskets), caught in Sterilin 30 ml universal containers (Sterilin Limited, Cambridge, UK) and kept individually. For stress protein experiments returning forager bees were caught at their hive (Hive 4_{2015} subscript indicates collection year; Fig. 35) entrance during afternoons in the summer of 2015. For SER experiments returning forager bees were caught from 3 hives during afternoons in the summer of 2017 (Hive 1_{2017}, Hive 2_{2017}, Hive 7_{2017}; Fig. 35. For

PER experiments bees were collected from Hive 4_{2014}. They were transported on site into the insectary in the Institute for Life Sciences at the University of Southampton, where they were immobilized on wet ice and transferred into the appropriate harness for experimentation.

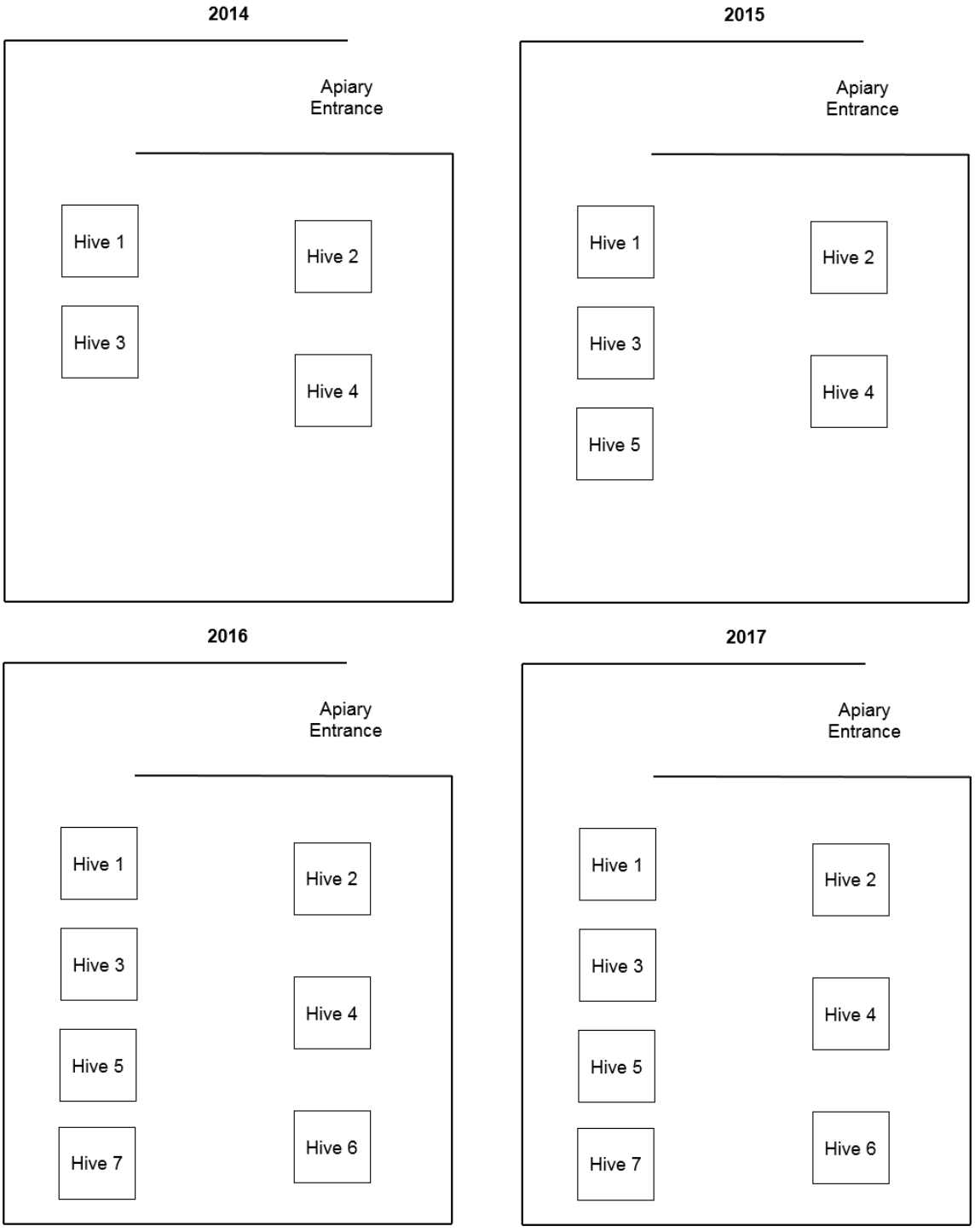

Figure 35. Map of apiary for seasons 2014-2017. All colonies were placed in Langstroth hives. Queens were regularly replaced in hives in between seasons. If data for an experiment was collected over multiple seasons, to avoid pseudoreplication different Langstroth hives were used in the second season, such that between any two seasons the same hive was not studied twice for the same experiment, whether or not the queen had been replaced.

For SER experiments bees were harnessed in SER cradles cut from Perspex. Bees were placed ventral side upwards in the metal fork of the cradle, such that the fork held the bee by the thorax, with prongs in place around the petiole and neck of the bee (Fig. 36). This fork will later serve as an electrode for a shock aversive stimulus during the SER assay. Tape was then applied laterally across the cradle and between the prongs of the fork across the thorax of the bee, holding it in place in the cradle. Once harnessed appropriately for respective experiments, and recovered from immobilization, bees were fed to satiation with a 40% w/v sucrose solution and were then ready for overnight treatment (17 hrs).

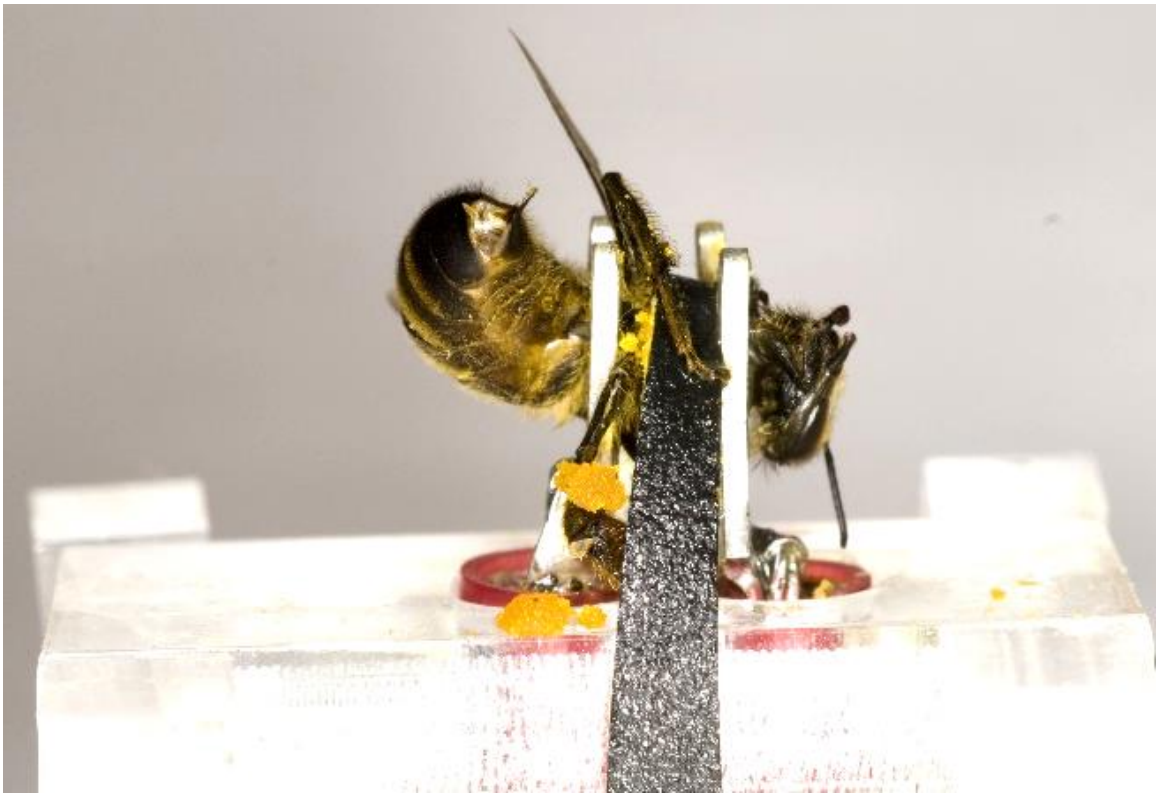

Figure 36. Harnessing of bee in SER cradle for EMF exposure in SER experiments. Above is the appropriate method for fastening a bee into an SER cradle. Tesa tape is applied around the thorax to hold the bee in between for fork prongs. Image taken by C. Jackson.

For stress protein experiments and PER experiments bees were harnessed in PER tubes crafted from 1 ml pipette tips to the design from Bitterman et al. (1983) with head, antennae and forelegs free to move (Fig. 37). Once recovered from immobilization, bees were fed to satiation with a 40% w/v sucrose solution and were then ready for overnight treatment (17 hr).

Figure 37. Harnessing of bee in PER tube for stress protein experiments and appetitive associative learning experiments. Tesa tape was wrapped over the thorax around the back of the head, to hold the bee in place, whilst allowing the bee free movement of the head, antennae and forelegs.

3.3.1.3 Odour delivery system

For PER and SER assays an experimental arena (W×D×H= 60cm×45cm×55cm) was used with a custom built odour delivery system at one end and an extraction fan at the other to remove any odours from the arena. The delivery system provided a constant airflow to be supplied to the arena from Teflon tubing which flowed into a glass tube. The conditioned stimulus (CS) was delivered though a multichannel system that ended in the same glass tube as the fresh air, before it discharged into the arena. Channels were made of electronic valves and Teflon tubing that was connected to 10 ml glass tubes, in which the stimuli were placed on a piece of filter paper. The CS used was 8 µl of 97% linalool (Sigma-Aldrich, UK) which

was pipetted onto the filter paper to be placed in the delivery channel. A control channel with clean air was always open when no odour was delivered. To deliver the CS, airflow was switched from the control channel to the odour delivery channel such that bees were supplied with a constant airflow, and would associate any stimulus with the odour and not any changes in airflow.

3.3.1.4 Stress Protein Levels

3.3.1.4.1 Treatment arrangement

To analyse stress protein levels bees were kept in a circular glass container (Diameter: 14 cm, Height: 7 cm) and exposed overnight (17 hrs) to EMFs at 10 µT, 100 µT, 1000 µT and 7000 µT in the magnetotherapy applicator. As there was no heating at 10 µT, 100 µT, and 1000 µT, the control setting for comparison with these EMF exposures was a room temperature (22.2ºC ± 1ºC) for 16 hr, which was labelled 'low control'. Due to heating at 7000 µT EMF the control for comparison with this high EMF exposure was heated to 29.3ºC ± 1ºC for 16 hr exposure, which was labelled 'high control' (Table 2). In total 210 bees were used in experiments for analysis (35 per treatment).

Table 2. Temperature settings for treatments in stress protein analysis. Below is a breakdown of the various temperatures bees were exposed to in the stress protein experiments.

EMF Exposure	Temperature	Label
Control	22.2 ± 1ºC	Low Control
20 µT	22.2 ± 1ºC	20 µT
100 µT	22.2 ± 1ºC	100 µT
1000 µT	22.2 ± 1ºC	1000 µT
Control	29.3 ± 1ºC	High Control
7000 µT	29.3 ± 1ºC	7000 µT

3.3.1.4.2 Sample preparation

Western blotting was used to detect the stress protein levels that had accumulated in the bees during exposure. Immediately following 17 hr exposure bees were snap frozen in liquid nitrogen and stored in a freezer (-80ºC) until dissection of material for western

blotting. For dissection the bee was placed in plastacine to stabilise it. The brain of the bee was then dissected following instruction from Carreck et al (2015); standard methods for Apis Mellifera anatomy and dissection, p31, section 3.3.

The brain was removed and placed in a 1.5 ml safe-lock centrifuge tube on dry ice. The total time for each dissection was < 2 min. To generate a sufficient tissue sample for Western-Blotting, 5 brains (i.e. from 5 bees, given the same treatment conditions) were added to each Eppendorf to form a sample, i.e. 35 bees with 5 brains/sample is 7 samples per treatment. As a result for low level treatments (low control, 20 µT, 100 µT and 1000 µT) 140 bees were used (35/treatment) and for high level treatments (high control, and 7000 µT) 70 bees were used (35/treatment).

3.3.1.4.3 Western-blotting

Once bee samples were prepared, the same Western-blotting protocol from Chapter 2 was used to determine levels of stress proteins from ELF EMF exposure.

3.3.1.5 Sting extension response

3.3.1.5.1 SER assay

For SER experiments bees were exposed to Control, 100 µT or 1000 µT treatments for 17 hrs in the Helmholtz coil apparatus. As no heating occurred in the Helmholtz coil only 1 control treatment was required. Following treatment SER trials immediately began. An SER cradle containing a harnessed bee was placed in the experimental arena of the odour delivery system. The bee was exposed to clear airflow for 20 s. During this time period, wires connected to the metal holding fork of the SER cradle were attached to a 12V DC stimulus generator, such that when the DC stimulus was activated a 12 V shock could be applied through the holding fork above and below the thorax of the bee. When this 20 s period was complete the airflow in the arena was then switched from clean air to linalool airflow, representing the CS. The CS lasted 8 s. For the final 2 s of the CS the bee was shocked with the 12 V DC stimulus, representing the unconditioned stimulus (US) and pairing US and CS for 2s. The US and CS finished at the same time (28 s into the trial). The clear airflow was then continued for 32 s with the bee in the arena to reinforce the association of the CS with

the US, and to allow the extractor to remove linalool from the arena. The length of 1 complete conditioning trial for a bee was, therefore, 1 min (Fig. 38).

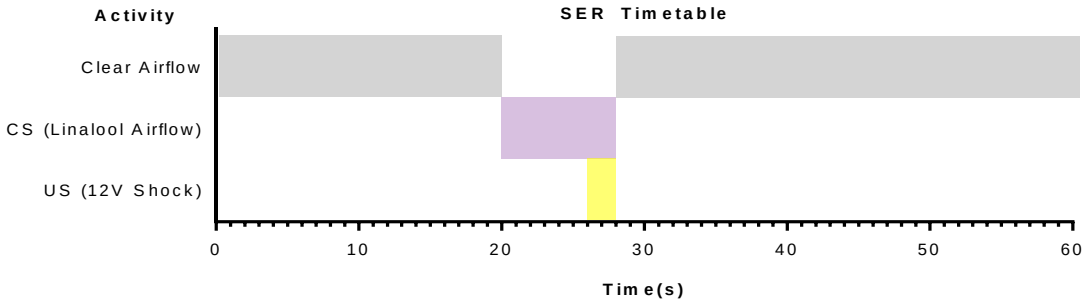

Figure 38. SER Timetable – Representation of an individual conditioning trial in SER experiments. The bee was acclimatised to the arena for 20 s, before CS (linalool) application. After 6 s of CS, CS and US (12 V shock) were paired for 2 s, after which both CS and US were switched off. A further 32 s of clear airflow was allowed for odour to be removed from the arena.

Conditioning trials were repeated 5 times for each individual bee with an inter-trial interval of 10 min. If a bee did not respond in any way during linalool delivery or electric shock then a 'failed response' was recorded. Bees that failed to respond more than once in conditioning (n = 16, 4.5% of 357) were excluded from statistical analyses. No bees exhibited a pre-learned aversive response to linalool in the first conditioning trial, and therefore no bees had to be excluded from analysis for this reason. After all exclusions described above were made 341 bees were left that completed the SER assay for inclusion in statistical analyses (Table 3).

Table 3. The number of bees in sting extension response analyses (after exclusions) for each hive and treatment

Treatment	Hive	Bees/Hive	Bees/Treatment
Control	1	39	113
	2	37	
	7	37	
100 µT	1	39	114
	2	38	
	7	37	
1000 µT	1	38	114
	2	36	
	7	40	

If a bee responded only after the shock stimulus then a non-conditioned sting extension response was recorded (i.e. the bee has responded to US but not CS). In previous aversive learning studies responses to the conditioned stimulus have been described only when a bee extends its sting during the CS application. Here this specific aversive response to the CS was defined as a 'sting extension response' (Fig. 39, A-B). Many bees responded to the CS by flexion of the abdomen such that they were aggressively primed to extend the sting, but without extending their stings (Fig. 40, A-B). This abdominal flexion response to the CS was defined as an 'abdominal flexion response'. The proportions of these conditioned sting extension and abdominal flexion responses over the 5 trials were analysed in detail to assess the effects of short-term ELF EMF exposure on aversive learning in honey bees.

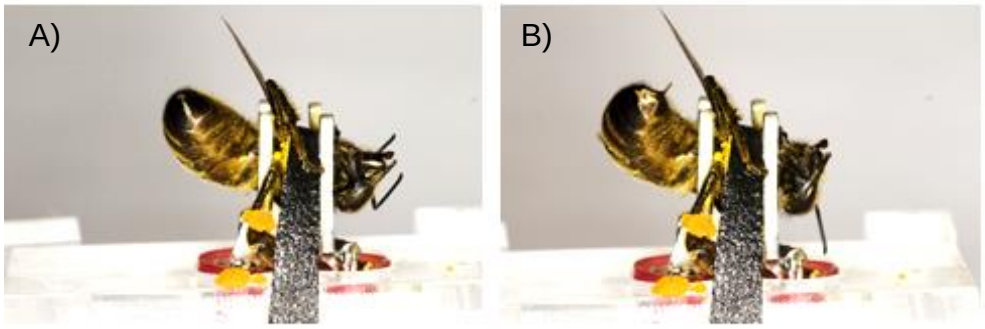

Figure 39. Example of a sting extension response. A) Honey bee at rest in a cradle. B) Aversive sting extension response to the CS in conditioning trials. Image taken by C. Jackson

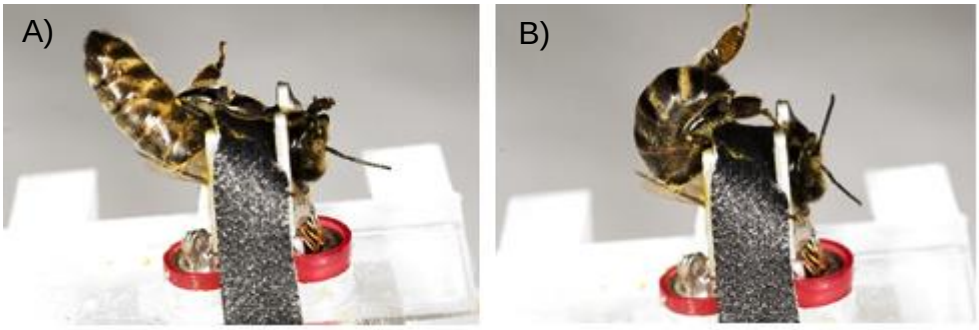

Figure 40. Example of an abdominal flexion respnose. A) Honey bee at rest in a cradle. B) Aversive abdominal flexion response, with no extension of sting, but flexion of abdomen in response to the CS in conditioning trials. Image taken by C. Jackson

3.3.1.6 PER Assay

3.3.1.6.1 Experimental set-up

To determine the effects of short-term ELF EMFs on olfactory appetitive learning acquisition, conditioning was conducted during the morning after a 17 hr primary treatment. In total, 10 conditioning trials were conducted under the described format for conditioning trials and following the PER assessment criteria, with a secondary 3 hr treatment occurring in the middle, between trials 5 and 6. There was therefore a primary phase of conditioning (trials 1-5) followed by a 3 hr secondary treatment and then a secondary phase of conditioning (trials 6-10) in the PER assay.

3.3.1.6.2 Conditioning trials

A PER arena with custom made odour delivery system and extractor to remove odour from experimental arena was used for this assay. 8 µl of 97% linalool (Sigma-Aldrich, UK) was pipetted onto filter paper that was placed in one of the airflow delivery channels and used as the CS. Bees were initially tested for gustatory responsiveness with a drop of 40% sucrose and bees that failed that test were excluded at this stage along with bees that did not survive overnight. During a conditioning trial bees were placed individually in the arena and allowed to adapt to the arena for 15 s before being presented with the CS for 10 s (Fig. 41). 5 s after the onset of the CS a 40% w/v sucrose solution reward (unconditioned stimulus (US)) was applied to an antenna. The bee was allowed to feed on the sucrose solution for 10 s (pairing CS and US for 5 s), given 30s clear airflow and then removed from the arena. A complete conditioning trial for a bee therefore lasted for 1 min. An optimal inter-trial interval for olfactory conditioning of 10 minutes (Menzel et al., 2001) was used for each bee in this assay.

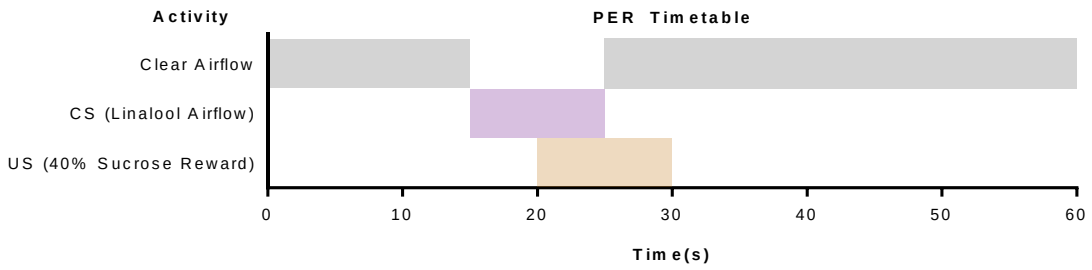

Figure 41. Protocol for a single conditioning trial in PER assays in short-term exposure experiments including application and pairing of CS and US.

3.3.1.6.3 PER criteria

A learned proboscis extension response was recorded if a bee extended its proboscis during the CS application but before presentation with the US (Fig. 42, C). If a bee did not extend its proboscis at any point during the linalool delivery or sucrose presentation (i.e. failed proboscis extension gustatory responsiveness) then 'no response' was recorded (Fig. 42, A). Bees that failed gustatory responsiveness were excluded from analysis. If a bee only extended its proboscis after the sugar reward (US) application began, then a 'gustatory response' was recorded (Fig. 42, B). Bees that exhibited a learned proboscis extension response to linalool in the first conditioning trial were excluded from analysis as learning cannot be recorded in bees that already respond to the CS. In total 579 bees survived overnight. Of these, 30 (5.2%) were excluded from trials for failing gustatory responsiveness and 16 (2.8%) for a pre-learned response to the CS. Therefore 533 bees were included in short-term exposure analyses.

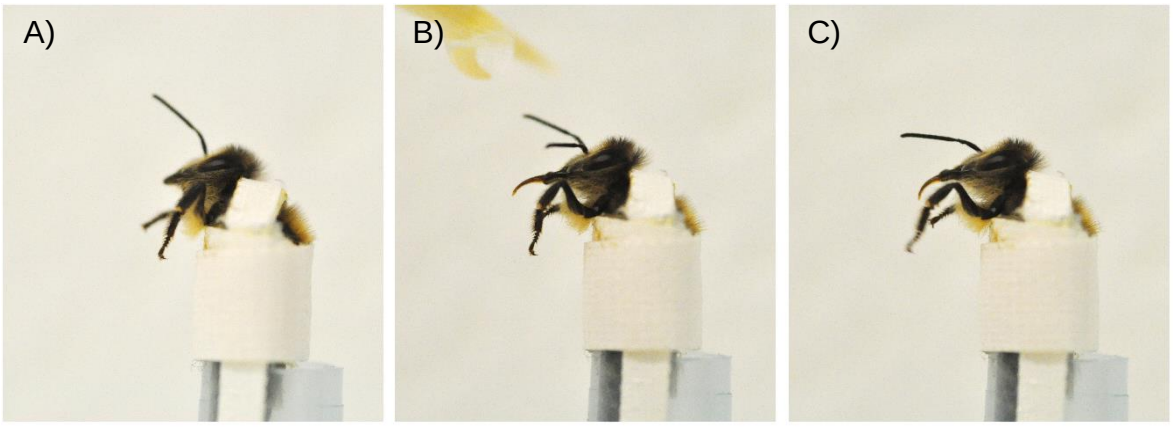

Figure 42. Criteria for responses in the PER assay. A) 'No response' recorded when bee did not extend its proboscis during both CS and US application i.e. failed gustatory responsiveness. B) 'Gustatory response' recorded when bee extended its proboscis but only after the US application began i.e. not a learned response to the CS. C) 'Proboscis extension response' recorded when bee extended its proboscis during the CS application and before the US application began i.e. a learned response to the CS

3.3.1.6.4 EMF exposure

Bees were exposed to a primary of either 100 µT or 7000 µT ELF EMF, or respective temperature controls, overnight for 17 hrs (Table 4) to determine short-term impacts of ELF EMFs on cognitive behaviour. Conditioning trials 1-5 were conducted the following morning to assess the effects of EMF exposure on learning acquisition. On completion of

these trials a secondary 3 hr exposure treatment was then applied, and was followed by 5 more conditioning trials (6-10) to determine the compounding effects of ELF EMFs on PER acquisition. Bees given the high control or 7000 µT EMF primary treatment, were exposed to a high control or 7000 µT EMF in the secondary treatment, while bees exposed to the low control or 100 µT EMF in the primary treatment were exposed to either low control or 100 µT EMF in the secondary treatment (Table 4).

Table 4. Numbers of bees exposed to 17 hr primary treatment groups and 3 hr secondary treatment groups in the analysis of the effects of ELF EMFs on appetitive learning

Primary treatment (17 hr)	**Bees (n)**	**Secondary treatment (3 hr)**	**Bees (n)**
High control	123	High control	58
		7000 µT	65
7000 µT	116	High control	57
		7000 µT	59
Low control	150	Low control	90
		100 µT	60
100 µT	144	Low control	67
		100 µT	77

3.3.2 Effects of acute ELF EMF exposure on appetitive learning

3.3.2.1 Magnetic fields

For assessment of the acute effects of 50 Hz EMFs on honey bee appetitive learning, the Helmholtz coil (2.3.3.1) was used (Fig. 43, A) to deliver ELF EMFs at 20 µT, 100 µT and 1000 µT (Fig. 43, B-D).

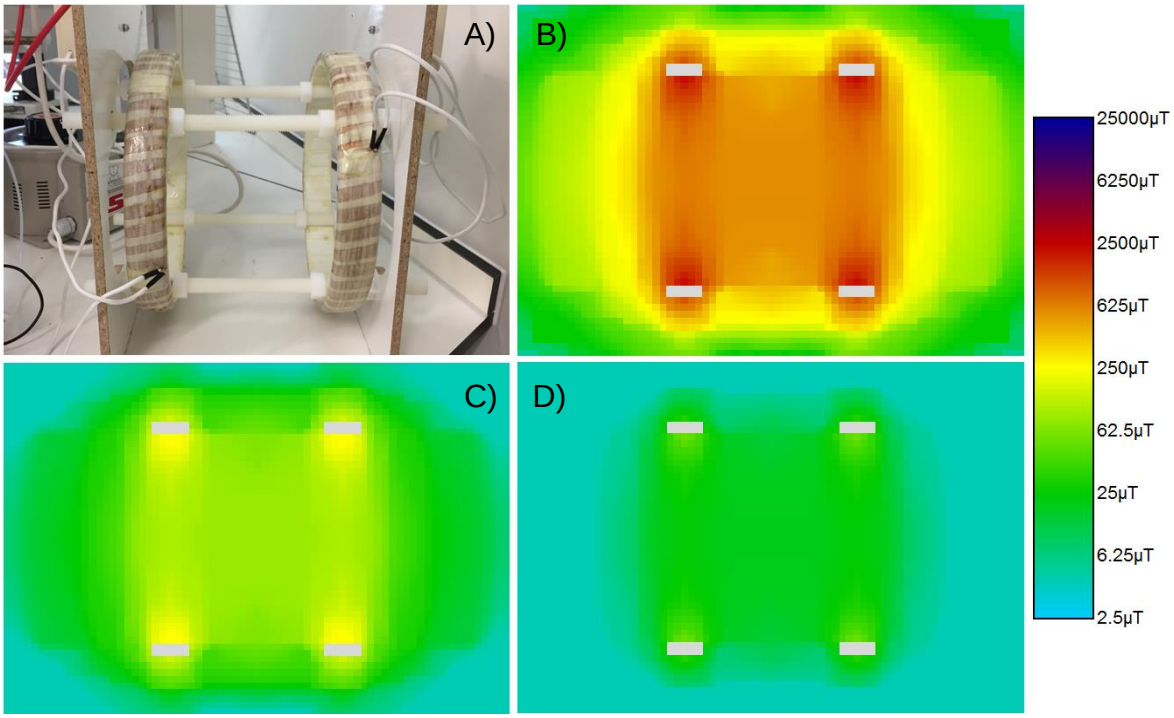

Figure 43. (A) Custom-made Helmholtz coil with solenoid electromagnets for generating homogenous 50 Hz sinusoidal AC electromagnetic fields. (B-D) Magnetic field mapping for recorded magnetic flux densities in lateral cross-section of the Helmholtz coil for (B) 1000 µT, (C) 100 µT and (D) 20 µT EMFs

3.3.2.2 Bee collection and harnessing

Bees were collected, harnessed in PER tubes, and fed to satiation following the same methods as bees for short-term exposure experiments. For acute exposure experiments bees were collected from 4 hives (Hive 4_{2015}, Hive 1_{2016}, Hive 2_{2016}, and Hive 3_{2016}; subscript is collection year). Bees were stored in perforated containers overnight at 29 ± 1ºC and PER assays were conducted the following day. In total 549 bees survived overnight from the 4 hives for acute exposure assessments. Of these 74 (13.5%) were excluded for failing gustatory responsiveness and 37 (6.7%) for a pre-learned response to the CS. 438 bees were, therefore, included in acute exposure analyses.

3.3.2.3 PER assay

For acute exposure experiments one phase of PER conditioning with 5 trials was conducted with the same format of conditioning trials as described previously, and the same criteria for PER and exclusions. Bees were given 5 conditioning trials and for each bee, 1 hr after the 5th conditioning trial, a single trial was conducted to assess PER retention.

3.3.2.4 EMF exposure

Bees were exposed to ELF EMFs for 1 min immediately following each of the 5 conditioning trials (Fig. 44). Bees were exposed to 3 different ELF EMF levels (20 µT, 100 µT, and 1000 µT) or control treatment. These levels represented those found in the natural environment at ground level below a powerline to those 1 m from a cable. Bees were exposed to EMFs by removing them from the PER arena, and placing them in the centre of the Helmholtz coil apparatus for one minute at the appropriate treatment setting. This simulated in the laboratory a realistic scenario of EMF exposure of bees in the field crossing an EMF boundary of an overhead powerline immediately after locating/returning to a food source.

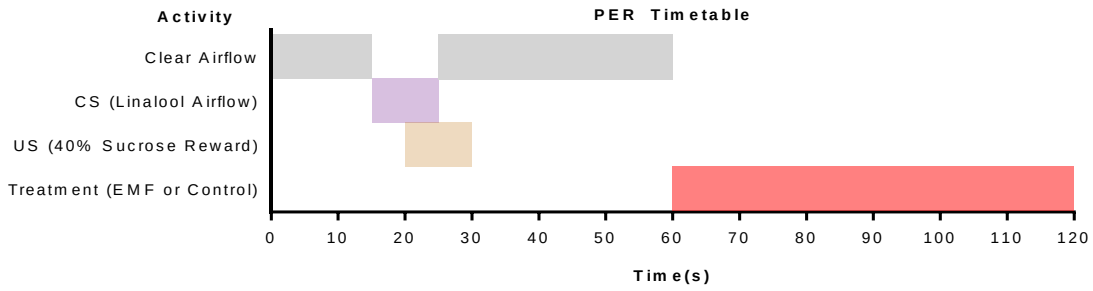

Figure 44. Protocol for acute exposure experiments with treatment (Control, 20 µT, 100 µT or 1000 µT) occurring for 1 min following each 1 min conditioning trial

3.3.3 Statistical analysis

Data were analysed in SPSS (v.24, IBM SPSS Inc.) and Graphpad Prism (v.7, Graph Pad Software Inc.). Where appropriate, homogeneity of variance and normality assumptions were tested. If required, data transformations were made, or alternative statistical tests, chosen. For all models assessing the impacts of treatments on binomial PER data, binomial error structure and logit link function were used, and where appropriate pairwise contrasts with Bonferroni adjusted significance were used in *post-hoc* analyses.

To analyse the effects of short-term ELF EMFs on stress protein levels data were first normalised. For data normalisation low control (22.2 ± 1ºC) or high control (29.3 ± 1ºC) 17 hr treatment effects on Hsp70 levels were compared. The low control Hsp70 signal was 0.036 ± 0.013 fluorescence units and the high control Hsp70 signal was 0.031 ± 0.010 fluorescence units. There was no significant difference between the Hsp70 signal between high and low control treatments (paired t-test, t = 0.628, d.f. = 7, P = 0.56). To allow for better data normalisation, all Hsp70 signals were normalised to the average signal between low and high control for each western-blot, described as the 'overall control signal'. For analysis of low-level treatments (low control, 20 µT, 100 µT and 1000 µT) a one-way repeated-measures ANOVA was used, matching recorded Hsp70 signals from the same blot as repeated measures, to determine the effects of different ELF EMF treatments *vs.* low control treatment, on Hsp70 levels relative to the overall control signal. For analysis of high-level treatments (high control and 7000 µT) a paired t-test was used to determine the effect of 7000 µT ELF EMF exposure *vs.* high control exposure on Hsp70 levels relative to the overall control signal.

In statistical anlysis of the short-term effects of ELF EMFs on aversive learning, to determine whether ELF EMF exposure or 'hive or origin' affected the initial aversive responsiveness of bees a generalized linear model (GLM) with logit link function was used with 'EMF treatment' and 'hive of origin' as interacting factors. To determine if the abdominal flexion response was produced in response to the CS a generalized mixed effect model (GLMM) was used to determine whether 'conditioning trial' as a factor affected the proportion of abdominal flexion responses. To analyse the effect of ELF EMF exposure on primary responses, abdominal flexion responses, and total responses (primary and abdominal flexion responses combined), GLMMs were use with 'EMF treatment', 'hive of origin', and 'conditioning trial' as interacting factors. For GLMMs considering the effects of ELF EMF exposure on aversive learning acquisition trial 1 was not included in analysis (i.e. trials 2-5 were used), as learning cannot occur in the first trial, as well as to improve model fit. In all GLMs and GLMMs of SER data binomial error structure with logit link function was used. Where appropriate pairwise contrasts with Bonferroni adjusted significance were used in *post-hoc* analysis of significant effects.

To determine the effect of short-term ELF EMF exposure on appetitive learning, generalized mixed effect models (GLMM) were used to analyse the effects of primary treatment on learning acquisition in trials 1-5 and secondary treatment on learning acquisition in trials 6-10. In the analysis of the impacts of primary treatment on learning

acquisition, analyses were separated by temperature (7000 µT *vs.* High control, and 100 µT *vs.* Low control). 'Primary treatment' was considered as a main factor, with 'trial number' as a repeated interactive factor. In the analysis of secondary treatment, analyses were also separated by high and low temperature settings. 'Secondary treatment' was a main factor and was nested within 'primary treatment'. 'Trial number (6-10)' was considered as a repeated interactive factor. 'Bee ID' was considered as a random effect.

To determine the effect of acute ELF EMF exposure on appetitive learning a GLMM was used to analyse the effects of EMF treatment on learning acquisition (PER), including 'hive of origin' and 'trial number' as interactive factors, while generalized linear models (GLM) were used for the analysis of final learning level and 1 hr post conditioning levels, comparing 'EMF exposure' and 'hive of origin' interactions. To determine the effects of EMFs on memory the proportion of PER responses in the final learning trial were compared to the proportion of PER responses in the PER retention trial within each treatment group, using a pairwise repeated samples McNemar test.

3.4 Results

3.4.1 Short-term exposure

3.4.1.1 Stress protein levels

3.4.1.1.1 High-level treatments

Analysis of the effects of high-level treatments of ELF EMF (high control and 7000 µT) on stress protein levels, Hsp70 levels relative to overall control were higher for bees exposed to 7000 µT ELF EMFs compared to temperature matched high controls. 17 hr high control exposure generated an Hsp70 signal of 0.85 ± 0.11 relative to controls, whereas 7000 µT exposure generated an Hsp70 signal of 1.43 ± 0.29 relative to control (Fig. 45, A-B). Exposure to 7000 µT EMF significantly increased Hsp70 levels in honey bee brains from levels generated in temperature matched controls (paired t-test, $t = 2.45$, d.f. = 6, $P = 0.0495$).

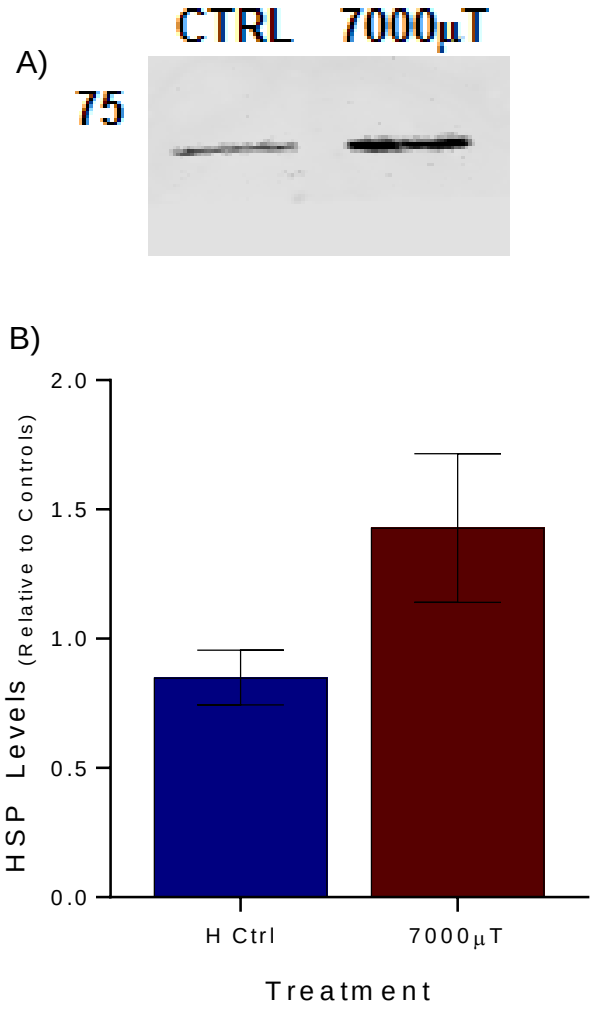

Figure 45. Effect of exposure to high-levels of EMF (high control, 7000 µT) on Hsp70 levels. A) Example western-blot of Hsp70 signal for high-level treatments. B) Measured Hsp70 levels relative to overall control from Western blot. Mean and SEM are plotted (arbitrary units).

3.4.1.1.2 Low-level treatments

Analysis of the effects of low-level exposure (low control, 20 µT, 100 µT and 1000 µT) on stress protein levels, showed that Hsp70 levels were consistent across all low-level treatments. 17 hr low control exposure generated an Hsp70 signal of 1.15 ± 0.11 (mean ± SEM) relative to controls, whereas 20 µT exposure generated an Hsp70 signal of 1.31 ± 0.17 relative to controls, 100 µT generated an Hsp70 signal of 1.22 ± 0.17 relative to controls, and 1000 µT generated an Hsp70 signal of 1.27 ± 0.25 relative to controls (Fig. 46, A-B). Low-level EMF exposure did not significantly affect Hsp70 levels in honey bee brains (One-way RM ANOVA, $F_{1.974,11.85} = 0.29$, $P = 0.75$).

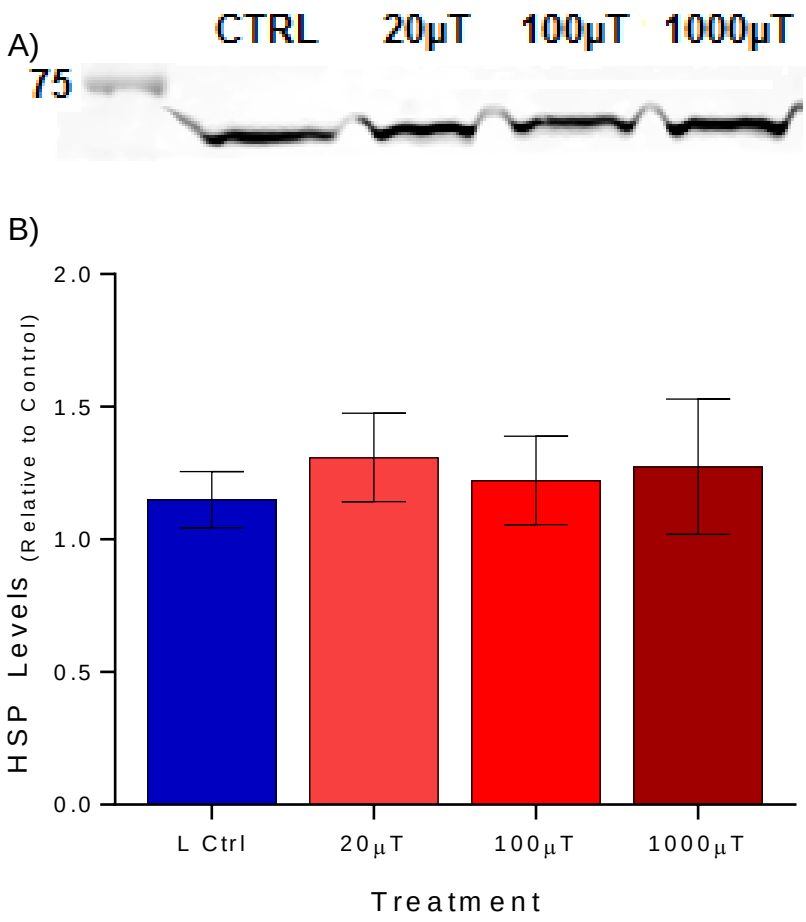

Figure 46. Effect of low-level ELF EMF exposure on Hsp70 levels in honey bee brains. A) Example western-blot showing Hsp70 signal for low-level treatments (low control, 20 µT, 100 µT and 1000 µT). B) Hsp70 levels relative to overall control levels. Mean and SEM are plotted (arbitrary units).

3.4.1.2 Sting extension response

3.4.1.2.1 Effect of ELF EMFs on initial aversive responsiveness

To determine if there were any effects of short-term exposure (Control, 100 µT, or 1000 µT) on the ability of bees to aversively respond with a full extension of the sting, the proportions of sting extensions between each treatment were compared. After 17 hr control exposure 95.0% of bees exhibited aversive responsiveness, whereas for 100 µT exposed bees 96.6% exhibited aversive responsiveness. For bees exposed to a 1000 µT EMF 95.0% of bees exhibited aversive responsiveness (Fig. 47). Thus there was no 17 hr short-term 'treatment' * 'hive of origin' interaction (GLM, χ^2<0.001, d.f. = 4, P > 0.99) effect on aversive responsiveness, nor were there any main effects of 'treatment' (GLM, χ^2<0.001, d.f. = 2, P > 0.99) or 'hive of origin' (GLM, χ^2<0.001, d.f. = 2, P > 0.99) on aversive responsiveness.

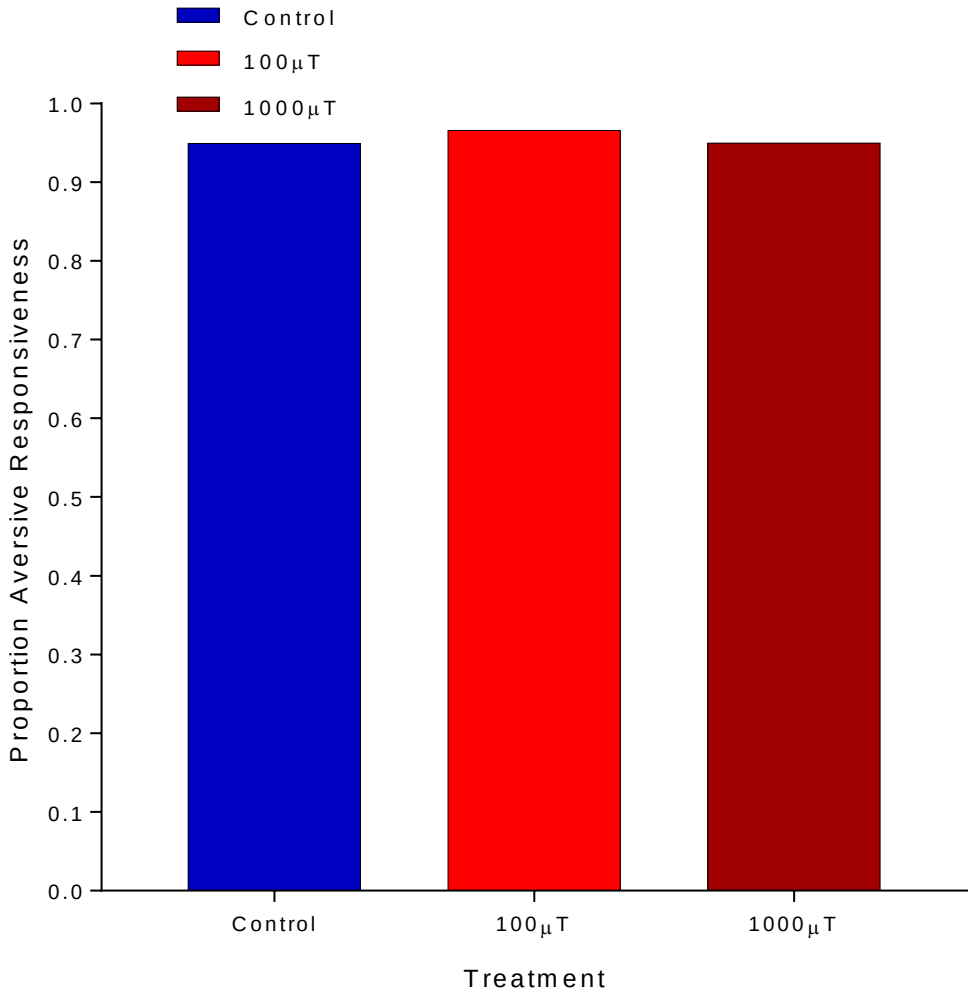

Figure 47. The effect of ELF EMF treatment on the proportion of aversive responsiveness to 12 V electric shock aversive stimuli. Exact proportions are plotted.

3.4.1.2.2 Abdominal flexion responses to conditioned stimuli are learned

Many bees exhibited an abdominal flexion response to the CS in SER trials that has been previously unreported in aversive learning literature, despite this being a well-known behaviour in priming for sting extension (Ogawa, 1995). To test whether bees could learn to produce abdominal flexion responses the impact of trial number on the proportion of abdominal flexion responses to the CS was measured. Only control exposed bees were assessed at this stage to remove any confounding effects of treatment, meaning that 113 bees were assessed. In trial 1, as the CS (linalool) and US (electric shock) have not yet been paired, it is expected that if abdominal flexion responses are learned, then bees would not exhibit this response during this trial, and of the 113 bees 0 (0.0%) exhibited abdominal flexion responses in trial 1 (Fig. 48). Furthermore it would be expected that if abdominal flexion responses are learned aversive responses to the CS, then in each of the proceeding trials (as the CS and US begin to become associated) the number of abdominal flexion

responses to the CS would increase as bees begin associate the CS with the US. In trial 2, 4 bees (3.5%) exhibited abdominal flexion responses, trial 3 11 (9.7%), trial 4 12 (10.6%), and in trial 5 20 (17.7%) (Fig. 48). Increasing trial number caused the number of abdominal flexion responses to increase significantly (GLMM, $F_{5,560}$ = 78,192,935, P < 0.0001) showing that these abdominal flexion responses are a learned response in association with the negative CS in the SER assay.

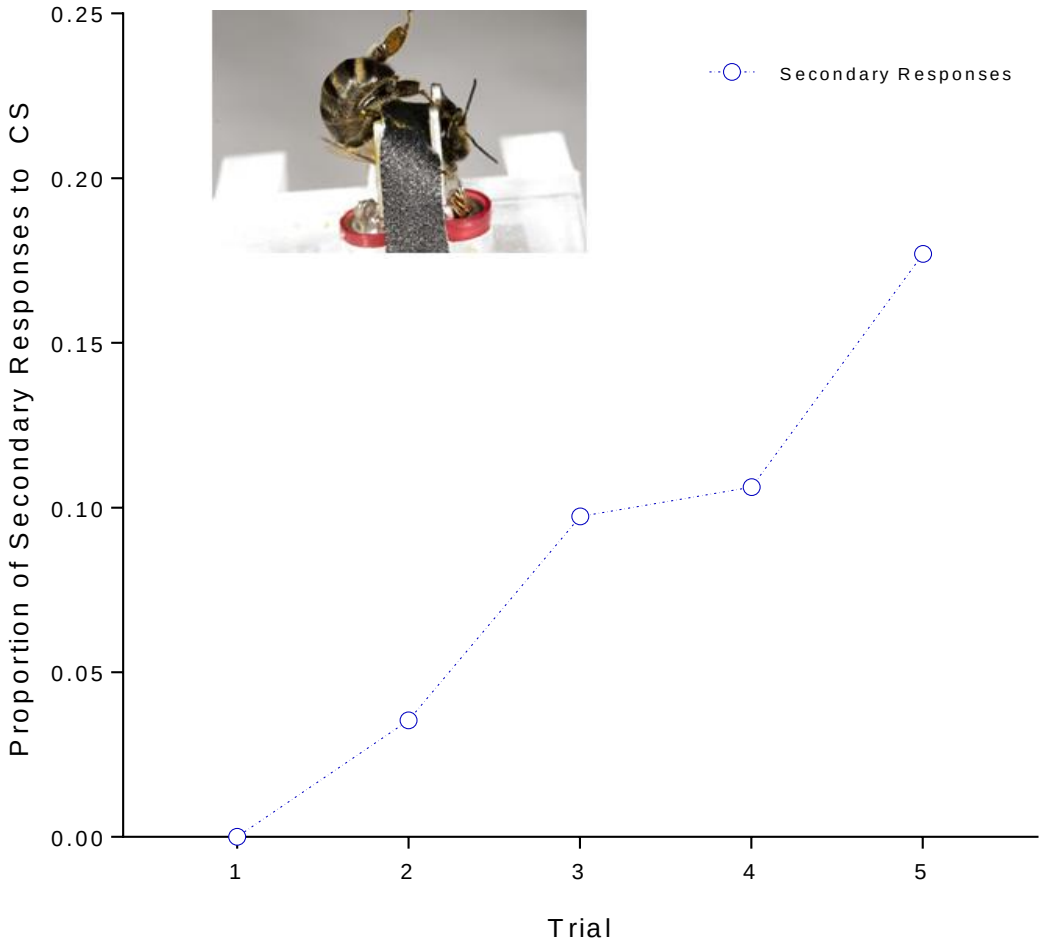

Figure 48. Proportion of bees producing abdominal flexion in response to the conditioned stimulus (linalool) for each of the five trials (1-5). Inset shows a photograph of a bee exhibiting abdominal flexion. The exact proportion of responses is plotted.

3.4.1.2.3 Effect of short-term EMF exposure on total aversive responses

As abdominal flexion responses to the CS have not been reported before with the SER assay, these responses were analysed separately from primary responses to ensure comparability of data and to determine the effect of ELF EMF exposure on sting extension responses. Abdominal flexion responses to the CS were also analysed together with primary sting responses (as total aversive responses to CS) to determine the overall effect of EMF exposure on aversive learning in honey bees. Hive of origin was considered as a factor in early model stages but was found to have no significant effect on aversive learning (GLMM, $F_{2,1328} = 0.17$, $P = 0.84$) nor any interaction with 'treatment' (GLMM, $F_{4,1328} = 1.38$, $P = 0.24$) 'conditioning trial' (GLMM, $F_{6,1328} = 0.24$, $P = 0.96$) or three-way interaction (GLMM, $F_{12,1328} = 0.33$, $P = 0.99$). Hive of origin was consequentially removed from the mixed model to improve model fit in subsequent analyses.

For all treatments (Control, 100 µT and 1000 µT) the proportion of aversive responses to the CS increased in every trial (Fig. 49) showing a clear impact of repeated conditioning trials on aversive learning (GLMM, $F_{3,1352} = 26.08$, $P < 0.0001$). 29% of control bees exhibited aversive learning after trial 3 and 50% by trial 5 (Fig. 49). In contrast, following 100 µT exposure 12% of bees exhibited aversive learning after trial 3 and 32% after trial 5. Following exposure to 1000 µT 19% of bees exhibited aversive after trial 3 and 27% after trial 5 (Fig. 49). EMF treatments were found to significantly reduce the number of total aversive responses to the conditioned stimulus in honey bees (GLMM, $F_{2,1352} = 15.01$, $P < 0.0001$). A significantly greater proportion of control bees exhibited aversive learning than both 1000 µT (Pairwise comparison, Bonferroni adjusted $P < 0.001$) and 100 µT (Pairwise comparison, Bonferroni adjusted $P < 0.001$) exposed bees. There was no 'treatment' * 'trial' interaction (GLMM, $F_{1,1352} = 0.82$, $P = 0.56$).

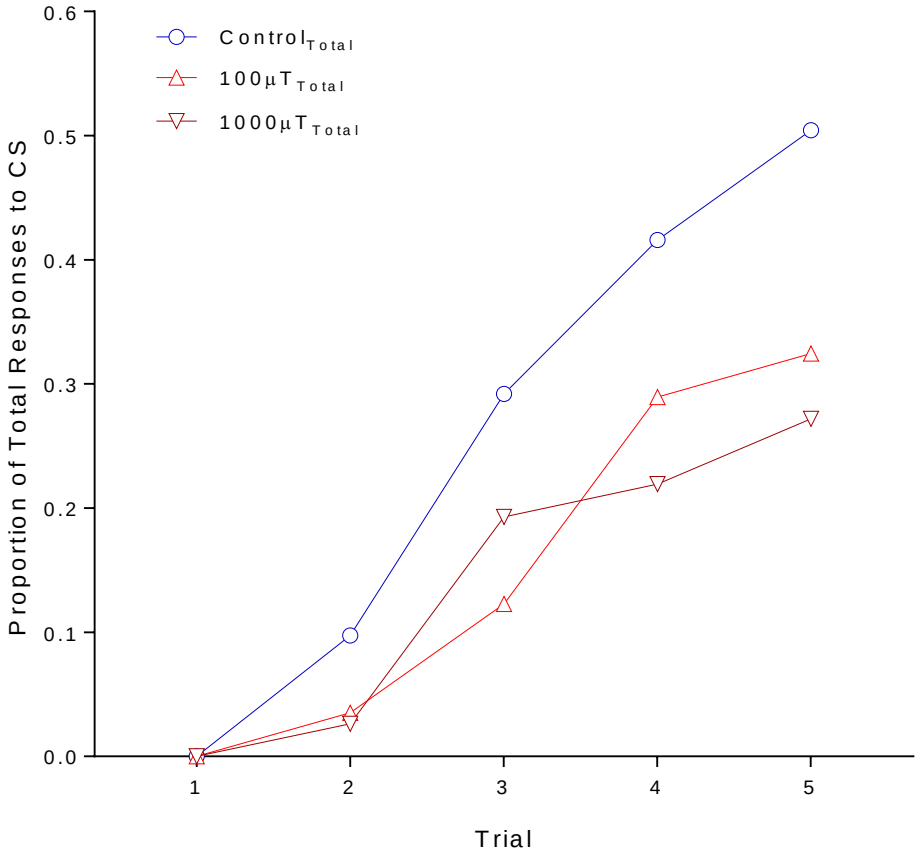

Figure 49. Effect of short-term ELF EMF exposure on the proportion of total aversive responses (primary sting extension responses and abdominal flexion responses) to the conditioned stimulus (linalool) for each of the trials. The exact proportion of responses is plotted.

3.4.1.2.4 Effect of short-term EMF exposure on primary responses

In an analysis of the effects of ELF EMF exposure on primary sting extension responses hive of origin was again removed as a factor to improve model fit, as it was found to have no effect on the proportion of SER to the CS (GLMM, $F_{2,1328} = 0.32$, $P = 0.73$) nor any interaction with 'treatment' (GLMM, $F_{4,1328} = 1.18$, $P = 0.32$) 'conditioning trial' (GLMM, $F_{6,1328} = 0.21$, $P = 0.97$) or three-way interaction (GLMM, $F_{12,1328} = 0.78$, $P = 0.70$).

For all treatments the proportion of bees exhibiting SER increased in every conditioning trial (GLMM, $F_{3,1352} = 14.738$, $P < 0.0001$). 19% of control bees showed SER after trial 3 and 33% after trial 5 (Fig. 50). In contrast, after bees were exposed to 100 µT only 9% of bees showed SER after trial 3 and 19% after trial 5. Following exposure to a 1000 µT ELF EMF 11% showed an SER after trial 3 and 17% after trial 5 (Fig. 50). EMF treatments were found to significantly reduce the proportions of SER in honey bees (GLMM,

$F_{2,1352}$ = 10.24, P < 0.0001). A significantly greater proportion of control exposed bees exhibited SER than both 1000 µT (Pairwise comparison, Bonferroni adjusted P < 0.001) and 100 µT (Pairwise comparison, Bonferroni adjusted P = 0.001) exposed bees. There was no *'treatment'* * *'trial'* interaction (GLMM, $F_{1,1352}$ = 0.25, P = 0.96).

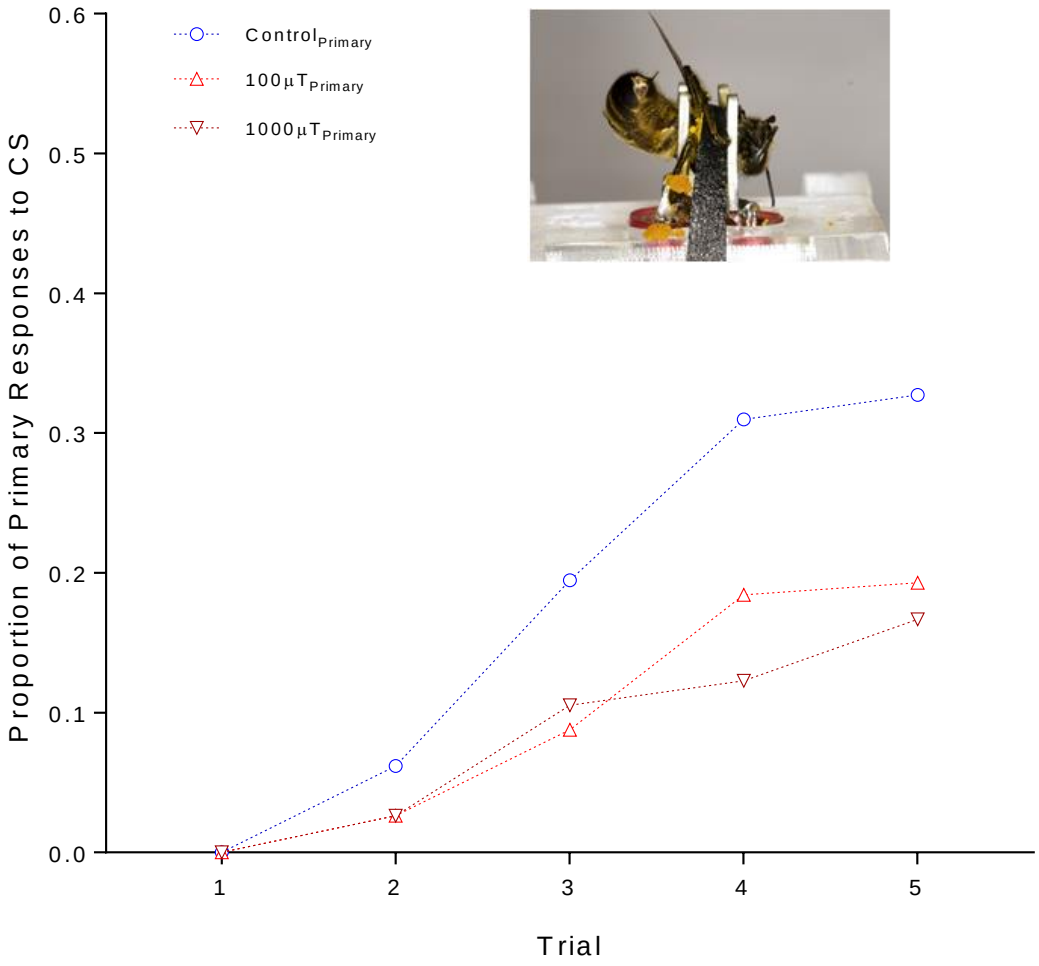

Figure 50. Effect of short-term ELF EMF exposure on the proportion of primary aversive responses (sting extension responses) to the conditioned stimulus (linalool) for each of the five trials in the SER assay. Inset shows photograph of sting extension. The exact proportion of responses is plotted.

3.4.1.2.5 Effect of short-term EMF exposure on abdominal flexion responses

The effects of short-term exposure of EMFs on abdominal flexion responses to the CS were also analysed separately. Hive of origin was removed as a factor in analysis to improve model fit, as it was found to have no effect on the proportion of abdominal flexion responses to the CS (GLMM, $F_{2,1328}$ = 0.17, P = 0.84) nor any interaction with 'treatment' (GLMM,

$F_{4,1328}$ = 1.39, P = 0.24) 'conditioning trial' (GLMM, $F_{6,1328}$ = 0.24, P = 0.96) or three-way interaction (GLMM, $F_{12,1328}$ = 0.33, P = 0.99).

The proportion of bees showing this abdominal flexion response increased significantly with the number of conditioning trials (Fig. 51) as bees associated the CS with the US (GLMM, $F_{3,1352}$= 2.78, P=0.04). 10% of control bees exhibited abdominal flexion responses after trial 3 while 18% of bees showed abdominal flexion after trial 5 (Fig. 51). In contrast, in bees exposed to 100 µT EMFs 4% showed abdominal flexion after trial 3 and 13% after trial 5. In bees exposed to 1000 µT EMF 9% showed abdominal flexion after trial 3 and 11% after trial 5 (Fig. 51). The reduction in the proportion of abdominal flexion responses after ELF EMF exposure approached significance (GLMM, $F_{2,1352}$ = 2.79, P = 0.06). There was no *'treatment'* * *'trial'* interaction (GLMM, $F_{1,1352}$ = 0.86, P = 0.53).

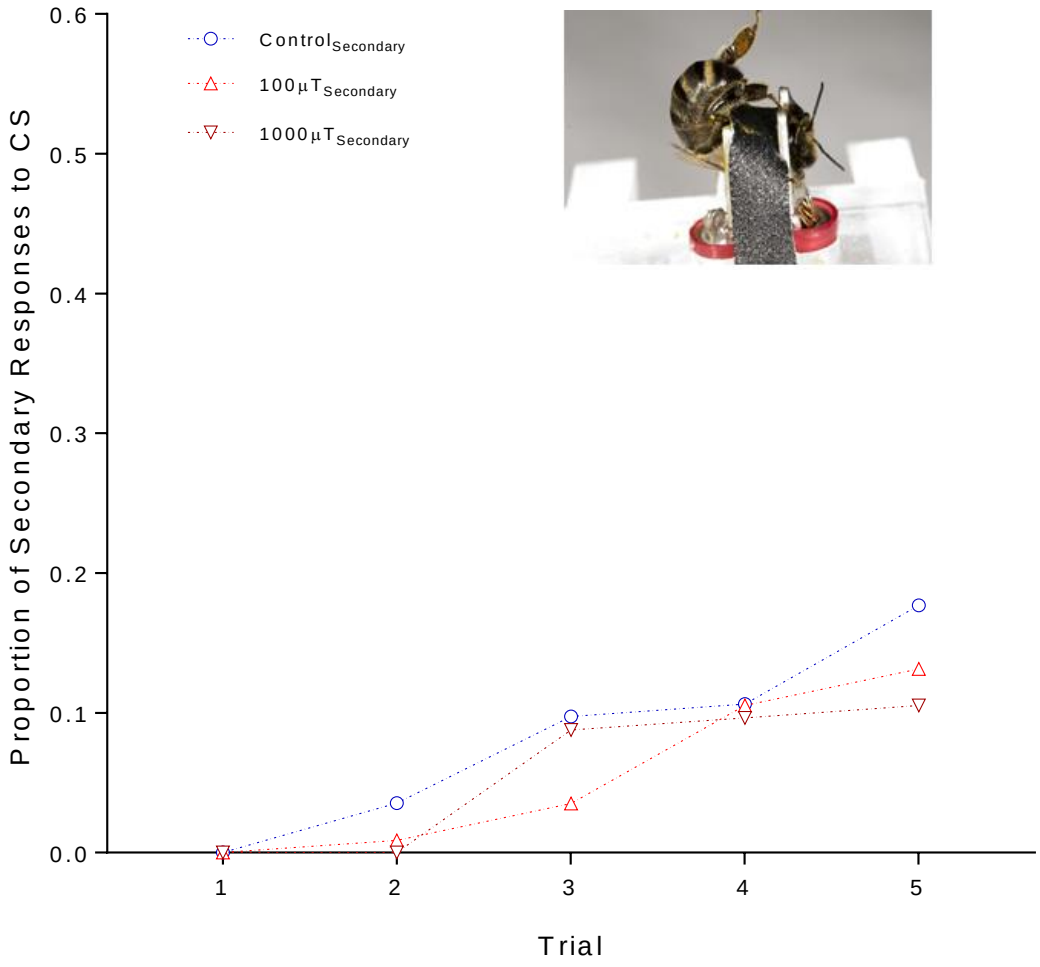

Figure 51. Effect of short-term ELF EMF exposure on the proportion of abdominal flexion responses to the conditioned stimulus (linalool) for each of five trials in the SER assay. Inset shows photograph of abdominal flexion response. The exact proportion of responses is plotted.

3.4.1.3 PER Assay

3.4.1.3.1 High-level treatments

3.4.1.3.1.1 Primary treatment

To determine the primary effect of a high ELF EMF exposure on PER acquisition, learning acquisition was measured after overnight (17 hr) control or 7000 µT EMF exposure. Between control and 7000 µT treatment, learning acquisition in trials 1-5 was similar (Fig. 52). As trial number increased more bees responded to the CS with PER (GLMM, $F = 11.79$, d.f. = 3, 948, $P < 0.001$). By the 3rd conditioning trial control bees and 7000 µT exposed bees had equal acquisition of 54%. By the end of primary conditioning control bees exhibited slightly higher acquisition of 64% versus 59% in 7000 µT exposed bees. Statistical analysis showed that there was no significant effect of short term 7000 µT EMF treatment on conditioning in trials 1-5 (GLMM, $F = 2.00$, d.f. = 1, 948, $P = 0.16$) nor any *'trial' x 'primary treatment'* interaction (GLMM, $F = 0.27$, d.f. = 3, 948, $P = 0.85$).

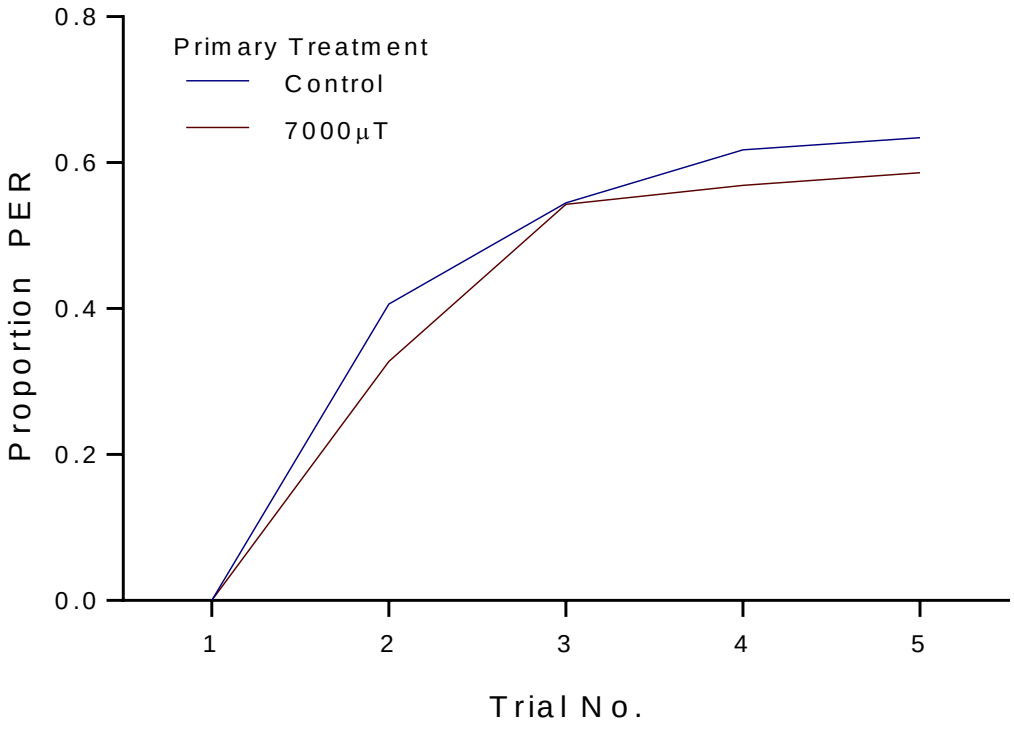

Figure 52. Effect of 17 hr 7000 µT ELF EMF exposure on PER acquisition. For control and treated bees the proportion of bees exhibiting PER increased with conditioning trial.

3.4.1.3.1.2 Secondary treatment

Secondary EMF treatments (3 hr) were applied to determine the impact of an interference with continued acquisition of the CS in trials 6-10, and further understand of the effects of ELF EMFs on cognitive behaviour. 7000 µT EMFs had differential effects on continued acquisition through trials 6-10. Control bees that were not exposed to EMF overnight, nor in the 3 hr secondary treatment period, retained acquisition at 58% following secondary treatment (trial 6) and had a final learning level (trial 10) of 68%. For bees that were not exposed to EMF overnight, but were exposed to a secondary treatment of 7000 µT EMF for 3 hrs, retained acquisition at 54% and had a final learning level (trial 10) of 66% (Fig. 53, A). In contrast, for bees that were exposed to a primary treatment of 7000 µT EMF for 17 hr and held under control conditions as a secondary treatment for 3 hr retained acquisition at 58% in trial 6 and had the highest final learning level of all combinations of treatment of 74% (Fig. 53, B). Finally, bees exposed to 7000 µT EMF for primary and secondary treatments retained acquisition at only 46% after the primary treatment and had the lowest final learning level of 47%.

There was a significant *'primary treatment' x 'secondary treatment'* interaction effect on learning, (GLMM, F = 5.80, d.f. = 1, 1175, P = 0.016) i.e. the primary 17 hr exposure impacted how the secondary treatment would affect learning in trials 6-10. Bees exposed to 7000 µT EMF for a 17 hr primary treatment and the 3 hr secondary treatment exhibited lower learning levels than other treatment permutations (Bonferroni adjusted P = 0.019). There was no 3-way *'primary treatment' x 'secondary treatment' x 'trial'* interaction effect on learning (GLMM, F = 0.60, d.f. = 4, 1175, P = 0.66). There was no increase of learning with trials 6-10 (GLMM, F = 1.94, d.f. = 4, 1175, P = 0.10) or *'trial' x 'primary treatment'* interaction effect on learning in trials 6-10 (GLMM, F = 0.40, d.f. = 4, 1175, P = 0.81) nor a *'trial' x 'secondary treatment'* effect on learning in trials 6-10 (GLMM, F = 0.11, d.f. = 4, 1175, P = 0.98)

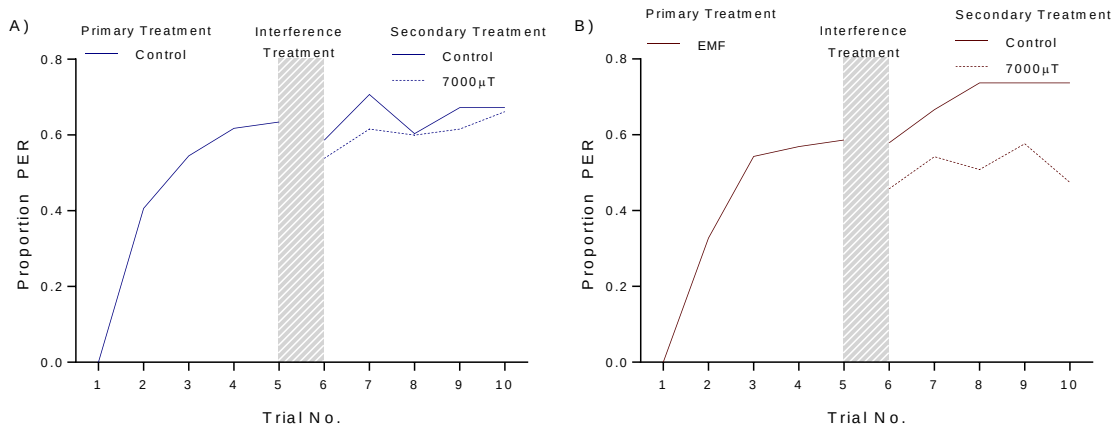

Figure 53. The effect of secondary 3 hr 7000 µT treatments in between trials 5 and 6 on PER acquisition for bees pre-treated for 17 hr under control and 7000 µT conditions. Data duplicated from Fig. 52 to clearly show initial effect, and then secondary effect.

3.4.1.3.2 Low-level treatments

3.4.1.3.2.1 Primary treatment

Bees that were not exposed to EMF for 17 hr had a higher learning acquisition in trials 1-5 than bees exposed to 100 µT EMF for 17 hr (Fig. 54). By the 3rd conditioning trial 58% of control bees responded to the CS while 53% of bees exposed to 100 µT EMF responded. By the end of primary conditioning (trial 5) 72% of control bees responded to the CS whilst 66% of bees exposed to 100 µT EMF responded. Seventeen hour exposure to 100 µT EMF significantly reduced learning responses in trials 2-5 (GLMM, F = 4.58, d.f. = 1, 1168, P 0.033). As trials progressed learning significantly increased with trial number (GLMM, F = 11.38, d.f. = 3, 1168, P < 0.001). There was no *'trial' x 'primary treatment'* interaction effect of learning in trials 2-5 (GLMM, F = 0.029, d.f. = 3, 1168, P = 0.99).

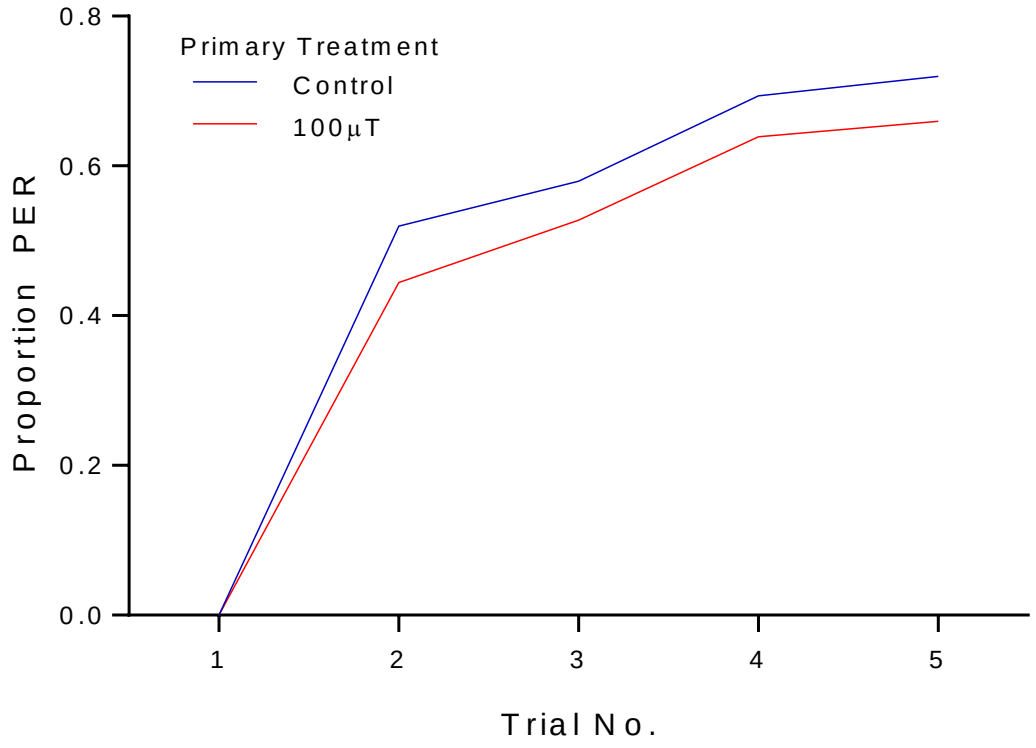

Figure 54. Effect of 17 hr 100 µT ELF EMF exposure on PER acquisition. For control and treated bees the proportion of bees exhibiting PER increased with conditioning trial.

3.4.1.3.2.2 Secondary treatment

Secondary treatments of a 3 hr exposure to 100 µT EMF were applied, and learning acquisition measured in trials 6-10. Control bees that were not exposed to EMF for both the primary and secondary treatments retained acquisition (trial 6) at 62% and had a final learning level (trial 10) of 79%. By comparison, bees exposed to a primary treatment of 17 hr and a secondary treatment of 100 µT EMF for 3 hrs had poorer retention (trial 6) at 53% and had a lower final learning level (trial 10) of 67% (Fig. 55, A). Bees that were exposed to a 100 µT EMF for 17 hr and no exposure during the secondary treatment between trial 5 and 6 retained acquisition at only 46% in trial 6 but recovered in secondary conditioning to 72% in trial 10. Bees exposed to 100 µT EMF repeat treatments retained learning at 60% and exhibited a final learning level of 72% (Fig. 55, B).

For bees exposed to 100 µT EMF in primary treatments there was a significant *'primary treatment' x 'secondary treatment'* interaction between the 17 hr treatment bees were exposed to, and the effect of the secondary 3 hr treatment on learning (GLMM, F = 6.22, d.f. = 1, 1175, P = 0.013). Bees given an 17 hr control treatment had significantly lower

learning in trials 6-10 when exposed to a 100 µT EMF as a secondary 3 hr treatment (Bonferroni adjusted P < 0.001). There was no significant 3-way *'primary treatment' x 'secondary treatment' x 'trial'* interaction effect on learning in trials 6-10 (GLMM, $F = 0.53$, d.f. = 4, 1450, P = 0.71). Learning did increase with trial number in trials 6-10 (GLMM, $F = 5.89$, d.f. = 4, 1450, P < 0.001) but there was no *'trial' x 'primary treatment'* interaction effect (GLMM, $F = 0.50$, d.f. = 4, 1450, P = 0.74) nor a *'trial' x 'secondary treatment'* interaction effect on learning in trials 6-10 (GLMM, $F = 0.62$, d.f. = 4, 1450, P = 0.65).

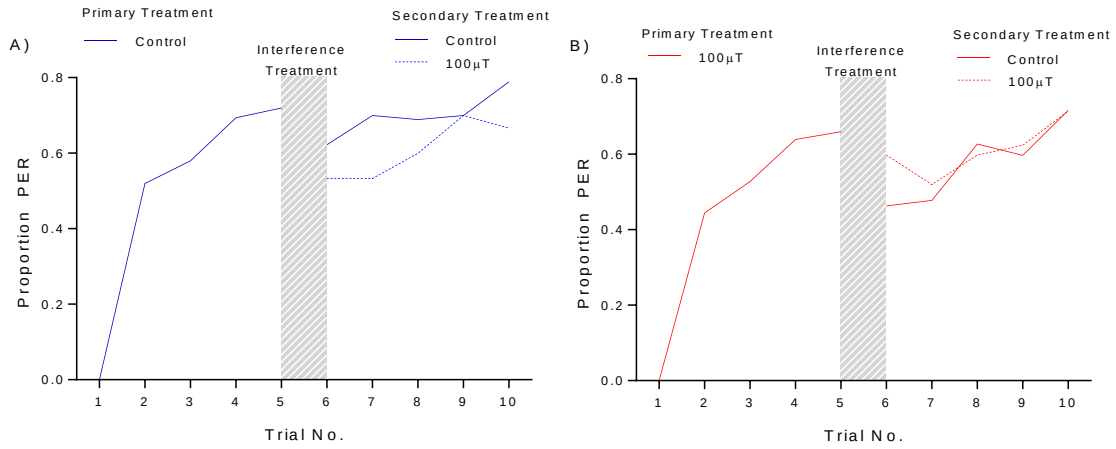

Figure 55. The effect of secondary 3 hr 100 µT treatments in between trials 5 and 6 on PER acquisition for bees pre-treated for 17 hr under control and 100 µT conditions. Data duplicated from Fig. 54 to clearly show initial effect, and then secondary effect.

3.4.2 Acute exposure

The effects of acute exposure of bees to ELF EMF were carried out in order to begin to understand how bees could be impacted by EMFs in the field. Moreover, for flying bees which can fly as high as the transmission line conductors, there is a greater chance they can encounter higher levels of EMF compared to bees held in hives at ground level. Both control and treated bees learned the CS as the proportion of bees exhibiting PER increased with trial number. Control bees showed the highest levels of PER, compared to bees exposed to EMFs for 1 min following each conditioning trial (at 20, 100 and 1000 µT exposures) (Fig. 56). Control bees reached a peak of 77% learning in trial 4 and attained a final level of 73% PER during trial 5. Bees exposed to 20 µT EMF showed similar levels of learning acquisition to control bees for trials 2-3, reaching 68% PER in trial 3. During trials 4 and 5, however, these bees exhibited lower levels of PER compared to control, of 59% and 63% respectively. Bees exposed to 100 µT and 1000 µT EMFs after each conditioning trial exhibited lower levels of PER than control or 20 µT exposed bees for all trials (Fig. 56). Bees exposed to 100 µT

EMF reached a peak learning level of 43% in trial 3 and a final learning level of 42%. Bees exposed to 1000 µT EMFs had a peak learning level of 51% in trial 3, and the lowest final learning level of all treatments at 36%.

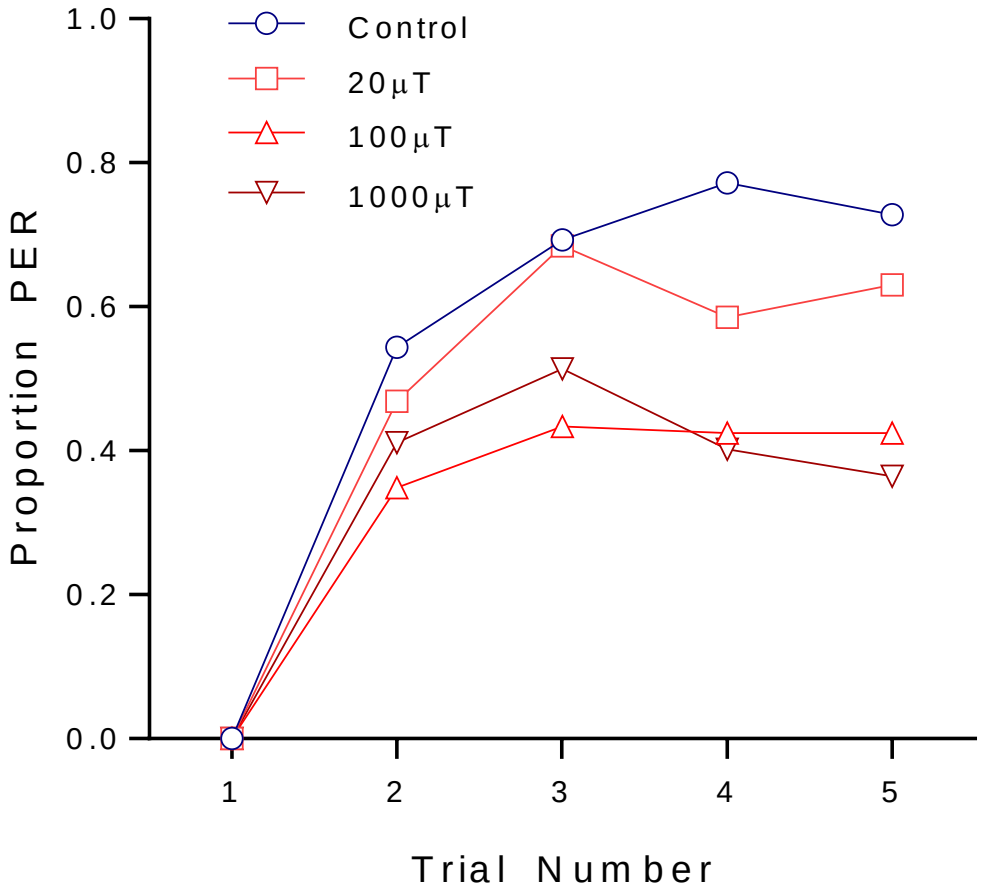

Figure 56. The effects of acute EMF exposure on PER. PER acquisition over 5 conditioning trials following exposure to differing levels of ELF electromagnetic fields or control treatment. The exact proportions of bees that exhibited PER (response to the CS before US reward) are plotted.

A GLMM was used to compare the effects of EMF exposure on learning acquisition. There was no significant three-way *'EMF' x 'hive of origin' x 'trial'* interaction effect on learning acquisition (GLMM, F = 0.56, d.f. = 27, 1688, P = 0.97). Similarly, there was no significant two-way *'hive of origin' x 'trial'* interaction effect on learning acquisition (GLMM, F=0.68, d.f. = 9, 1688, P = 0.72) or '*EMF' x 'trial'* interaction effect (GLMM, F = 1.76, d.f. = 9,1688, P = 0.07) on learning acquisition.

There was also a significant *'EMF' x 'hive of origin'* interaction effect on learning (GLMM F = 3.68, d.f. = 9, 1688, P < 0.001) with all hives having reduced learning

acquisition following EMF exposure (Bonferroni: $P < 0.001$) apart from hive 2. The reduced learning acquisition following EMF exposure of bees from hive 2, however, approached significance (Bonferroni: $P = 0.052$).

EMF exposure for 1 min following each conditioning trial significantly reduced learning acquisition across trials 2-5 (GLMM, $F = 27.97$, d.f. = 3,1688, $P < 0.001$) with control exposed bees acquiring significantly higher levels of learning than all EMF exposures across the four trials (54 - 77% acquisition, Bonferroni: $P < 0.001$). Bees exposed to a 20 µT EMF attained lower learning than control bees but significantly higher learning acquisition than 100 µT and 1000 µT exposed bees (47 - 68% acquisition, Bonferroni: $P < 0.001$). Learning acquisition did not differ significantly between 100 µT and 1000 µT exposed bees (100 µT: 35-43%; 1000 µT: 36-51% acquisition, Bonferroni: $P = 0.29$). These results show that acute EMF exposure during conditioning impacts on the cognitive abilities of bees by reducing olfactory learning acquisition, and that the magnitude of the effect was dependent on magnetic flux density.

EMF exposure in the one minute period following each conditioning trial significantly reduced the final learning level (after 5 trials) acquired by bees (GLM: $\chi^2=34.02$, d.f.=3, $P<0.001$). 73% of control bees exhibited PER in the final learning trial (Fig. 56), which was not significantly higher than the 63% acquisition for bees exposed to 20 µT EMF (Bonferroni: $P = 0.051$). The proportion of learned responses in the final trial was significantly reduced to 42% following exposure to 100 µT EMFs (Bonferroni: $P < 0.001$), and 36% after exposure to 1000 µT EMFs (Bonferroni: $P < 0.001$) from control levels (Fig. 56).

One hour after the final learning trial a single response test to the CS was carried out to assess how acute EMF exposure affected memory retention. During this trial EMF exposed bees still exhibited significantly lower responses to the CS than control bees (GLM: $\chi^2=9.80$, d.f. = 3, $P = 0.02$). For all EMF exposures however, the proportion of CS responses 1 hr after learning was not significantly different to the proportion of CS response in the final learning trial (pairwise repeated sample McNemar tests, $P = 0.32$-1.00), indicating that memory retention over the 1 hr time period was not affected by EMF exposure during conditioning (Fig. 57).

Bees from different hives again showed significant variation in the proportion of PER in the final learning trial (GLM: $\chi^2 = 13.42$, d.f. = 3, $P = 0.004$) and in the 1 hr retention trial (GLM: $\chi^2 = 27.829$, d.f. = 3, $P < 0.001$), although there was no significant '*hive of origin' x*

'EMF' interaction effect on the final level of learning (GLM: $\chi^2 = 16.88$, d.f. = 9, P = 0.06) or on 1 hr retention levels (GLM: $\chi^2 = 10.319$, d.f. = 9, P = 0.33).

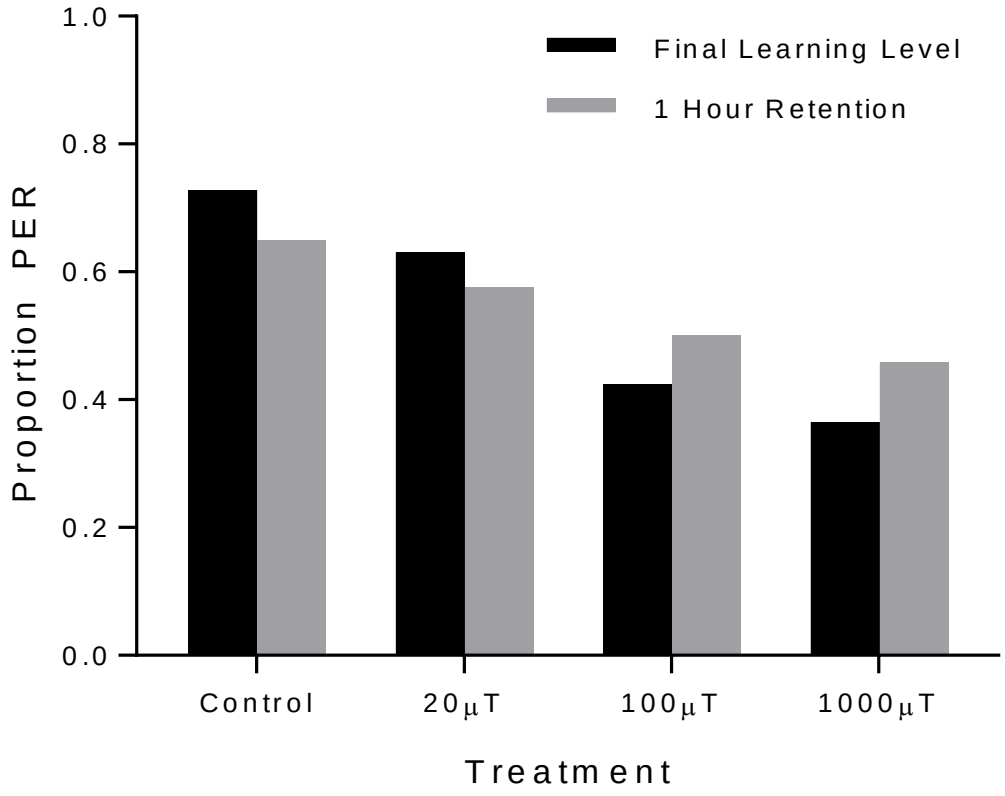

Figure 57. The effect of EMF exposure on memory retention. Proportions of bees showing PER after the final conditioning trial (trial 5) and the retention trial (1 hr after conditioning) are plotted for each EMF/control treatment.

3.5 Discussion

Short-term 50 Hz ELF EMF exposure increased stress protein levels at high EMF levels, and affected aversive learning at levels as low as 100 µT. Acute ELF EMF exposure as low as 20 µT reduced appetitive learning acquisition in honey bees. This shows for the first time directly, that short-term and acute ELF EMF exposure can have biological effects on an insect pollinator, and can affect insect cognitive behaviour. A new abdominal flexion response in the SER assay was also characterized.

3.5.1 Stress proteins

There was no evidence that exposure to 100 µT and 1000 µT EMFs had an effect on stress protein levels, however exposure to 7000 µT EMFs led to an increase in Hsp70 levels. The results suggest that there may be a threshold for activation of stress responses in honeybees between exposures of 1000 µT and 7000 µT EMFs, which would indicate that quite high magnetic flux densities would be required to induce Hsp70 expression. Indeed, Li et al. (2013) found in *Drosophila* that short- and long-term exposure to 3000 µT 50 Hz EMFs increased heat-shock levels. A similar increase in Hsp70 levels was also found in locusts following exposure to 7000 µT EMFs (Chapter 2).

As has been discussed previously, there are multiple methods for inducing an increase in heat shock proteins. One mechanism is through direct interactions with DNA H-bonds (Blank and Goodman, 2004) in specifically electromagnetic responsive elements (EMREs) of the Hsp70 gene promoter (Lin et al., 2001). This would lead to the unwinding of DNA in the Hsp70 promoter region and could directly increase heat-shock protein expression increased transcription (Goodman and Blank, 2002; Blank and Goodman, 2004). The other pathway with which EMFs may increase heat shock expression could be via induced molecular/physiological stresses (King and MacRae, 2015). If applied EMF forces could affect biological processes leading to damaged proteins, or caused them to become misfolded/aggregated, this could lead to increased Hsp70 levels. In this case it would be very difficult to determine what processes are affected, especially when EMFs have such a large capability to exert forces on charged molecules.

The functional relevance of an increase in Hsp70 levels after ELF EMF exposure may be related to their role as heat-shock chaperones to provide a buffer for further stresses as energy is expended in the transcription, translation and exportation of these proteins in

biological systems (King and MacRae, 2015). Whilst the initial protective function of increased heat-shock protein levels may confer an advantage to a stressed bee (or other organism) if it encounters a secondary environmental stressor, the indication is that biomolecular stress has occurred, and energy has been expended in responding to this. There is the possibility that with increased ELF EMF exposure, or with the addition of another stressor, the heat-shock repair system may be overloaded and begin to fail, leading to larger scale biological damages and crossing a tipping point in terms of stress responses (Staveley et al., 2014; Gill et al., 2012).

3.5.2 Aversive and appetitive learning

The results of this study clearly show that the cognitive abilities of *A. mellifera*, through aversive learning, are impacted by short-term exposures to ELF EMFs at 100 µT and 1000 µT. Appetitive olfactory conditioning in bees was affected by both short-term and acute exposure to 50 Hz ELF EMFs. Short-term exposure at a low temperature level to 100 µT EMFs overnight before conditioning slightly reduced learning levels from controls, but exposure at a high temperature level to 7000 µT did not. Short-term exposure to 7000 µT EMFs during conditioning for 3 hrs reduced learning levels as a repeat EMF exposure. Changing electromagnetic field generating equipment from the magnetotherapy applicator to the Helmholtz coil allowed for better temperature controls to compare different ELF EMF exposures in further experiments with bees. This effect of ELF EMFs on cognitive behaviour was more strongly shown by acute exposure experiments where temperature was controlled for all exposures. In acute exposure experiments all EMF exposures 20-1000 µT reduced learning, with higher level EMFs (100-1000 µT) having a greater impact than 20 µT. These findings establish that ELF EMFs at levels which can be encountered regularly around HVTLs affect cognitive behaviour of honeybees. This appears to be the first time that ELF EMFs have been shown to affect cognitive behaviour in an invertebrate species, as most studies have focussed on mammals.

Whilst the only available literature regarding cognitive effects of ELF EMFs is with mammals, specific timing and duration of exposure appears to be critical. A study by Sienkiewicz et al. (2001) found that acute ELF EMF exposure did not affect recognition memory of mice, however Mostafa et al. (2002) found that chronic exposure did affect recognition memory. Here, acute exposures were applied directly after conditioning, and as this may occur for bees foraging in proximity to HVTLs, this indicates that field-realistic simulated exposures may affect behaviours critical to successful foraging. Chronic exposure

to ELF EMFs was not tested here, and these impacts on cognitive behaviour are potential routes to extend the findings of this study. In-between chronic and acute ELF EMF exposures, short-term EMF exposures may occur. In the environment bees are unlikely to encounter short-term ELF EMF exposures of 1000-7000 µT that may affect aversive or appetitive learning, and these levels were considered to determine potential thresholds for ELF EMF effects. However bees may be exposed to short-term ELF EMFs in the range of 100 µT for hives that are placed at ground level under powerlines. Single and repeat short-term exposures can occur from hives that are moved regularly, or for hives that are kept under powerlines for long term periods, where ELF EMF pollution levels fluctuate with energy demands. When expanding to chronic ELF EMF exposure studies it will be important to determine what biological effects long-term consistent chronic EMF exposure has on insects, as well as what the far more dyanamic field scenario (of intermittent and repeated short-term ELF EMF exposures) has on insects.

Here, the levels with which short-term ELF EMF exposure affected bee aversive learning (100 µT and 1000 µT exposures) draw parallels with some mammalian of the mammalian studies. For example, there are multiple reports of reduced task performance in mice and rats for ELF EMF exposures above 100 µT (Kavaliers et al., 1996; Lai, 1996, Lai et al., 1998; Sienkiewicz et al., 1998a; Sienkiewicz et al., 1998b). There are also a few studies in humans such as Preece et al. (1998), which found that a 50 Hz 600 µT EMF reduced performance in numerical memory and work recognition tasks. Keetley et al. (2001) also found reduced performance in a visual-motor memory task when exposure to a 50 Hz 28 µT EMF. Podd et al. (2002) found reduced performance in a recognition memory test for 50 Hz 100 µT EMF exposure. Trimmel and Schweiger (1998) found with a 1000 µT 50 Hz EMF there were reductions in visual attention, perception and verbal memory.

3.5.3 Mechanisms for impacts of ELF EMFs on cognitive behaviour

While there are a number of reported effects of ELF EMFs on cognitive behaviour, the mechanisms that underpin these effects are poorly understood. Most studies with mammals attribute ELF EMF-induced changes to reduced cholinergic function from EMF induced effects on endogenous opioid activity, which has critical roles in response to stress and pain

in humans (Lai and Carino, 1999, Thomas and Persinger, 1997). The mechanisms which may underpin cognitive behavioural effects of ELF EMFs on invertebrates are likely to vary from those which occur in mammals.

As honey bee learning pathways are well characterised, there may be some indication as to the mechanisms of ELF EMF effects on cognitive behaviour here, given the effects of different ELF EMF exposure types on learning. For example, for acute ELF EMF effects on PER and short-term EMF effects on SER, the immediate learning acquisition was affected by ELF EMF exposure. This suggests ELF EMF effects on the neurophysiological pathways involved in the acquisition of information and/or the formation of short-term memory. In contrast, for short-term impacts of ELF EMFs on appetitive learning, there were no primary effects of ELF EMFs on PER levels, and only when secondary ELF EMF treatments were applied in the 3 hr period after 5 repeated trials were any reduced levels of PER observed. This potentially indicates some ELF EMF effects on memory formation pathways further downstream, such as effects on medium-term and long term memory formation. Potential impacts of ELF EMFs on the downstream consolidation of memory are further supported by changes in stress protein levels, as increased protein synthesis is required for long-term memory formatin (Menzel, 2001). Further approaches to determine the mechanistic understanding of ELF EMF effects on cognitive behaviour could explore changes in memory formation pathways after ELF EMF exposure, including impacts on neural systems involved in short-term memory formation, as well as impacts on protein synthesis and activities of enzymes critical to medium-term and long-term memory formation such as protein kinases and NO synthase (Menzel, 2001).

A variety of studies investigating the effects of EMFs on invertebrates have reported increased activity in study organisms, and have suggested increased octopamine levels for these behavioural changes (Wyszkowska et al., 2006; Todorovic et al., 2013; Jankowska et al., 2015). The levels of the biogenic amine octopamine, the homolog of mammalian norepinephrine in insects, can change under stress, and it is involved in insect fight or flight responses (Davenport and Evans, 1984; Farooqui, 2012). Octopamine has a crucial role in honey bee olfactory conditioning (Hammer, 1997; Hammer and Menzel, 1998; Scheiner et al., 2006). Menzel et al. (1999) showed that for bees treated with reserpine (which depletes biogenic amine levels and consequentially impairs learning/memory formation) octopamine treatment caused a recovery of conditioning, but did not cause a recovery in memory retention, highlighting that the role of octopamine is more related to conditioning (memory formation) than memory retention. An effect of ELF EMFs on octopamine would therefore

match the effects on honey bees found in this study for acute ELF EMF exposure and its effects on appetitive learning, where memory formation, but not memory retention, was reduced by ELF EMF exposure. Furthermore, 7000 µT ELF EMF exposure has been shown to increase activity in cockroaches, the effect of which is suppresed by phentolamine, an octopamenergic inhibitor, suggesting a key role of octopamine in the effects of ELF EMFs (Wyszkowska et al., 2006). The potential effects of ELF EMFs on octopamine, as well as other biogenic amines, are an essential future area of research that may elucidate how ELF EMFs may affect physiological processes that underpin behaviour.

Dopamine is another biogenic amine which alongside octopamine has a critical role in associative learning in honey bees (Hammer and Menzel, 1998) and insects in general (Barron et al., 2010). Schwaerzel et al., (2003), when investigating catecholamine levels in different learning behaviours in *Drosophila*, found different catecholamines were required for memory formation under different learning pathways, dopamine for aversive learning and octopamine for appetitive conditioning. This differential modulation of appetitve learning by octopamine, and aversive learning by dopamine, appeared be consistent in different insect taxa including crickets (Unoki et al., 2005, 2006; Mizunami et al., 2009) and bees (Farooqui et al., 2003; Vergoz et al., 2007). For example Vergoz et al. (2007) found that aversive learning is impaired after the injection of dopaminergic antagonists, suggesting that dopamine may have a primary role in memory formation in aversive learning. However, this may be a slight oversimplification of the roles of these biogenic amines in associative learning pathways (Barron et al., 2010). More targeted genetic manipulations have shown that dopamine signals can inhibit mushroom body neurons enabling the expression of food-associated conditioned responses (Krashes and Waddell, 2008; Krashes et al., 2009), or in other words, dopamine also has important roles in appetitive reward learning. A more recent consensus on the topic in *Drosophila* is that dopamine confers a dual-role in signalling value in aversive and appetitive pathways in insects, and octopamine confers appetitive reinforcement of sweet taste through dopamine neurons (Wadell, 2013). Given the link of octopamine changes from ELF EMF exposure, and the key roles of dopamine and octopamine in associative learaning, the functions of these biogenic amines appear to be important areas for further exploration of the mechanisms of ELF EMFs effects on insect cognitive behaviour.

A direct mechanism for the observed cognitive behavioural effects found here is currently unknown. ELF EMF exposure may elicit a stress response in bees, or lead to detrimental molecular biological effects. ELF EMFs have been shown to increase heat-shock

protein expression in locusts (Chapter 2) and *Drosophila* (Li et al., 2013), and were found to increase stress protein levels here after 7000 µT exposures. As heat-shock proteins are upregulated in response to environmental stress, it is possible that ELF EMF exposure may cause molecular damage, and/or directly interact with the promoter regions of heat-shock proteins, to up-regulate heat-shock expression (Goodman and Blank, 2002). It is essential for future research to consider effects of ELF EMF pollution on molecular biology, and how this may underpin changes in cognitive behaviour, when considering biological effects of ELF EMFs.

ELF EMFs may affect honey bee physiology in a variety of other ways to cause the behavioural effects observed in this study. Neural pathways involved in honeybee conditioning are well characterised (Menzel and Müller, 1996; Hammer, 1997) including neurons associated with octopamenergic activity such as VUMmx1 (Hammer, 1997). Exposure to 7000 µT ELF EMF in locusts causes abnormal motor neuron signalling by increasing action potential duration and latency (Chapter 2). It is entirely possible, therefore, that ELF EMFs may also affect VUMmx1 activity to underpin the effects of ELF EMFs on cognitive behaviour, as well as locomotory behaviour. It is well established that ELF EMFs induce electric fields and currents within organisms, causing excitability of neurons (Dimbylow, 1998; Jacobson, 2005; WHO, 2007; Halgamuge et al., 2009), which is a potential mechanism for this kind of effect. Furthermore it has been described by Menzel and Sugawa (1986) that olfactory conditioning in honey bees is highly sensitive to weak sinusoidal 18 Hz electrical stimulation of the median-frontal head capsule of the honey bee brain, in a fashion that erases memory formation, but only if electrical stimulation occurs immediately after the learning trial. Here, ELF EMF exposure in conditioning occurred immediately after each conditioning trial, and electric currents may have been induced in the brains of treated bees that lead to the impairment of honey bee learning via a similar disruption of memory formation as described by Menzel and Sugawa (1986). To further determine effects of ELF EMFs, further electrophysiological analyses are needed, and it is clear that the timing of ELF EMF exposure in relation to critical memory formation may be important.

3.5.4 Abdominal flexion responses

Results show for the first time an abdominal flexion response to the CS, where bees do not extend their sting, but complete the rest of the behavioural response that occurs in the sting extension response by flexing the abdomen under the thorax, such that the bee is primed

to extend the sting. All the abdominal flexion responders extended their sting apparatus immediately once the US (electric shock) was applied (only a 2s window was allowed for this), as this was a criteria for inclusion in the SER assay to ensure that only bees which could show a full conditioned response to the CS were tested. This is despite being given 6s to repond to the CS in each successive conditioning trial. The gradual increase in these abdominal flexion responses to the CS (6s window) alongside consistent fast extensions of the sting apparatus by the same bees in reponse to the US (2s window) throughout conditioning trials, suggests that this is more likely a lower-level response to the CS than a slower response to the CS. Abdominal flexion behaviour was not observed in unconditioned bees and only occurred with repeated conditioning trials, and is therefore a clear learned response to the aversive CS. Abdominal flexion is a well described aggressive aversive behaviour in bees, for example Rittschof et al. (2015) used abdomen flexion 'when the abdomen is flexed but the stinger is not extruded', as an element of aggression assessment in an intruder assay. In Avalos et al. (2014) abdominal flexion is described as an aversive response in drones that do not have a stinger. Ogawa (1995) describes the motor pattern of the sting response, which includes the flexion of the abdomen priming the sting, before the sting is actually extended.

In locusts ELF EMFs have been shown to impact motor neuron circuits, and muscular force (Chapter 2). During the stinging response the protraction of the abdomen tip, and the alternate sliding of barbed lancets of the stinging apparatus, are coordinated by 4 large abdominal muscles (Snodgrass, 1956; Dade, 1962; Ogawa, 1995) which is regulated by the activity of a central pattern generator in the terminal abdominal ganglion (Nouvian et al., 2016). There were no differences in the abilities of bees to extend their stings after ELF EMF exposure (i.e. bees could still extend their sting in response to the electric shock, the lack of response was specifically to the CS), and therefore the differences observed in this study are solely down to reduced ability to learn the aversive stimuli, and not the motor pattern involved in responding to the stimuli.

Whilst the SER assay has utility in measuring and studying aversive learning in a controlled laboratory environment, there may be difficulty to translate findings to ecological scenarios, as realistic responses of bees in the environment to aversive stimuli are not binary (i.e. when presented with a threat a bee does not simply respond by either stinging or not stinging the threat). Instead an array of aversive responses are exhibited when a threat is detected. Cunard and Breed (1998) found that only 25% of honey bees pursuing intruders

actually sting the target, as in many cases harassment, flying directly at the threat, and increased buzzing are sufficient to remove the threat. In a dynamic field scenario both the range of environmental stimuli present and the physiological state of the bee determine the level of engagement with a threat (Breed et al., 2004; Nouvian et al., 2015). As a result, this characterization of abdominal flexion responses in the SER assay may increase the utility of the SER assay by adding qualitative detail when measuring aversive learning.

Recording the range of aggressive responses exhibited by bees when learning could not only provide information about the quality of learning/responses of bees, but also determine whether bees respond with appropriate or variable aggression when learning about aversive stimuli. In other associative learning studies attempts have been made to characterize/quantify the level of responsiveness to conditioning stimuli. For example, Chabaud et al. (2006) quantified the level of proboscis protrusion along an ordinal scale of 0-5, with 0 being no movement of the proboscis, to 5 being full extension of the haustellum and rostrum with a 180º angle between the two. As examples of intermediate responses, on the scale in that study trembling of maxillary palps with slight extension of the labellum producing an angle between the palps and labellum was characterised as a '2', and slight extension of the haustellum producing an angle between the labellum and head was characterised as a '3' on the scale. In this way, clear conditioned behavioural responses of *Drosophila* to the CS were not lost in the assay. By classifying abdominal flexion as a learned aversive response in the SER assay, an important conditioned behavioural response will not be missed, and this may allow for more realistic comparisons between lab findings and actual dynamic field scenarios of aversive learning and appropriate responses to environmental threats.

3.5.5 Ecological implications and conclusion

This is the first time that ELF EMFs have been shown to affect appetitive olfactory learning in a pollinating insect. A reduced learning performance can be very detrimental to bees. For example, in their natural environment honey bees must learn and process locations of food, resource quality, resource type (e.g. colour, smell, shape of flowers), geographic landmarks, hive location as well as the distance and direction of food sources from the hive to communicate to the colony (Hammer, 1997). Differing abilities to learn through olfactory conditioning are highly correlated with natural foraging success in bees (Raine and Chittka, 2008), and therefore a reduction in learning performance (as observed here with ELF EMF exposure) can lead to stress on both the individual and colony levels. Electromagnetic field

levels of 1000 µT can be experienced < 1 m from a HVTL, and levels of 20-100 µT can be experienced at ground level. It is, therefore, highly likely that bees could experience similar cognitive effects of ELF EMF exposure in the field, where HVTLs are located between bee hives and food sources.

Overall aversive learning decreased with EMF exposure, while at high exposure levels stress responses increased. These findings have potentially large implications for bee ecology. It is imperative that honeybees are able to perceive, learn and avoid threats in the environment (Hammer and Menzel, 1998; McNally and Westbrook, 2006). Reductions in the ability to learn about negative stimuli could have implications for the abilities of bees to deal with predatory/invader threats (Cappa et al., 2016; Nouvian et al., 2016), detecting/avoiding deleterious stimuli (Wright et al., 2010) and responding to negative stimuli that require action e.g. attacking/removing diseased individuals from the hive (Cappa et al., 2016), all of which could have detrimental effects for bee colonies. Although it is not yet known how bees will actually respond in the field, it is clear that the reduction in aversive learning seen here with short-term 1000 µT and 100 µT exposures could be detrimental to honeybees on an ecological level.

A number of studies have described bee colonies failing that are hived under HVTLs, where EMF levels can reach 100 µT (Wellenstein, 1973; Greenberg et al., 1981; Rogers et al., 1982; Morse and Hooper 1985). There is the possibility that with hives located under HVTLs, the long-term chronic exposure to ELF EMFs could continually reduce cognitive abilities both with regards to aversive and appetitive learning, potentially leading to some of the negative effects found in these studies. Stress protein levels were found to be increased here, but only at levels much higher than ground level under HVTLs. However it remains to be seen whether longer term exposures to lower level ELF EMFs, such as would occur for bees hived under HVTLs, could elicit the same molecular responses as observed here with shorter exposures at higher levels. Further studies should consider whether ELF EMF pollution may affect other important honey bee behaviours such as locomotory behaviours or communication (e.g. waggle dance).

ELF EMF exposures that can be encountered at ground level and within the range < 1 m from HVTLs have been shown to reduce honey bee learning acquisition. Reductions in learning over time could be detrimental to individual and colony survivability. There are large potential ecological consequences for reduced ability to learn about aversive and appetitive stimuli for bees. Future studies should focus on whether there are ecological effects of ELF EMF exposure, with direct measurements of chronic EMF exposure under

power lines, as well as determining what physiological/molecular processes may be affected by this kind of exposure. These effects may not be confined to managed honey bees as the may be much wider implications for wild bees and even other pollinators that require powerline strips for critical habitat refuge (Russell et al., 2005; Wojcik and Buchmann, 2012; Wagner et al., 2014; Berg et al., 2016; Hill and Bartomeus., 2016). The underlying mechanisms, as well as the potential ecological implications of ELF EMF pollution in the field must be further investigated to determine the effects of ELF EMF pollution on insect biology and ecology, including crucial pollination ecosystem services

Chapter 4 ELF EMF effects on honey bee flight and feeding

4.1.1 Abstract

Flight is an essential behaviour for many insects, and especially for pollinators when foraging. Here the impacts of ELF EMFs on honey bee flight in lab and field scenarios were explored. In a tethered flight lab assay ELF EMF exposures that can be encountered within the range < 1 m from HVTLs increased flight activity. In a field cage flight study ELF EMFs that can be found at ground level under HVTLs reduced feeding abundance and flight success. Extensive reductions of these behaviours over time could be detrimental to

individual and colony survivability. The mechanistic understanding of these effects, as well as the potential ecological implications of ELF EMF pollution in the field must be further investigated to determine the effects of ELF EMF pollution on insect biology and ecology, including crucial pollination ecosystem services.

4.2 Introduction

4.2.1 Importance of flight as a locomotory behaviour

Flight is the primary method of locomotion, and an essential motor behaviour, in bees. Bees fly when finding/collecting food (Beekman and Ratnieks, 2000) and water (Kühnholz and Seeley, 1997), when swarming/finding nesting sites (Camazine et al., 1999), when defending their colonies (Nouvian et al., 2016), when exploring and orienting their environment (Degen et al., 2015) and even when mating in nuptial flights (Koeniger et al., 1979). Insect flight is an intense, energy-demanding motor behaviour (Brembs et al., 2007). Depending on environmental conditions honey bees expend 0.3-0.8 W/g in flight (Harrison and Fewell, 2002), which can be over 40 times the 0.02 W/g energy expenditure seen at rest (Rothe and Nachtigall, 1989). Factors when foraging, such as a heavy pollen load, can increase flight attributes such as wingbeat frequency or stroke amplitude, and consequentially increase energy expenditure (Harrison and Roberts, 2000). As a flight intensive behaviour, foraging involves just 10% of the bees in a colony, but expends 30% of the total colony energy (Harrison and Fewell, 2002). As a result it is critical that flight behaviours can be performed efficiently by bees, for the overall success of these behaviours and consequential health of the colony.

4.2.2 Consequences of factors that affect flight

Factors that impact upon flight, and the efficiency of flight intensive behaviours, can be extremely detrimental to bees. For example tracheal bee mites, *Acarapis woodi*, reduce the safety margin for oxygen delivery for high metabolic activities such as flying in cool weather, with evidence that these infections a related to failure of bees to return to the hive during late winter flights (Harrison et al., 2001). In an extreme example, bees infected with deformed wing virus (DWV) have extremely malformed wings and are unable to fly (Highfield et al., 2009). Whilst this symptom of DWV is not the only cause for severely reduced lifespan (< 48 hrs) in infected bees, eliminated flight ability causes a major reduction in health for individuals, which may contribute to losses of colonies with high levels of DWV

(Berthoud et al., 2010; Le Conte et al., 2010). Where geographic (Dillon et al., 2006) or anthropogenic barriers make flight difficult, fragmentation can occur leading to reduced gene flow in pollinators (Jha, 2015) as well as limiting ability to access different food sources and consequentially pollinate plants (Wratten et al., 2003). In contrast, factors which improve flight mobility between habitats can improve pollination success (Cranmer et al., 2012). Factors that can act as flight barriers can have large ecological impacts, as the ability to access high quality and nutritious food sources is critical to pollinator health (Brodschneider and Crailsheim, 2010).

4.2.3 Potential for ELF EMFs to impact upon locomotory behaviour

As a critical behaviour to pollinator success, flight is important to analyse in terms of the effects of ELF EMFs. There is very little literary evidence of the impacts of ELF EMFs on locomotory behaviour in different animals. There are several indications that short-term exposure can lead to changes in locomotory activity in mammals (Janać et al., 2005; Rostami et al., 2016). In insects, Zmejkoskia et al. (2017) found short-term EMF exposure at 500 µT decreased locomotor activity in *Drosophila subobscura*. There are extremely few published examples where controlled ELF EMFs have been acutely applied to organisms to measure locomotory behaviour, let alone flight. Low-frequency EMFs have been shown to diversely affect insect groups causing positive taxis for some taxa, and negative taxis for others (Wijenberg et al., 2013). Bergh (1979) found that natural fluctuations in atmospheric low frequency electromagnetic radiation from storms stimulates take-off behaviour in the desert locust *Schistocerca gregaria*. Earlier (Chapter 2) acute ELF EMF exposure was shown to impact up wingbeat frequency of locusts. It is possible the ELF EMFs could acutely affect critical behaviours such as flight in pollinators and other flying insects.

4.2.4 Potential for bee flight to be affected by ELF EMFs

As mentioned previously, there is a range of observational evidence that bees may be affected by magnetic fields, as bees hived under HVTLs are not successful (Wellenstein, 1973, Greenberg et al., 1981; Rogers et al. 1982; Morse and Hooper, 1985), and it has been recommended that bee hives are not placed under HVTLs (Lee, 1989). Bees may be also be able to respond to ELF EMFs through magnetosensory processes, for example Kirschvink et al. (1997) found a lower limit of detection via magnetite-based magnetoreception in bees was 100 µT with a 60 Hz EMF, as well as being able to detect fields as strong as at least 2200 µT from static-60 Hz (Kirschvink et al., 1992). These limits are frequency dependent,

with bee responses to magnetic fields above 60 Hz becoming random (Kirschvink et al., 1992), whereas with lower EMF frequencies (closer to Earth's static geomagnetic field) bees have a higher sensitivity regarding magnetoreception. This would suggest that with a 50 Hz EMF, as emitted from powerlines in the UK, the lower limit of honey bee sensitivity to ELF EMFs via magnetite-based magnetoreception would be < 100 μT. Not only are these ELF EMFs levels equivalent to what can be encountered at ground level under HVTLs, but much higher levels 100-14,000 μT can be encountered above ground level by bees, as flying insects which regularly travel above ground level (Gary, 1963; Loper et al., 1992; Osborne et al., 1999).

As well as this the range of land where bees can come into contact with ELF EMFs is very large, as many bee species, dependent on body size, forage from 1-10 km of their colonies (Greenleaf et al., 2007). Even with a conservative estimate that bees forage up to 1.5 km, a 3 km corridor is present around a HVTL that could be within foraging distance of bees. With 22,643 km (ENA, 2011) of HVTLs alone, not including the hundreds of thousands of kilometers of distribution lines that emit weaker ELF EMFs, this gives a 67,929 km^2 area of land (equating to 28% of the land in the UK) within bee foraging range (for most bee species) of HVTLs where high levels of EMF pollution can be experienced. For honey bees this range could be even larger, e.g. Beekman and Ratnieks (2000) found mean foraging flights were 5.5 km and median flights were 6.1 km, with only 10% of bees foraging within 0-5 km of the hive. Given the high likelihood that bees can be exposed to high levels of ELF EMFs, and that ELF EMFs may stress bees physiologically and/or affect behavior as a sensory stimulus, it is pertinent to determine whether or not ELF EMFs affect the critical behavior of flight, in bees. Here impacts of ELF EMFs on wingbeat frequency will be assessed in lab conditions, as well as effects on free flight in the field in a foraging scenario.

4.2.5 Aims and Objectives

Previous chapters have shown that ELF EMFs can affect locomotory flight behaviour of locusts, and cognitive behaviour of bees. It is pertinent to determine to what extent acute ELF EMF exposure may affect honey bee locomotory flight behaviour. Furthermore, as locomotion and cognitive processing are critical to honey bee pollination, it is important to expand studies to a field realistic scenario where acute effects of ELF EMFs on both locomotion and cognitive behaviours can be assessed (e.g. in foraging).

The aim of this chapter is to determine whether ELF EMFs affect honey bee locomotion, as a critical behaviour for colony success, alongside cognitive behaviours that

were assessed in Chapter 3. Secondly, this chapter aims to expand flight analyses to a semi-field scenario where acute effects of ELF EMFs on flight can be assessed in a setting where cognition and locomotion are important (foraging).

This chapter addresses the following questions:

Do ELF EMFs affect honey bee flight?

Do ELF EMFs affect flight and feeding in a foraging scenario?

4.3 Methods

4.3.1 Tethered flight

4.3.1.1 Magnetic fields

For assessment of the effects of 50 Hz EMFs on honey bee flight, the Helmholtz coil apparatus (2.3.3.1) was used (Fig. 58, A). 20 µT, 100 µT and 1000 µT exposures were used (Fig. 58, B-D) as well as a control treatment.

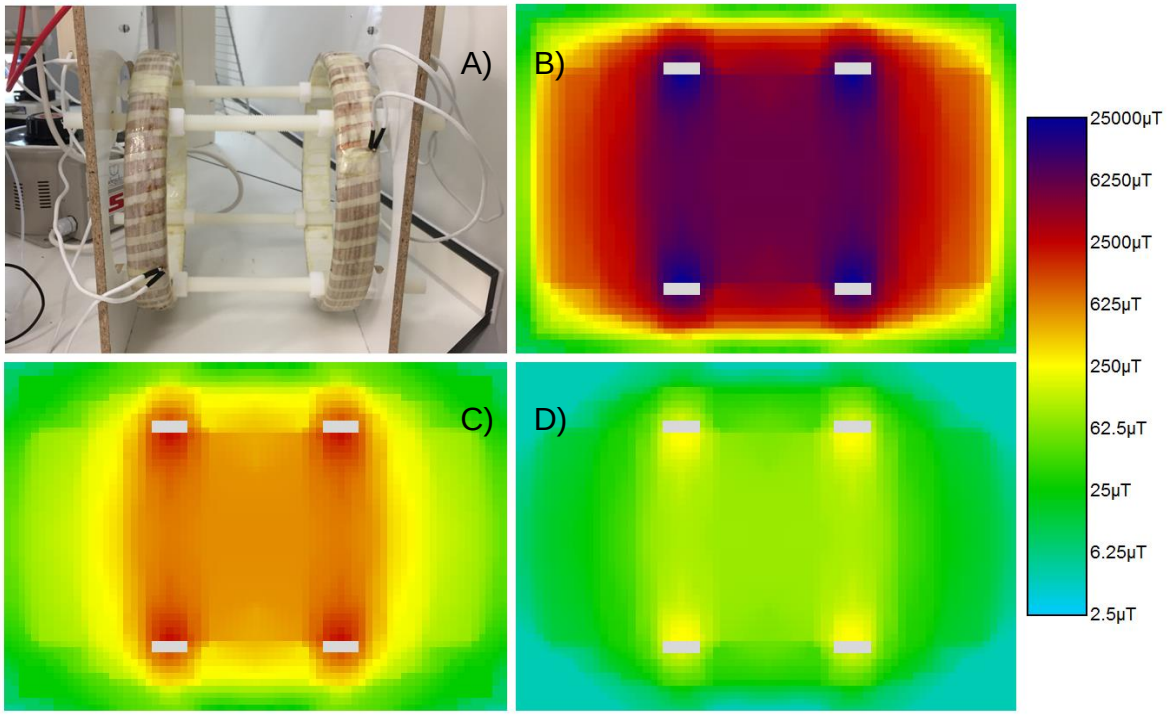

Figure 58. (A) Custom-made Helmholtz coil on stand with solenoid electromagnets for generating homogenous 50 Hz sinusoidal AC electromagnetic field placed 14 cm apart. (B-D) Magnetic field mapping for recorded magnetic flux densities in lateral cross-section of the Helmholtz coil for various EMF exposures. (B) 7000 µT. (C) 1000 µT. (D) 100 µT

4.3.1.2 Bee collection and harnessing

For lab-based flight dynamics experiments returning forager honey bees from the University of Southampton Highfield Campus Apiary were collected from 3 hives (Hive 1_{2016}, Hive 2_{2016}, and Hive 3_{2016}). Bees were transferred to free-feeding cages in the lab (Fig. 59), with 15 bees per cage, and kept overnight at 29 ± 1 ºC with a food source of 40% w/v sucrose solution. Tethered flight assays occurred 24 hrs after collection.

Figure 59. Disposable cage used for collecting bees and keeping overnight in tethered flight analyses. Two 1.5ml Eppendorf tubes were placed within the cage with holes for feeding, containing 40% w/v sucrose.

24 hrs after collection, bees were immobilised on wet ice. Body hair on the dorsal side of the thorax was shaved using a scalpel, to provide a tether attachment point. Bees were then attached to a tether using UV activated glue (Bug-Bond™, Veniard Ltd., Croydon, UK). The tether allowed bees to be placed on, and raised from, a platform in the centre of the Helmholtz coil apparatus (Fig. 60).

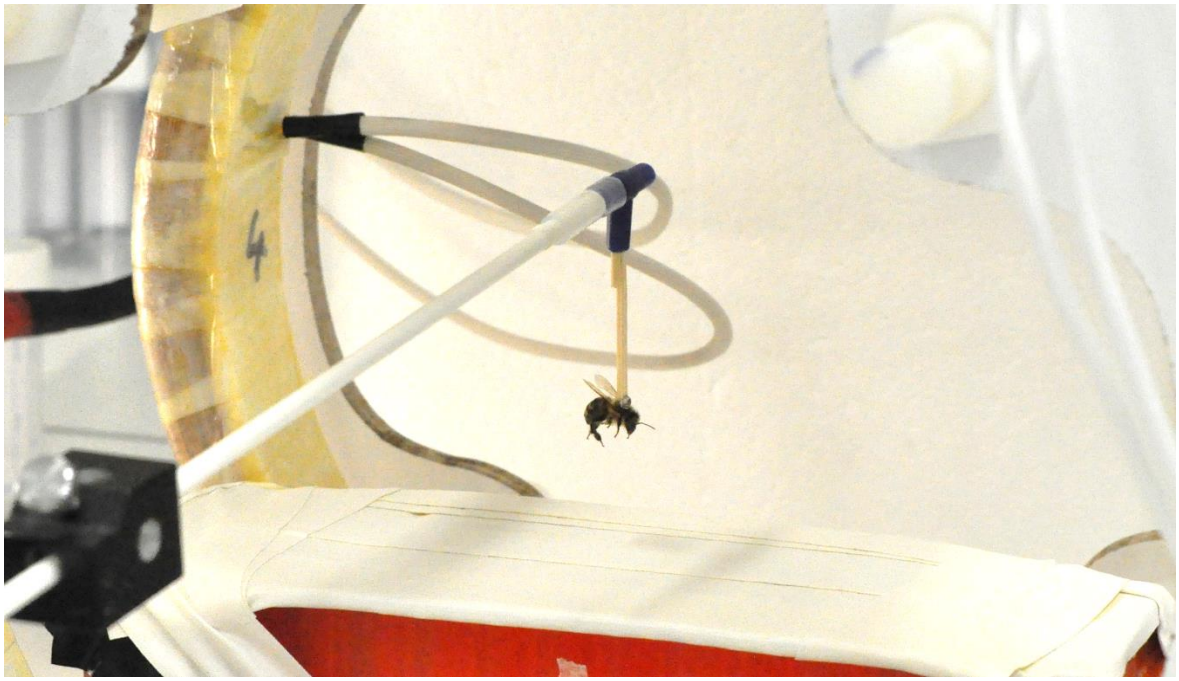

Figure 60. Bee suspended on the tether raised above the resting platform in the centre of the Helmholtz coil apparatus. The bee is attached to the tether on the dorsal side of the thorax

Raising bees vertically from the platform initiated flight. A high-speed video camera (MotionScope 1000S, Redlake Imaging, CA, USA) was used to record flight at 1000 fps. Following 5 s of consistent flight an EMF was applied and the video camera triggered to capture flight 1 s prior to EMF exposure and 3 s after exposure (Fig. 61). Bees (120 in total, n = 30 for each treatment, from 3 hives) were exposed to 1 of 3 different EMF (100 µT, 1000 µT, and 7000 µT) or control treatments. High-speed video was analysed to determine wingbeat frequencies of bees 0.5 s before EMF exposure (pre-treatment) and 2.5 s after the EMF onset (treatment). To determine the impact of acute EMF exposure on honey bee flight, the change in wingbeat frequency from pre-treatment to treatment was calculated for each bee, for both control and exposed bees.

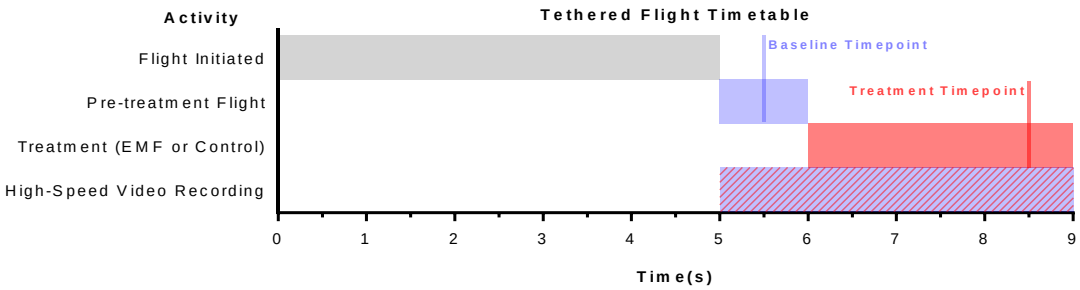

Figure 61. Flight trial for tethered flight assay. Flight was initiated for 5 seconds. 1000 fps high-speed video recording (4 s) captured pre-treatment period (1 s) and a treatment (Control or EMF) period (3 s). Baseline wingbeat frequency was recorded 0.5 s before the initiation of the treatment and treatment wingbeat frequency was recorded 2.5 s after the initiation of treatment.

4.3.2 Free flight and feeding

4.3.2.1 Magnetic fields

To assess the impacts of ELF EMFs on free flight and feeding, experiments were conducted in the field. In this setting electromagnetic fields were still generated with the custom-made Helmholtz coil consisting of two solenoid electromagnets (Faculty of Engineering and the Environment, University of Southampton). In these field experiments the electromagnets were paired together 20 cm apart on a vertical (instead of horizontal) axis, on an adjustable custom stand (Fig. 62, A). Coils were powered with a Ring RPP210 12 V 40ah portable power pack (Ring Automotive, UK) through RS Pro 1 Phase 1.92kVA 1 Output 240 V Variacs (RS Components, UK) to generate homogenous 50 Hz sinusoidal AC electromagnetic fields. In field experiments one EMF treatment at 100 µT (Fig. 62, B) was used simulating the EMF magnetic flux at ground level under a HVTL, as well as a control treatment.

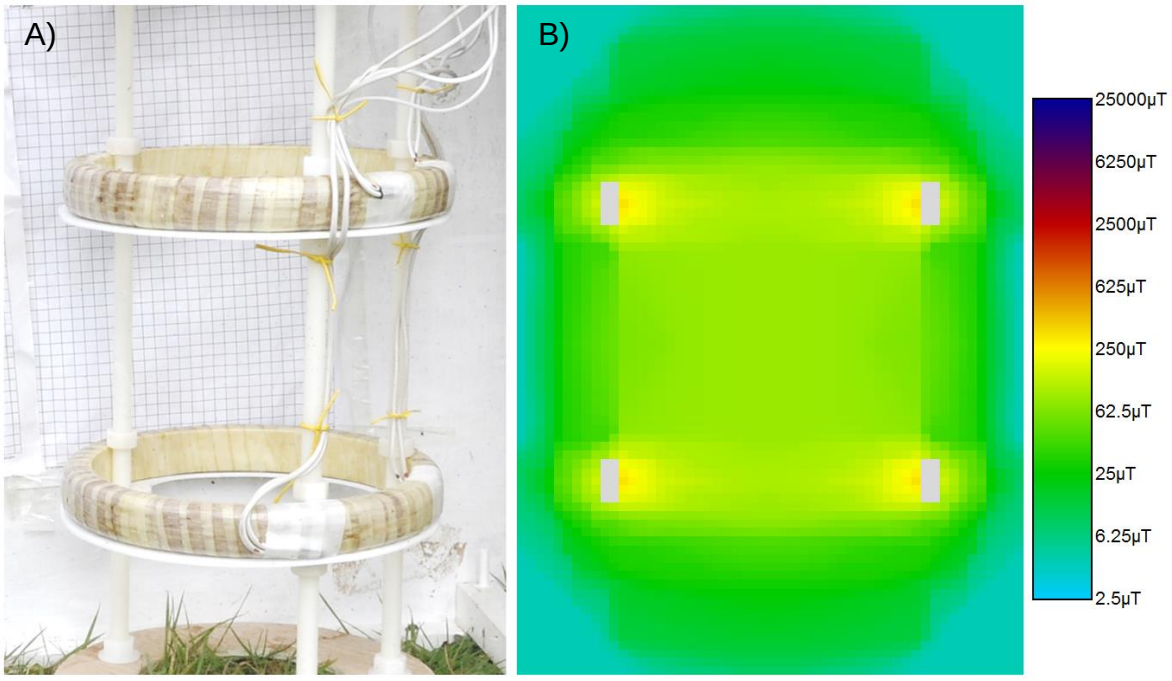

Figure 62. (A) Custom-made Helmholtz coil vertically aligned on stand with solenoid electromagnets for generating homogenous 50 Hz sinusoidal AC electromagnetic field placed 20cm apart. (B) Magnetic field mapping for recorded magnetic flux densities in lateral cross-section of the Helmholtz coil for 100 µT field exposure.

4.3.2.2 Experimental set up

Foraging experiments were conducted with bees from 6 nucleus hives in the field at Southampton Science Park (Chilworth, 50.963787, -1.422833) in summer 2016 (Fig. 63).

Figure 63. Field site at Southampton Science Park with 6 nucleus hives used for free flight and feeding experiments.

Flight cages (W×L×H= 40 × 140 × 69cm) were attached to the front of hives so that bees could fly freely in the cage (Fig. 64). A feeder was placed at the end of the tunnel, and electromagnet coils were placed 20 cm from the hive and 80 cm from the feeder, so that bees had to fly through the coils to get to and from the feeder. The feeder was filled with 100 ml of honey, and 700 ml of 50% w/v sucrose solution.

Figure 64. Flight cage attached to the front of a nucleus hive. A feeder is located at the far end of the cage. The Helmholtz coil and stand are placed between the hive and feeder such that bees must fly past the Helmholtz coil to reach the feeder, and to return from the feeder to the hive.

4.3.2.3 Recordings

Bees were given 30 min to locate the feeder, and for activity in the cage to increase. Subsequently, a 15 min recording of baseline feeding and flight levels was made with no EMF exposure (Fig. 65). Following this, 15 min recordings of flight and feeding levels during control or 100 µT EMF treatments were made, allowing the change in feeding and flight from baseline levels during each treatment to be determined. Both treatments (control or 100 µT) were applied to each nucleus hive, with experiments conducted at least 7 d apart, and the order in which they were applied alternated for each nucleus hive (hive 1,3,5: control first, EMF 1 wk later; hive 2,4,6: EMF first, control 1 wk later).

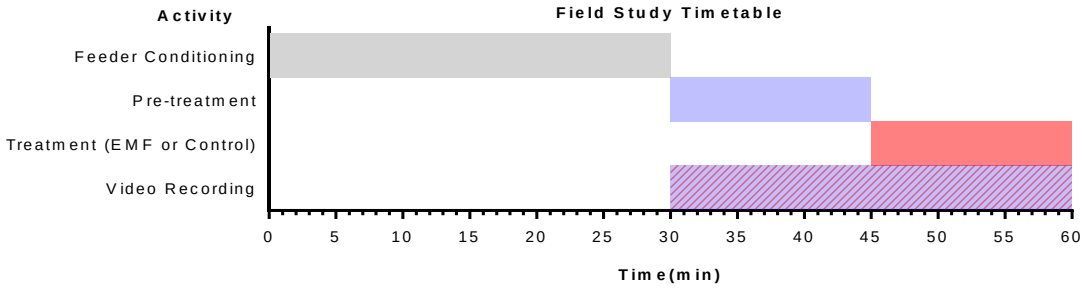

Figure 65. Timetable for flight and feeding experiments in the field. Each hive was given 30 minutes for conditioning to the feeder. The video recording (30 min) and pre-treatment time period (15 min) were then initiated. After 15 minutes the treatment (Control or EMF) then began. For feeding data the baseline amount of feeding bees was taken from the last 1min time point before treatment, and for flight data flights from the entire pre-treatment period were used for baseline flight outcome percentages.

4.3.2.4 Feeding

The number of bees feeding every minute during pre-treatment and treatment times was recorded. For analysis the number of feeding bees in the final time point before treatment initiation was used as a baseline value, and all changes relative to the baseline value for each minute in the treatment time period recorded, such that changes in feeding activity in the treatment time period for control *versus* EMF treatments could be compared. The effects of exposure to EMFs on feeding were analysed on 3,699 feeding events recorded during pre-treatment and treatment periods for bees from the 6 hives combined.

4.3.2.5 Free flight

A digital video camera (HDR-HC1, Sony, Japan) was used to record all bee flights in an area (40 × 50 × 69 cm) around the coils both in the pre-treatment and treatment periods. Videos were subsequently analysed and the outcomes of all flights were recorded. Bee flights that began on one side of the area and exited from the opposite side were defined as *successful passes*. All other flights that did not fulfil this criterion (i.e. turned around in flight/landed), were defined as *failed passes*. The direction of the flight was also recorded. Changes in the percentages of successful/failed passes between pre-treatment and treatment periods for control and EMF exposures were compared to determine the effects of EMFs on flight performance. 2,919 bee flights were recorded from bees from 6 nucleus hives to analyse the effect of EMFs on free foraging flight.

4.3.3 Statistical analysis

Data were analysed in SPSS (v.24, IBM SPSS Inc.) and Graphpad Prism (v.7, Graph Pad Software Inc.). Where appropriate homogeneity of variance and normality assumptions were tested. If required, data transformations were made, or alternative statistical tests were chosen. Where appropriate pairwise contrasts with Bonferroni adjusted significance were used in *post-hoc* analyses.

Analysis of Variance (ANOVA) was used to assess the effects of EMFs on tethered flight responses, with a two-way structure to include 'hive of origin' as an interaction factor, and change in wingbeat frequency as the dependent variable. For field-based experiments, nucleus hives were used as replicates to avoid pseudoreplication. To determine the effects of EMFs on free flight the percentage change in flight outcomes were compared for control *versus* EMF treatments using a paired-samples t-test. To determine the effects of EMFs on feeding a two-way repeated-measures ANOVA was used, with time and EMF treatment as repeated measures, and the number of bees feeding relative to the pre-treatment period as the dependent variable.

4.4 Results

4.4.1 Tethered flight

To determine the effects of acute EMF exposure on flight, bees were exposed to EMFs during steady tethered flight and changes in wingbeat frequency were analysed. In these experimental conditions, with a control setting, the wingbeat frequency of bees on average increased by 2.4 ± 0.9 Hz relative to the pre-treatment wingbeat frequency. During flight, insects generate higher temperatures in flight muscles, which increases maximum power output and also increases optimal wingbeat frequency (Heinrich, 1974; Stevenson and Josephson, 1989). The increased temperature of bee flight muscles as flight continued in this assay may explain the slight general increase in wingbeat frequency for control bees. All EMF exposures caused a greater increase in wingbeat frequency than the increase seen in control treatment. Furthermore, the magnitude of this effect was greater with higher EMF levels (Fig. 66). 100 µT exposure caused an increase of 2.9 ± 0.8 Hz, 1000 µT caused an increase of 5.6 ± 0.9 Hz, and 7000 µT caused an increase of 6.4 ± 1.1 Hz from pre-treatment wingbeat frequency. There was no *'hive of origin' x 'treatment'* interaction effect on change in wingbeat frequency (ANOVA, $F_{6,108} = 1.10$, $P = 0.37$). Exposure to EMFs during flight significantly increased the wingbeat frequency of bees (ANOVA, $F_{3,108} = 4.42$, $P = 0.006$). There was no main effect of 'hive of origin' on change in wingbeat frequency (ANOVA, $F_{2,108} = 1.63$, $P = 0.20$). These results provide evidence that high EMF exposures have the ability to affect bee locomotory behaviour immediately during flight, by increasing wingbeat frequency.

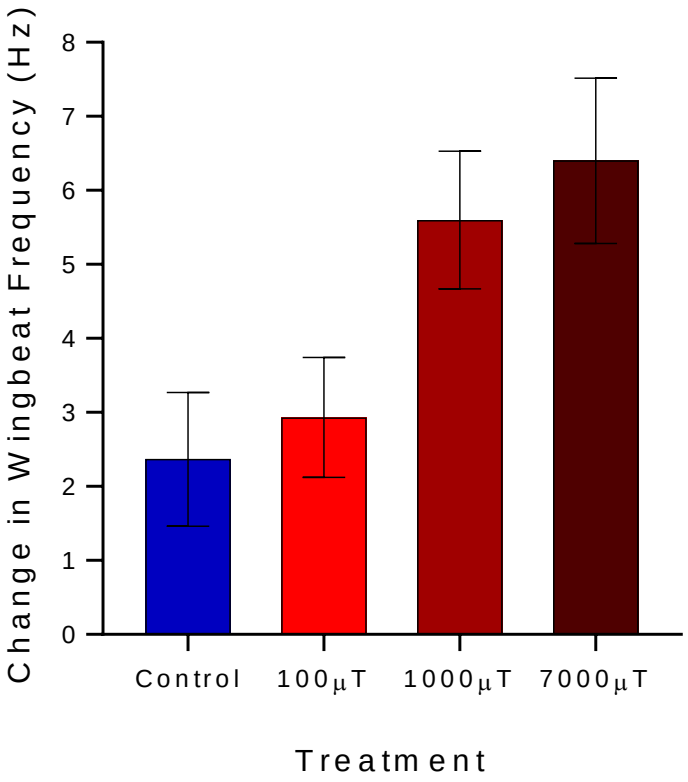

Figure 66. Changes in wingbeat frequency (Hz) of bees from baseline levels in pre-treatment to treatment levels 2.5 s after treatment began for all EMF/Control treatments. Mean and SEM are plotted.

4.4.2 Free flight

In field experiments, after bees were trained to a feeder in a flight cage, a baseline recording of flight activity was made, followed by a treatment recording of flight activity for control and 100 µT EMF exposure (intensity found at ground level below HVTLs). Treatment was applied in the middle of the flight cage between the hive and feeder, to determine the impacts of EMF exposure on free flight of bees between hive and food source. Under control conditions the percentage of successful outgoing flight passes from hive to feeder increased by 3.8 ± 1.8%. In contrast exposure to 100 µT EMFs reduced the percentage of successful outgoing flying passes from the hive to the feeder by -6.6 ± 3.2% (Fig. 67, A). The relative increase in successful outgoing passes under control conditions was significantly different from the relative decrease in successful outgoing passes with 100 µT EMF exposure (t-test, d.f. = 5, P = 0.026). Exposure to a 100 µT EMF also led to an increase in failed outgoing passes by 11.6 ± 5.6% in comparison to a reduction of -2.5 ± 5.1% in control conditions (Fig. 67, B), which approached significance (t-test, d.f.=5, P=0.076).

Exposure to a 100 µT EMF had no significant effects on the returning passes from the feeder to the hive, for both the percentage of successful passes (control -0.5 ± 1.5%; 100 µT -4.9 ± 1.9%) (Fig. 67, C) across the treatment area (t-test, d.f. = 5, P > 0.14) and the percentage of failed passes (control -0.8 ± 5.1%; 100 µT -0.1 ± 5.0%) (Fig. 67, D) across the treatment area (t-test, d.f. = 5, P = 0.90). Overall these results suggest that 100 µT EMF exposure has the ability to affect flight success in a dynamic feeding scenario in the field. EMF exposure at this intensity and in this scenario specifically reduces the number of successful flights to a food source from the hive, but does not appear to affect flights of bees returning to the hive from a food source.

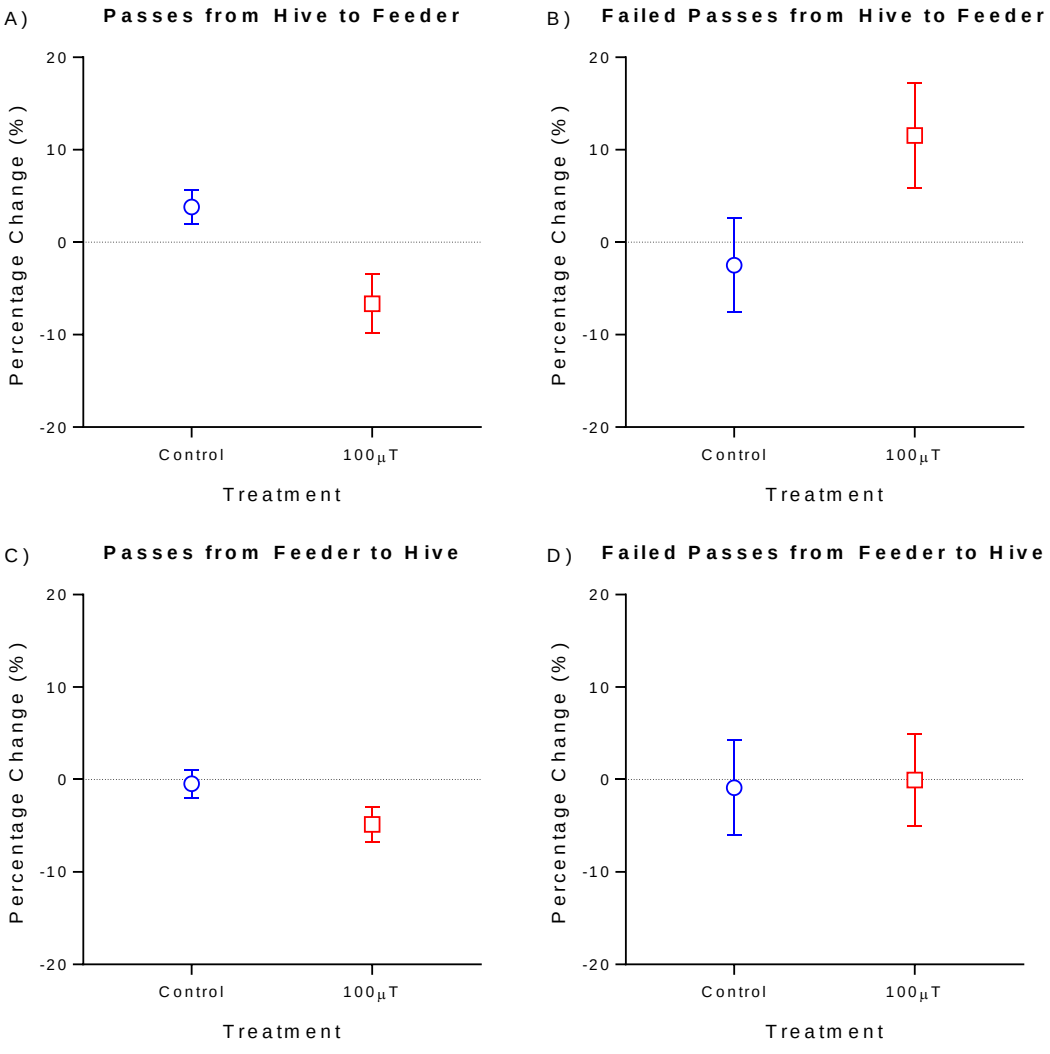

Figure 67. Change in percentages of flight outcomes (*successful passes* or *failed passes*) between pre-treatment baseline levels and treatment levels for control and 100 µT EMF treatment. N=6 (nucleus hives used as replicates). Mean and SEM are plotted. A) Successful outgoing passes from hive to feeder. B) Failed outgoing passes from hive to feeder. C) Successful returning passes from feeder to hive. D) Failed returning passes from feeder to hive

4.4.3 Feeding

In the same field scenario the number of feeding bees at the food source was quantified to determine the impacts of ELF EMF exposure on feeding. As time passed, under control conditions, the number of feeding bees relative to pre-treatment levels, increased steadily (Fig. 68) with an average recruitment rate of 1 bee every 231 s (linear regression). This is expected as more bees identify the food source or are recruited towards the feeder. As a result, after 15 mins in control experiments, feeding activity increased by 4.5 ± 1.7 bees relative to pre-treatment levels. In contrast, with 100 µT EMF treatment the number of feeding bees gradually decreased with time (Fig. 68) by approximately 1 bee every 257 seconds (linear regression). There was a significant *'EMF exposure' x 'time'* interaction effect on the number of bees feeding relative to pre-treatment levels (RM-ANOVA, $F_{14,70}$ = 2.79, P = 0.0024) as under control conditions the number of feeding bees increased with time, whereas with EMF exposure the number of feeding bees decreased with time significantly. After 15 minutes of EMF exposure between the hive and feeder the number of bees feeding had decreased by -3.0 ± 1.2 bees which was significantly lower than the 4.5 ± 1.7 bee increase observed under control conditions (Bonferroni: P = 0.0001). This suggests that an EMF interference at 100 µT causes a reduction in feeding capabilities in honeybees in this field scenario, by reducing the number of bees that feed over time. This may be related to flight results which suggest no effects of EMFs on bees returning from the feeder to the hive, but a reduction in the number of successful passes from the feeder towards the hive.

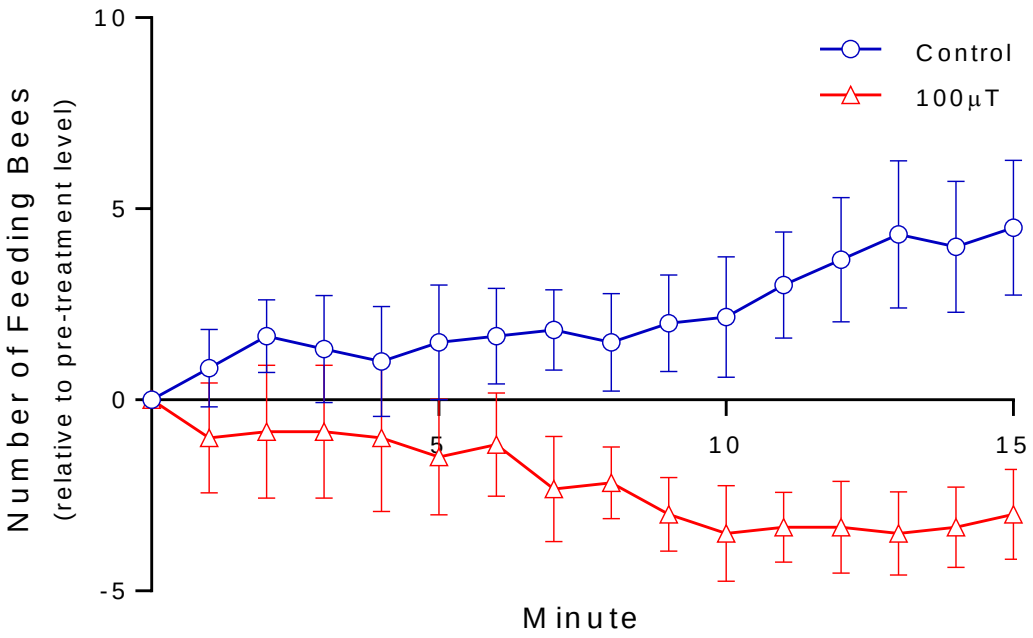

Figure 68. Amount of bees feeding each minute relative to baseline levels (the amount of bees immediately before treatment began) for 100 µT EMF and control treatments. Mean and SEM are plotted.

4.5 Discussion

4.5.1 Impacts of ELF EMFs on flight and feeding

Here it has been shown that ELF EMFs, at levels which can be encountered regularly around powerlines (100-7000 µT), can affect flight behaviour in bees. Magnetic field levels at 100 µT can be encountered at ground level below HVTLs, and EMFs higher than this can be encountered between ground level and the conductor. During lab-based assays immediate ELF EMF exposures of 1000-7000 µT increased flight activity (wingbeat frequency) of bees. When flight studies were expanded to the field, ELF EMF exposure of 100 µT reduced the percentage of successful outgoing flight passes from hive to food source but not the percentage of successful returning flight passes from the food source to hive, which likely led to the reduced number of bees feeding at the food source during ELF EMF exposures. These findings, that ELF EMFs cause reduced ability to feed (and potentially acquire important resources for the colony), and reduced flight performance (reducing movement of bees towards important food resources) as well as increased flight activity (likely leading to increased energy expenditure in flight), can be detrimental not just to the individual honey bee, but also the colony, in terms of efficiency of resource allocation, energy expenditure and overall robustness of colonies.

4.5.2 Consequences of ELF EMF effects on bees

As mentioned previously, insect flight is an intense, energy-demanding motor behaviour (Brembs et al., 2007) and resultantly honey bee lifespan is highly correlated with total flight performance and activity/energy turnover as shown by the dramatic seasonal relationship between increased honey bee mortality and increased energy expenditure during periods of increased foraging (Neukirch, 1982). Factors which reduce metabolic efficiency have been associated with poor overwinter survival of hives (Harrison et al., 2001). Consequentially, actions of bees during foraging behaviour are executed to maximise efficiency (Schmid-Hempel et al., 1985). Wingbeat frequency is a major attribute of flight dynamics, increases in which cause increased energy expenditure (Harrison and Roberts, 2000). It is not likely that ELF EMFs will cause long-term increased energy expenditure in flight, but rather if ELF EMFs make flight inefficient (for example by causing an increase in wingbeat frequency), these conditions could cause bees to avoid ELF EMFs. Bees did avoid ELF EMFs to an extent when foraging, as ELF EMFs here reduced outgoing flight success towards a food source. Return flights to the hive where not affected, and as a result

overall feeding decreased. Access to quality food sources is essential for pollinator health (Brodschneider and Crailsheim, 2010) and consequentially the potential of ELF EMFs to limit food sources for bees is concerning. Greenberg et al. (1981), found that bee hives exposed to HVTLs had increased motor activity, abnormal propolisation, reduced weight gain of hives, queen loss, impaired production of queen cells, decreased sealed brood and poor winter survival; effects that match the findings of ELF EMF exposure on increased flight activity and reduced flight success in this study, and could be caused by poor acquisition of food sources and foraging inefficiencies.

4.5.3 Potential mechanisms of locomotory effects of ELF EMFs

The increased wingbeat frequency observed in flight may be an excitatory increase in locomotory activity as a physiological response to the EMFs, or a sensory response to move quickly out of the EMF (which bees could not do in the lab, as they were tethered), whereas the reduction in flight passes observed in the field appears to be locomotory avoidance of the 100 μT ELF EMF. These flight results suggest a negative magnetotactic response of honey bees to ELF EMFs that can be found under HVTLs, which potentially could arise as an aversive response to the physical effects of ELF EMFs, or a magnetosensory response. Magnetoreception is well documented in honeybees, with two potential mechanisms: cryptochrome (Gegear et al., 2008; Bazalova et al., 2016) and magnetite (Kirschvink et al., 2001; Liang et al., 2016) based magnetoreception. Either mechanism for detecting ELF EMFs could explain avoidance behaviour observed in this study. As mentioned previously the magnetosensitivity thresholds for honey bees are < 100 μT at 50 Hz (Kirschvink et al., 1992; Kirschvink et al., 1997), and as a result a sensory response of bees to EMFs generated by HVTLs cannot be ruled out.

Species often exhibit negative taxis towards environmental stimuli to avoid stress, and it is possible that ELF EMF exposure will elicit stress responses in honey bees, or lead to detrimental biological effects. As mentioned previously ELF EMFs have been shown to increase heat-shock protein expression in drosophila (Li et al., 2013), in locusts (Chapter 2) and bees (Chapter 3). Heat-shock proteins are chaperone proteins that are upregulated in response to environmental stress, and it is possible that ELF EMF exposure may cause molecular damage, and/or directly interact with the promoter regions of heat-shock proteins, to up-regulate heat-shock expression (Goodman and Blank, 2002). It is essential for future research to consider effects of ELF EMF pollution on molecular biology, and how this may underpin observed behavioural changes, when considering biological effects of ELF EMFs.

Physiological effects of ELF EMFs may also underpin the behavioural effects observed in this study. Octopamine, as the homolog of norepinephrine in insects, is a biogenic amine, the levels of which can change under stress, and which is suggested to be involved in insect fight or flight responses (Davenport and Evans, 1984). Octopamine has a crucial role in locomotory behaviour including flight (Fussnecker et al., 2006), and in altering muscle contraction kinetics and flight muscle glycolysis (Brembs et al., 2007). As mentioned earlier, (Chapter 4) 7000 μT ELF EMF exposure has been shown to increase activity in cockroaches, the effect of which is suppressed by phentolamine, a octopamenergic inhibitor, suggesting a key role of octopamine in the locomotory effects of ELF EMFs (Wyszkowska et al., 2006). Effects of ELF EMFs on octopamine, as well as other biogenic amines, are an essential future field of research to determine how ELF EMFs may affect physiological processes that underpin behaviour.

7000 μT ELF EMF exposure in locusts causes abnormal motor neuron signalling by increasing action potential duration and latency (Chapter 2). As well as this ELF EMFs have been show to affect wingbeat frequency in locusts (Chapter 2). Effects on neuronal signalling could underpin impacts of ELF EMFs on flight observed in this study. It is well established that ELF EMFs induce electric fields and currents within organisms, causing excitability of neurons (Dimbylow, 1998; Jacobson, 2005; WHO, 2007; Halgamuge et al., 2009), which is a potential mechanism for this kind of effect. The neuromuscular system driving bee flight is not as well characterised as in the locust, however it is known that large fibrillar muscles, the dorsolongitudinal and dorsoventral muscles, of bees are used to drive the wings during flight (Hasselrot, 1960; Bastian, 1972; Esch and Goller; 1991). Action potential frequency in dorsolongitudinal and dorsoventral muscles, and wingbeat frequency, are highly related (Bastian and Esch, 1970), as increases in action potentials in these muscle systems increase wingbeat frequency and flight speed (Bastian, 1972). Due to the known excitability effects of ELF EMFs in neurons (Dimbylow, 1998; Jacobson, 2005; WHO, 2007a; Halgamuge et al., 2009), and the known impacts of ELF EMFs on neuromuscular systems (Chapter 2), it is possible that this neuromuscular system may be affected in honey bees to bring about the observed changes in honey bee flight. To further determine effects of ELF EMFs, electrophysiological parameters must be explored in further detail, and it is clear that the timing of ELF EMF exposure in relation to critical memory formation may be important.

4.5.4 Potential wider ecological implications

ELF EMF effects on avoidance or modified locomotor activity may not be limited to bees, but may affect a wide range of insects. For example Prolić et al. (2003) found that a 50 Hz 2000 µT ELF EMF exposure of 15 minutes, increased locomotor activity in longhorn beetles with low/medium initial activity, and decreased activity in individuals with high initial activity. As well as this, Wijenberg et al. (2013) found that static electromagnetic fields (500-1000 µT) had differing attractive/repulsive effects in different insect species. Exposures of 4000-7000 µT ELF EMFs for 24 hrs led to reduced locomotory performance in desert locusts (Chapter 2). It is clear from literature, and the findings here, that ELF EMFs at intensities that can be encountered around HVTLs can affect the locomotion of insects and can lead to taxis, both attractive and repulsive, in a range of insects. As mentioned previously, powerline strips have been suggested to be important refuges for insect pollinators (Russell et al., 2005; Wojcik and Buchmann, 2012; Wagner et al., 2014; Berg et al., 2016; Hill and Bartomeus, 2016). The impacts of this on distribution of insects in the environment could be vast, and vary between differing insect species and their responses to ELF EMF exposures. Reduced colony success and flight performance in honey bees alone can reduce the reproductive success of the plants that they pollinate (Cranmer et al., 2012), which can lead to the degradation of ecosystem services provided by honey bees. It will be crucial for future research into the effects of ELF EMF pollution on the environment to consider the complex population or even community level effects in diverse habitats.

Chapter 5 The combined effects of ELF EMFs and the neonicotinoid insecticide clothianidin on flight and learning

5.1 Abstract

A variety of combined environmental stressors are currently implicated in the declines of flying insects in general, and important pollinator species. Given the previous findings of ELF EMF impacts on neurophysiology, and locomotory and cognitive behaviour, there is a potential that ELF EMFs could interact with other stressors that affect these biological functions. Here the combined impacts of acute ELF EMF exposure in combination with the neonicotinoid insecticide clothianidin were assessed on olfactory appetitive learning in the PER assay, and flight in a tethered lab assay. Clothianidin at the 2.00 ng/bee level, which is below the level which can be consumed per bee in a day, dramatically increased mortality. Clothianidin also increased initial wingbeat frequency and reduced cognitive performance of bees from colonies that were more susceptible to clothianidin mortality. ELF EMF exposure at high intensities caused an increase in wingbeat frequency, the magnitude of which was reduced slightly by clothianidin exposure. ELF EMF exposures at levels that can occur regularly at ground level under HVTLs reduced learning performance, an effect reduced in bees from colonies more susceptible to clothianidin induced mortality. These findings indicate no evidence of synergy between clothianidin and ELF EMFs and environmental stressors, but a potential for ELF EMFs to affect the same susceptible fraction of the bee population that may have been affected by clothianidin. These results lay the foundation to further explore interactions of ELF EMFs with other environmental stressors, and consider the key factors which may make bees susceptible to ELF EMF exposure, as well as other combined environmental stresses.

5.2 Introduction

In their natural environment the most likely route for acute exposure of bees to ELF EMFs is during activities such as foraging. While it is invaluable to determine the effects of acute exposure to ELF EMFs alone, in realistic field scenarios bees undergo short-term/chronic stresses from a variety of combined stressors including, but not limited to, poor resource quality/availability, disease, variable/extreme weather, and pesticide exposure

(Goulson, 2015). A critical step in determining the impacts of ELF EMFs on bees is determining the complex interactions that ELF EMFs may have with other environmental stressors, including here neonicotinoid insecticides, a critical environmental stressor of bees (Jeschke et al., 2010; Blacquiere, 2012; Goulson, 2013).

5.2.1 Neonicotinoids background

Clothianidin is one of the most widely used neonicotinoid insecticides (Goulson, 2013). Neonicotinoids are systemic insecticides that are water soluble and when applied to crops and are absorbed and translocated into plant tissues, providing protection against insects and arthropods (Goulson, 2013; Bonmatin et al., 2015). The mode of action of neonicotinoids is through binding to nicotinic acetylcholine receptors (nAChRs), which have an essential role in mediating fast excitatory synaptic transmission in the insect central nervous system (CNS) (Matsuda et al., 2001; Jeschke and Nauen, 2008; Jeschke et al., 2010). Low level activation of nAChRs, by neonicotinoid binding, causes nervous stimulation, while high levels lead to hyperexcitation, and eventually paralysis and death (Matsuda et al., 2001; Jeschke and Nauen, 2008). Acetycholinesterase cannot break down neonicotinoids, making their binding irreversible (Matsuda et al., 2001) and consequently they are extremely effective insecticides, and toxic at very low doses.

5.2.2 Neonicotinoid history and usage

Most neonicotinoids were developed in the 1980's, and were commercially approved in the 1990's and 2000's, the first being imidacloprid (Table 5). Clothianidin was approved for commercial use in Europe in 2006 (EFSA, 2013). In application, neonicotinoids have a broad range of targets, and are used on a variety of crops including corn, cereals (wheat, barley, oats), mustard, oilseed rape, sunflower, beets and potatoes (EFSA, 2013; Goulson, 2013), to control various sucking/biting pests including aphids, whiteflies, thrips, leaf miners, beetles and lepidoptera (Elbert et al., 1998) . Application methods include spraying, but as a systemic insecticide the most common method is as a seed dressing, which comprises 60% of the global neonicotinoid usage (Goulson, 2013).

Table 5. List of commercially used neonicotinoid insecticides, patent dates (Tomizawa and Casida, 2005), their 24 hr contact LD_{50}'s (Iwasa et al., 2004), and first date of use in the UK (FERA, 2017) - note: unused neonicotinoids (Nitenpyram and Dinotefuran), have not been approved for EU use.

Insecticide	Patent	First Use (UK)	24 hr Contact LD_{50} (ng/bee)
Imidacloprid	1985	1994	18
Thiacloprid	1985	2001	14600
Nitenpyram	1988	-	138
Acetamiprid	1989	2007	7070
Clothianidin	1989	2006	22
Thiamethoxam	1992	2005	29
Dinotefuran	1994	-	75

Neonicotinoid usage increased dramatically in the early 2000's, with imidacloprid, clothianidin and thiamethoxam becoming the most commonly used neonicotinoids (Fig. 68, A-B), largely due to their high toxicity. While imidacloprid has been used longest, clothianidin has been the most widely used neonicotinoid in recent years in many countries, including the UK (Fig. 68, A) and the USA (Fig. 68, B). In addition, residues of the major neonicotinoid thiamethoxam are metabolised to the more toxic clothianidin within plants and insects (Nauen at al., 2003; Benzidane et al., 2010). As a result, the use of the active ingredient clothianidin (red – Fig. 68, A-B) and its precursor (orange – Fig. 68, A-B) makes up the majority of neonicotinoid use in the UK and USA. The total area of land treated with neonicotinoids in 2013 was nearly 6% of the UK (Carreck and Ratnieks, 2014).

Initially neonicotinoids were deemed safe for non-target insects, due to the systemic nature of neonicotinoid action. Concern began to develop that neonicotinoid residues could be translocated into the nectar and pollen of plants, leading to neonicotinoid exposure in bees and other pollinators (Blacquiere, 2012). Neonicotinoids are extremely toxic to bees with a 24 hr contact LD_{50} as low as 18 ng/bee (Table 5) and 24 hr oral LD_{50} of only 4 ng/bee for imidacloprid, making neonicotinoids 10,000 times more toxic to bees than dichlorodiphenyltrichloroethane (DDT) (Goulson, 2013). Moreover, the range of crops with which neonicotinoids are used are attractive to bees, and include alfalfa, beet, clover, corn, mustard, oilseed rape, sunflower, poppy chicory, and cereals (EFSA, 2013). Following the rise in evidence of the effects of neonicotinoids on bees (Blacquiere, 2012; Goulson, 2013) in December 2013, the European Commission imposed a two year moratorium on the use

imidacloprid, clothianidin and thiamethoxam as a seed dressing on crops attractive to bees (European Commission, 2013) with current expectations that this ban will be made permanent. These neonicotinoids, however, are still heavily used around the rest of the world.

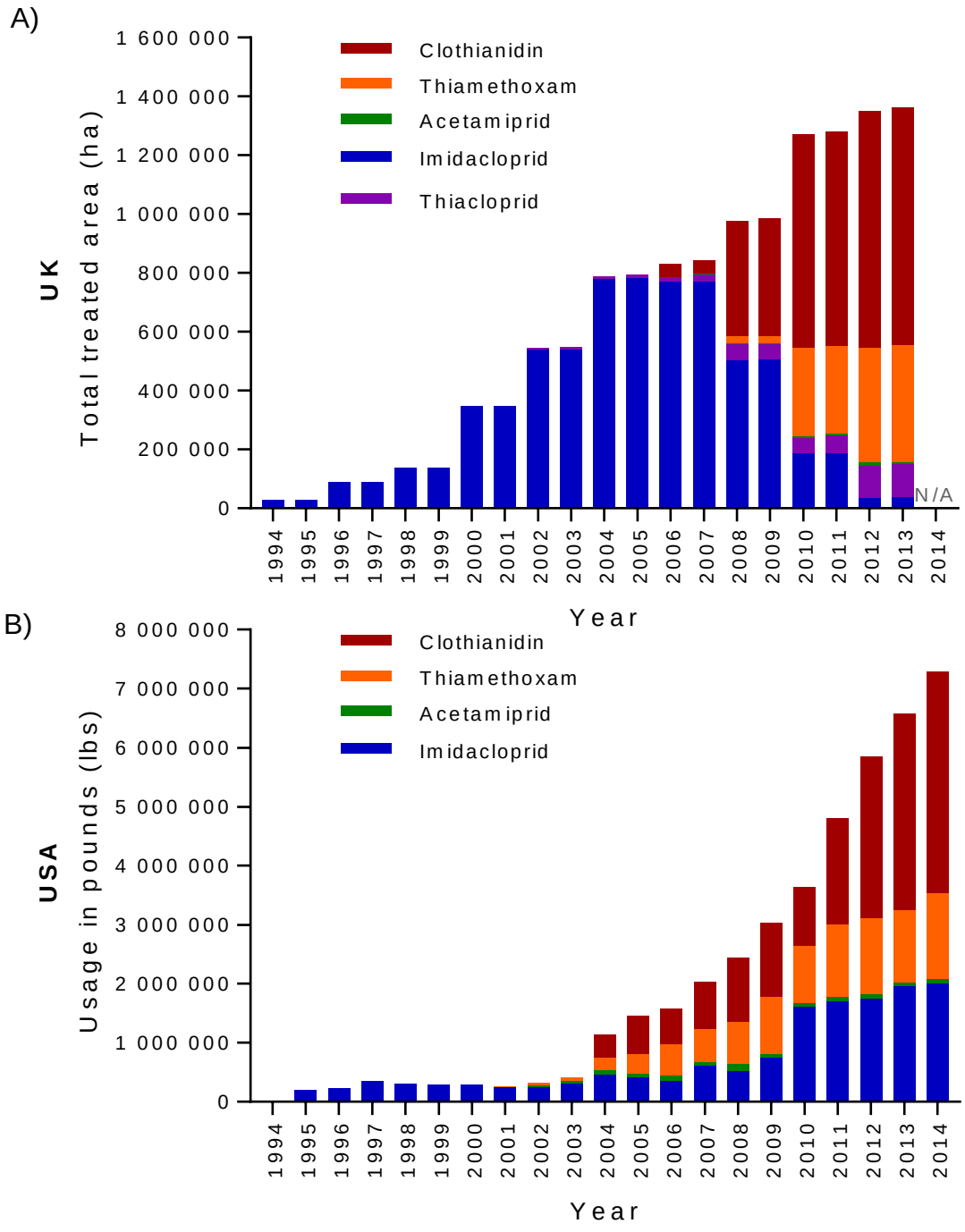

Figure 68. Examples of neonicotinoid use since 1994. A) UK annual use in land area (ha). Data collated from FERA (2017). B) USA annual usage in pounds applied (lbs). Data collated from USGS (2017).

5.2.3 Field exposure

In their natural environment bees can be exposed to neonicotinoids in a variety of ways. The greatest concern for bee exposure comes through the consumption of residues in nectar and pollen. Clothianidin, for example, is often found at levels above 10 ppb in pollen of treated crops, and has been measured at levels as high as 88 ppb in pollen carried by bees (Henry et al., 2012). The average range of field realistic doses of clothianidin in nectar range from 1-14 ppb (Sanchez-Bayo and Goka, 2014; Bonmatin et al., 2015; Botías et al., 2015; Rundlöf et al., 2015), but can be higher or lower depending on crop type. 1 ml of nectar at 14 ppb contains a 14 ng dose of clothianidin. Given the varied consumption rates of bees, the EFSA (2013) calculates a worst case scenario for bees feeding on oilseed rape of a 4.3-13.7 ng dose of clothianidin per day. This is alarming as the 24 hr oral LD_{50} of clothianidin is only 3.79 ng/bee (EFSA, 2013). Further, exposure routes other than nectar and pollen may provide far higher doses of neonicotinoids. Guttation fluid is xylem sap naturally secreted as droplets by plants, and due to the water solubility of neonicotinoids, they are found at extremely high concentrations in guttation drops of treated plants. For example, Tapparo et al. (2011) found clothianidin in guttation drops at concentrations from 76,200 – 101,700 ppb at the top, and 7,300 – 47,000 ppb in the crown cup, of corn leaves. Girolami et al. (2009) found clothianidin in guttation from seed treated corn constantly higher than 10,000 ppb, with a maximum of 100,000 ppb. The highest recorded concentration of clothianidin in guttation fluid is 717,000 ppb (EFSA, 2013). At these levels < 0.006 µl of guttation fluid contains the median lethal dose (LD_{50}) of clothianidin, and consumption can lead to death in minutes. This is alarming as bees commonly forage for water (Visscher et al., 1996) and have been observed drinking guttation drops (Shawki et al., 2006).

5.2.4 Biological effects

While the 'worst case scenario' for biological effects of neonicotinoids results in death, common exposures of bees to neonicotinoids are below lethal doses, and sublethal impacts of these insecticides are a major concern in assessing their environmental safety. The interaction of neonicotinoids with bee nAChRs is neurotoxic, and is likely to affect cognitive and motor neural processes, underpinning sub lethal effects. For example, clothianidin and imidacloprid cause a depolarization-block of neuronal firing and inhibit nicotinic responses in mushroom body Kenyon cells (Palmer et al., 2013). As Kenyon cells in the mushroom bodies of bee brains have a critical role in learning and memory formation (Menzel, 2012), this physiological effect is likely to lead to cognitive impairment in honey bees.

Neonicotinoids have been shown to cause cognitive effects in bees, including reduced olfactory learning acquisition in PER assays (Decourtye et al., 2004a; Decourtye et al., 2004b; Han et al., 2010; Williamson and Wright, 2013; Tan et al., 2015) and learning performance in semi-field foraging activities (Decourtye et al., 2004b; Han et al., 2010). Longer term impacts have been suggested as well, for example, winter bees surviving imidacloprid exposure had reduced learning performance in PER assays (Decourtye et al., 2003). For clothianidin, with 12 days of free feeding at 4 ppb, olfactory learning acquisition is reduced in honey bees (Piiroinen and Goulson, 2016). A 0.76 ng/bee dose of clothianidin interferes with solitary bee (*Osmia cornuta*) navigational memory (Jin et al., 2015). Fischer et al. (2014) found a dose of 2.5 ng/bee increased the length of homing flights, and Matsumoto (2013) found a 0.22 ng/bee dose reduced proportions of successful homing flights in honey bees. Doses $\geq$ 0.50 ng/bee increase the length of foraging flights (Schneider et al., 2012). Moreover, thiamethoxam, which is metabolised to clothianidin, reduces bumble bee learning and memory (Stanley et al., 2015), and failed honey bee homing flights (Henry et al., 2012).

The effects neonicotinoids have also been linked to reduced motor function in bees, leading to various behavioural effects, including knockdown, poor coordination, increased activity and tremors (Lambin et al., 2001; Nauen et al., 2001; Suchail et al., 2001; Medrzycki et al., 2003; Colin et al., 2004). Williamson et al. (2014) found that a 0.34 ng/bee dose of clothianidin reduced basic motor function, and Alkassab and Kirchner (2018) found motor effects above 0.10 ng/bee, with locomotory behavioural effects at 0.50 ng/bee. Thiomethoxam, as a precursor to clothianidin, has also been shown to cause locomotor deficit in honey bees after a 3.8 ng/bee dose (Charreton et al., 2015).

5.2.5 The combined effects of neonicotinoids and ELF EMFs

When neonicotinoids are consumed by bees at a sub lethal level, compounds which have not bound with nAChRs can be detoxified. Detoxification of neonicotinoids, however, has been shown to require the engagement of multiple molecular pathways including antioxidant and heat-shock responses as well as increased energy investment (Esther et al., 2015; Gong and Diao, 2017). Christensen et al (2016) also found transcriptional alternations from neonicotinoid exposure of genes for vitellogenin (stores of which have an essential role in bee health), apidaecin and densin-1 (which have immune functions). Esther et al. (2015) identifies this as one major potential cause for interactions with other environmental stressors. Moreover, the known impacts of neonicotinoids on cognitive behaviour and motor

function makes interactions of neonicotinoids with stressors that also affect these processes highly likely.

So far I have shown that ELF EMFs affect stress responses in insects (Chapter 2 and Chapter 3), motor neuron spike dynamics (Chapter 2), locomotory behaviour (Chapter 2 and Chapter 5), and cognitive behaviour (Chapter 3 and Chapter 4). HVTLs must be built on flat land, and in general away from residential areas, making farmland highly suitable for HVTL construction (Fig. 69). Consequently in the natural environment it is likely that insects that are exposed to ELF EMFs will have been exposed to agrochemicals, including potentially neonicotinoid insecticides, and that this exposure will occur during foraging activities. Here, the effects of ELF EMF on flight and associative appetitive learning will be investigated in combination with the neonicotinoid insecticide clothianidin, to determine how this well studied environmental stressor interacts with acute effects of ELF EMFs.

Figure 69. Photograph of a high-voltage transmission line bisecting a field used for oilseed rape crop in the United Kingdom

5.2.6 Aims and Objectives

The results of the previous chapter showed that exposure to ELF EMFs alone at field-realistic levels can affect cognitive and locomotory behaviour in honey bees. In the environment honey bees and other pollinators are commonly exposed to a variety of

environmental stressors, however ELF EMFs have previously been overlooked as a factor which could cause biological effects in combination with other environmental stressors.

The aim of this chapter is to determine whether ELF EMFs may have combined effects with another well-known environmental stressor, neonicotinoid insecticides. Therefore in this chapter bees will be pre-treated with neonicotinoids before acute exposure to ELF EMF occurs. Hive susceptibility to neonicotinoids will be monitored, however neonicotinoid pre-treatment will not be adjusted by concentration for each hive as the aim here is to observe how single field-relevant doses of neonicitoids affect varied populations of bees, and how a specific neonicotinoid dose may interact with ELF EMFs as an environmental stressor. The main aim of monitoring hive susceptibility is to increase the information available that may explain differences between ELF EMF and neonic effects on bees from different hives. If any effects exist, then a further aim of this chapter is to elucidate whether these effects may occur at an ELF EMF exposure level which a bee may encounter in the environment, and to begin to determine any potential causes and thresholds for effects.

This chapter addresses the following questions

To what extent does clothianidin as a neonicotinoid insecticide, and a known environmental stressor, affect bees from different hives?

Do ELF EMFs have combined effects with neonicotinoid insecticides?

5.3 Methods

5.3.1 Magnetic fields

In all experiments 50 Hz EMFs were produced using the Helmholtz coil apparatus previously described (2.3.3.1). For experiments testing the combined ELF EMF and clothianidin impacts on learning and flight, the same exposures were used as they were for each respective experiment testing the effects of ELF EMFs alone (Learning: 20 µT, 100 µT and 1000 µT – see Chapter 4; Flight: 100 µT, 1000 µT, 7000 µT – see Chapter 5).

5.3.2 Clothianidin treatment preparation

Analytical standard Clothianidin (PESTANAL - 33589; Sigma-Aldrich, UK) was used for all experiments. Clothianidin was dissolved in acetone, and then diluted to treatment concentrations by adding distilled water. For control treatment no clothianidin was added, but acetone was added and prepared to the same dilution as for clothianidin treatments. Sucrose was also added to solutions. All final solutions had a final concentration of 0.000125% (v:v) acetone and 50% (w:v) sucrose.

5.3.3 Clothianidin mortality

5.3.3.1 Collection and exposure

To determine the effects of clothianidin on honey bee mortality the LD_{50}'s were determined for each hive. Returning forager honey bees were collected from the University of Southampton campus apiary, Hive 1_{2016}, Hive 2_{2016}, and Hive 3_{2016}, in 1.5 ml Eppendorf tubes that were perforated so that bees could be fed solutions with a micropipette (Fig. 70), as well as to allow airflow. 15 forager bees were collected at a time from the same hive. Bees were fed 7 different doses of clothianidin ranging from 0.25-20.00 ng/bee (Table 6), dissolved in 10 µl of sucrose solution, while individually held in Eppendorf containers. Immediately after exposure bees were transferred to plastic containers (4.3.1) with feeders filled with 50% (w:v) sucrose, from which they could feed freely. 15 bees from the same hive and treatment were added to the same container, which in LD_{50} analyses formed a single cohort. For each of the 7 concentrations of clothianidin 3 replicate cohorts of 15 bees were used for each of the 3 hives, giving 9 cohorts (135 bees) from all hives for each treatment (Table 7). Containers were kept at 29 ± 1 ºC and assessed 24 hrs after exposure.

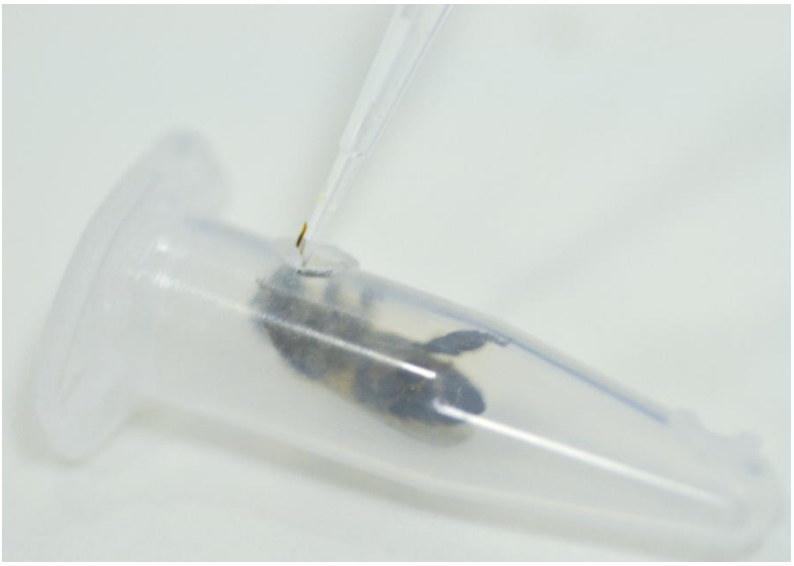

Figure 70. Honey bee in Eppendorf tube being fed solution with a 2-20 µl micropipette. Used for Clothianidin exposure in LD_{50} and flight experiments.

Table 6. Clothianidin doses used for all 7 treatments in toxicity analyses and delivery concentrations and cohort size for each treatment. Each treatment was applied to 15 bees from each hive 3 separate times, with 3 origin hives, giving a total of 135 bees for each treatment.

Dose	**In**	**Concentration**	**Cohort**		**Total bees**	
ng/bee	*µl*	*ppb*	*bees*	*reps/hive*	*per hive*	*all hives*
0.25	10	25	15	3	45	135
0.50	10	50	15	3	45	135
1.00	10	100	15	3	45	135
2.00	10	200	15	3	45	135
4.00	10	400	15	3	45	135
10.00	10	1000	15	3	45	135
20.00	10	2000	15	3	45	135

5.3.3.2 Mortality assessment

Twenty four hours after clothianidin treatment, bee mortality in each container was assessed using *COLOSS* standard methods for toxicology research in *Apis mellifera* (Medrzycki, et al., 2013). The number of dead bees in each container was totalled, and then analysed to determine the LD_{50} for each hive as well as all hives combined.

5.3.4 Appetitive learning

Returning forager honey bees were collected from the University of Southampton campus apiary from Hive 1_{2016}, Hive 2_{2016}, and Hive 3_{2016} (subscript indicates collection year) from the hive entrance in Sterilin 30ml universal containers (Sterilin Limited, Cambridge, UK). Bees were immobilized on wet ice and harnessed in PER tubes made from 1ml pipette tips to the design from Bitterman et al. (1983) with head, antennae and forelegs free, as for all other PER experiments (**Error! Reference source not found.**). Bees were then fed a single treatment dose (prepared via clothianidin treatment protocol) in a 10 µl volume solution. 3 different treatments were used, a control containing no clothianidin ('sucrose'), and two clothianidin treatments of 0.25 ng/bee (0.025 ng/ µl) and 2.00 ng/bee (0.200 ng/ µl). Over 110-150 bees were sampled for each clothianidin treatment from every hive (Table 7), giving a total of 1,131 bees collected for combined clothianidin/EMF PER experiments. After treatment application, procedures followed the protocol for PER assays for acute effects of ELF EMFs on learning (**Error! Reference source not found.**). PER assays were conducted 17 hrs after clothianidin treatment was applied. PER assays followed the same conditioning format, recording criteria, exclusion criteria and EMF exposures as for the acute effects of ELF EMFs on PER, where ELF EMFs were applied for 1 min after each conditioning trial. Within each clothianidin treatment bees were evenly grouped into one of 4 EMF treatments (control, 20 µT, 100 µT, and 1000 µT), giving a total of 12 combined treatments (*Clothianidin x EMF*: 3 x 4 = 12 treatments). 5 conditioning trials were conducted total for each bee.

Table 7. Clothianidin treatments used in PER assay for analysis of the combined effects of clothianidin and ELF EMFs. Numbers of bees for each treatment are given for each hive, as well as total numbers.

Treatment	**Dose**	**In**	**Concentration**	**Total bees**			
name	*ng/bee*	*µl*	*ppb*	*Hive 1*	*Hive 2*	*Hive 3*	*All*
Sucrose	0.00	10	0	130	114	120	364
0.25	0.25	10	25	121	115	121	357
2.00	2.00	10	200	135	132	143	410
-	-	-	-	**386**	**361**	**384**	**1131**

5.3.5 Flight

To determine the combined impacts of clothianidin and ELF EMFs on flight bees were collected via the same method as described as for LD_{50} (Table 8). Forager bees were collected from the University of Southampton Campus Apiary from Hive 1_{2016}, Hive 2_{2016}, and Hive 3_{2016} in perforated 1.5ml Eppendorfs. Bees were given 1 of 3 different treatments, a control containing no clothianidin ('sucrose'), and two clothianidin treatments of 0.25 ng/bee (0.025 ng/ µl) and 2.00 ng/bee (0.200 ng/ µl). Immediately after treatment bees were transferred to plastic containers and from this stage followed protocol for the acute impacts of ELF EMFs on flight (4.3.1). Flight assays were conducted 24 hr after clothianidin treatment was applied using the tethered flight assay, and high speed video recording to determine the impacts of ELF EMF exposure on wingbeat frequency. Within each clothianidin treatment bees were evenly grouped into one of 4 EMF treatments (Control, 100 µT, 1000 µT and 7000 µT), giving a total of 12 combined treatments (*Clothianidin x EMF*: 3 x 4 = 12 treatments). For each of the 12 combined clothianidin EMF treatments at least 10 bees were tested for each hive, giving a minimum a 30 bees per treatment from all hives, and a total of 369 bees sampled in combined effects of clothianidin and ELF EMFs on tethered flight. Experimental protocol and analyses were conducted in the same manner as for the acute effects of ELF EMFs on tethered flight (Chapter 5).

Table 8. Total numbers of bees for each of the combined clothianidin/EMF treatments used in the tethered flight assay

Treatment		**Total bees**			
Clothianidin	*EMF*	*Hive 1*	*Hive 2*	*Hive 3*	*All*
Sucrose	Control	10	10	10	30
	100 µT	10	10	10	30
	1000 µT	10	10	10	30
	7000 µT	10	10	10	30
0.25	Control	10	10	10	30
	100 µT	10	10	10	30
	1000 µT	10	10	10	30
	7000 µT	10	10	10	30
2.00	Control	10	10	15	35
	100 µT	10	10	10	30
	1000 µT	10	10	10	30
	7000 µT	10	10	14	34
-	-	**120**	**120**	**129**	**369**

5.3.6 Statistical analysis

Data were analysed in SPSS (v.24, IBM SPSS Inc.) and Graphpad Prism (v.7, Graph Pad Software Inc.). Where appropriate, homogeneity of variance and normality assumptions were tested. If required, data transformations were made, or alternative statistical tests were chosen. For all models assessing the impacts of treatments on binomial PER data (as well as mortality and gustatory responsiveness in the PER assay), binomial error structure and logit link function were used. Where appropriate pairwise contrasts with Bonferroni adjusted significance were used in *post-hoc* analyses.

LD_{50} analyses were conducted following standard methods for toxicology research in *Apis mellifera* (Medrzycki, et al., 2013). To determine LD_{50}'s for each hive and all hives combined, proportions of mortality were plotted in dose-response curves. Regression analyses were used to plot dose-response curves with 95% confidence limits, where clothianidin doses were logarithmically transformed and mortality proportions were logit transformed. LD_{50}'s and other median lethal doses (LD_{10} and LD_{90}) were interpolated from dose-response curves.

To determine the combined effects of ELF EMFs and clothianidin on learning in the PER assay bees were exposed to clothianidin 17 hrs before the assay was conducted. Clothianidin treatment could potentially cause more bees to be excluded from the PER assay by either increasing mortality at 17 hrs, or by decreasing gustatory responsiveness. To determine this the impact of clothianidin treatment (sucrose, 0.25 ng, or 2.00 ng) on the proportion of mortality, and the proportion of gustatory responsiveness in surviving bees, was assessed using GLMs, with 'hive of origin' as an interaction factor. A GLMM was used to analyse the effects of combined 'Clothianidin' and 'EMF' treatments on learning acquisition (PER) in trials 2-5 (trial 1 was not include as bees cannot exhibit conditioned PER in trial 1, and to improve model fit), including 'hive of origin' and 'trial number' as interactive factors.

To determine the combined effects of ELF EMFs and clothianidin on bee flight, bees were collected from 3 different hives, and bees were exposed to clothianidin 24 hrs before the flight assay was conducted. Hives may have had inherent variation in wingbeat frequency, and clothianidin treatment could potentially have initial impacts on the baseline wingbeat frequencies of bees, before EMF treatments were applied. To determine whether these factors affected wingbeat frequency the impact of 'clothianidin treatment' and 'hive of origin' on wingbeat frequency was assessed using a two-way ANOVA. To determine whether combined ELF EMF and clothianidin treatment affected wingbeat frequency the change in wingbeat frequency in tether flight assay was classified as the dependent variable, with 'clothianidin treatment', 'EMF treatment' and 'hive of origin' as interactive factors in a univariate ANOVA.

5.4 Results

5.4.1 Clothianidin mortality

To determine the effect of clothianidin on bee mortality dose-response curves for 7 clothianidin doses (0.25 ng, 0.50 ng, 1.00 ng, 2.00 ng, 4.00 ng, 10.00 ng, and 20.00 ng) and 24 hr mortality were plotted for each individual hive, and all hives combined. Some hives were more susceptible to clothianidin exposure than others (Fig. 71, A-D). The 24 hr LD_{50} for Hive 1_{2016} was 3.03 ng/bee (95% CI: 2.57-3.49 ng/bee) which was mid-range compared

to the other two hives (Fig. 71, A). Hive 2_{2016} was the most resilient to clothianidin exposure (Fig. 71, B) with the highest 24 hr LD_{50} at 4.22 ng/bee (95% CI: 3.82-4.62 ng/bee). In contrast, Hive 3_{2016} was the most susceptible to clothianidin exposure, with the lowest 24 hr LD_{50} at 2.59 ng/bee (95% CI: 2.10-3.08 ng/bee).

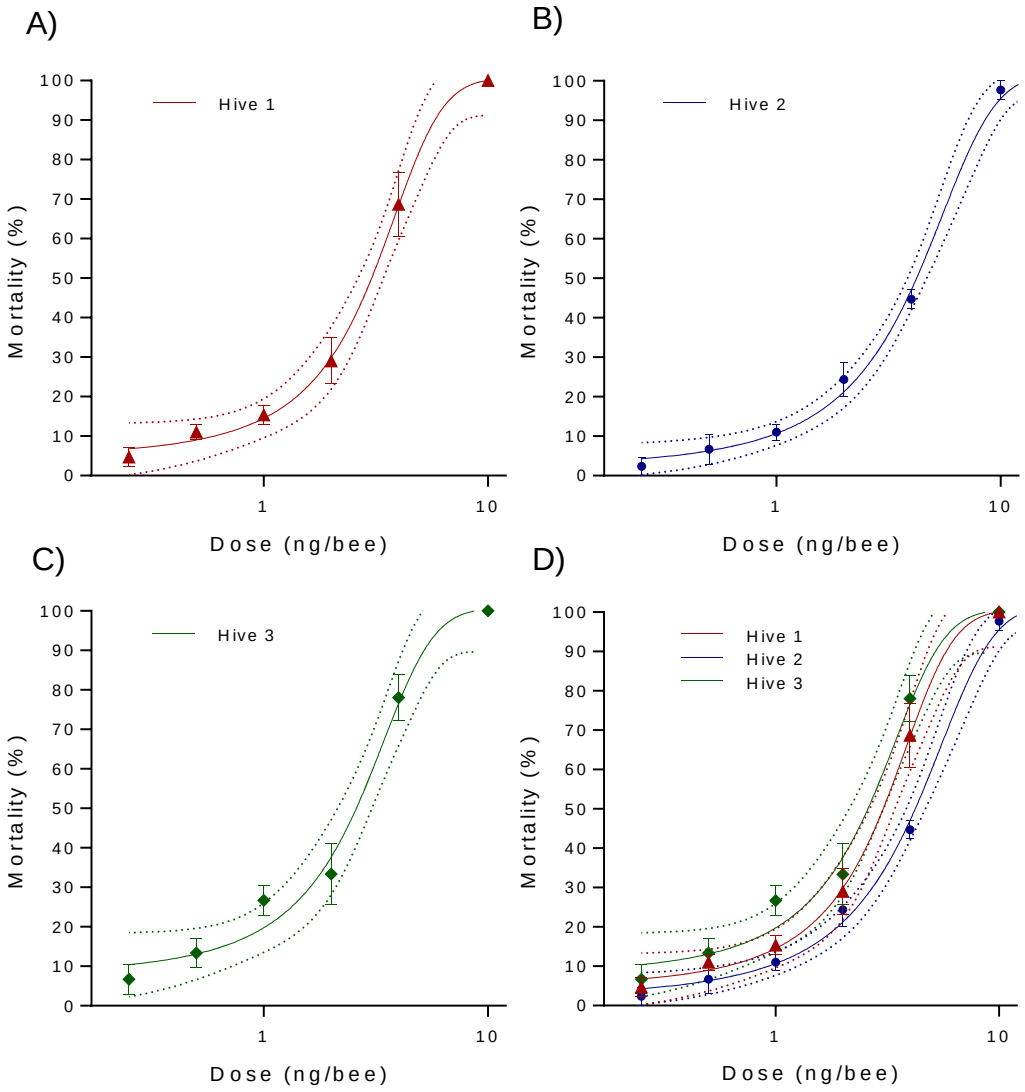

Figure 71. Dose-response curves for the effect of clothianidin on bee mortality for each of the individual hives as well as all three hives. Log dose is plotted. Dotted lines show 95% confidence intervals. Data points and error bars show mean and SEM. A) Hive 1. B) Hive 2. C) Hive 3 D) Hives 1, 2 and 3. Hives plotted separately for clarity, and together to allow comparison of data.

After pooling all hive data (Fig. 72) the 24 hr LD_{50} for clothianidin was 3.16 ng/bee (95% CI: 2.85-3.46 ng/bee). For all hives doses lower than 1.00 ng/bee were sufficient to cause 10% mortality (Table 9), and doses higher than 5.00 ng/bee were required to cause 90% mortality.

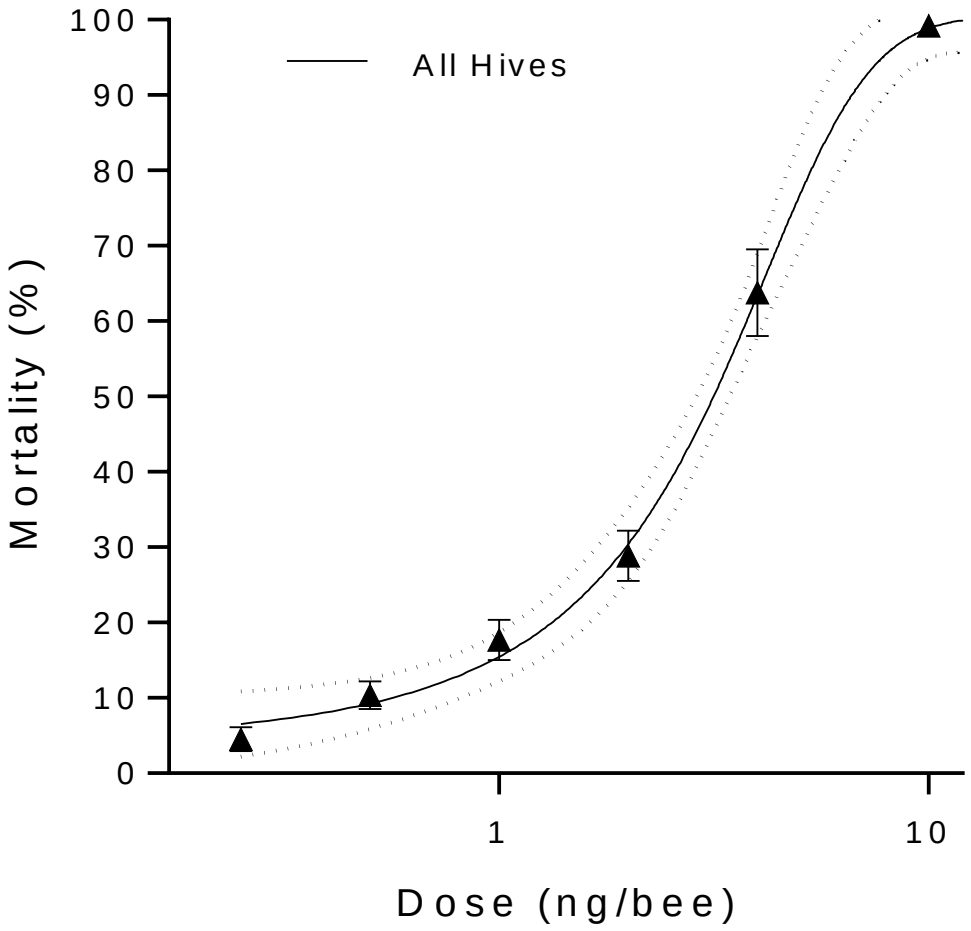

Figure 72. Dose-response curve for the effect of clothianidin treatment on mortality for all hives combined. Log dose is plotted. Dotted lines show 95% confidence intervals. Data points and error bars show mean and SEM

Table 9. Lethal doses (LD_{10}, LD_{50}, and LD_{90}) of clothianidin for each individual hive as well as all hives combined in mortality analysis

Hive	Lethal Dose (ng/bee)		
	LD_{10}	LD_{50}	LD_{90}
1	0.6	3.03	5.8
2	0.93	4.22	8.5
3	0.21	2.59	5.19
All Hives	**0.56**	**3.16**	**6.63**

5.4.2 Appetitive learning

5.4.2.1 Exclusions

For PER experiments the numbers of bees that died before or during trials was recorded, as these bees were excluded from the PER assay. In addition, bees were excluded for failing gustatory responsiveness tests. The impact of different clothianidin treatments may have affected the number of bees that were excluded from the PER assay, and thus impacted on results in the PER assay. Impacts of clothianidin treatment on mortality and gustatory responsiveness were analysed.

5.4.2.1.1 Mortality

Mortality in the PER assay was measured at 17 hrs, plus any bees that died during conditioning trials (Fig. 73). Slightly higher mortality was expected in these analyses as bees were held in PER tubes, and not in groups in holding cages with the ability to freely move and feed as they were in LD_{50} experiments. Results here mirrored the results from the LD_{50} analysis. Hive 3 (24 hr LD_{50}: 2.59 ng/bee) was again the most susceptible to clothianidin exposure with 14.2% mortality for sucrose treatment, 19.8% mortality for a 0.25 ng/bee dose, and 53.8% mortality for a 2.00 ng/bee dose. Hive 1 (24 hr LD_{50}: 3.03 ng/bee) again was intermediate in terms of susceptibility to clothianidin exposure, with 6.2% mortality for sucrose treatment, 15.7% mortality for a 0.25 ng/bee dose, and 24.4% mortality for a 2.00 ng/bee dose. Hive 2 (24 hr LD_{50}: 4.22 ng/bee) again was the most resilient to clothianidin exposure with 7.0% mortality for sucrose, 7.8% mortality for a 0.25 ng/bee dose, and 12.9% mortality for a 2.00 ng/bee dose.

For all hives combined sucrose treatment caused 10.4% mortality, a 0.25 ng/bee clothianidin dose caused 14.7% mortality, and a 2.00 ng/bee dose caused 30.7% mortality. There was a significant *'clothianidin' x 'hive of origin'* interaction effect on mortality (GLM: $\chi^2 = 10.58$, d.f. = 4, P = 0.032), as 2.00 ng/bee exposure caused significantly higher mortality in hive 3 than all other exposures in all hives (Bonferroni P = 0.001), and 2.00 ng/bee exposure in hive 1 caused significantly lower mortality than 2.00 ng/bee in hive 2, but higher mortality than all other exposures in all other hives (Bonferroni P = 0.001-0.007). In summary, hives had an inherent variation in clothianidin susceptibility in terms of mortality, with hive 3 being most susceptible, and hive 2 being least susceptible, to clothianidin induced mortality.

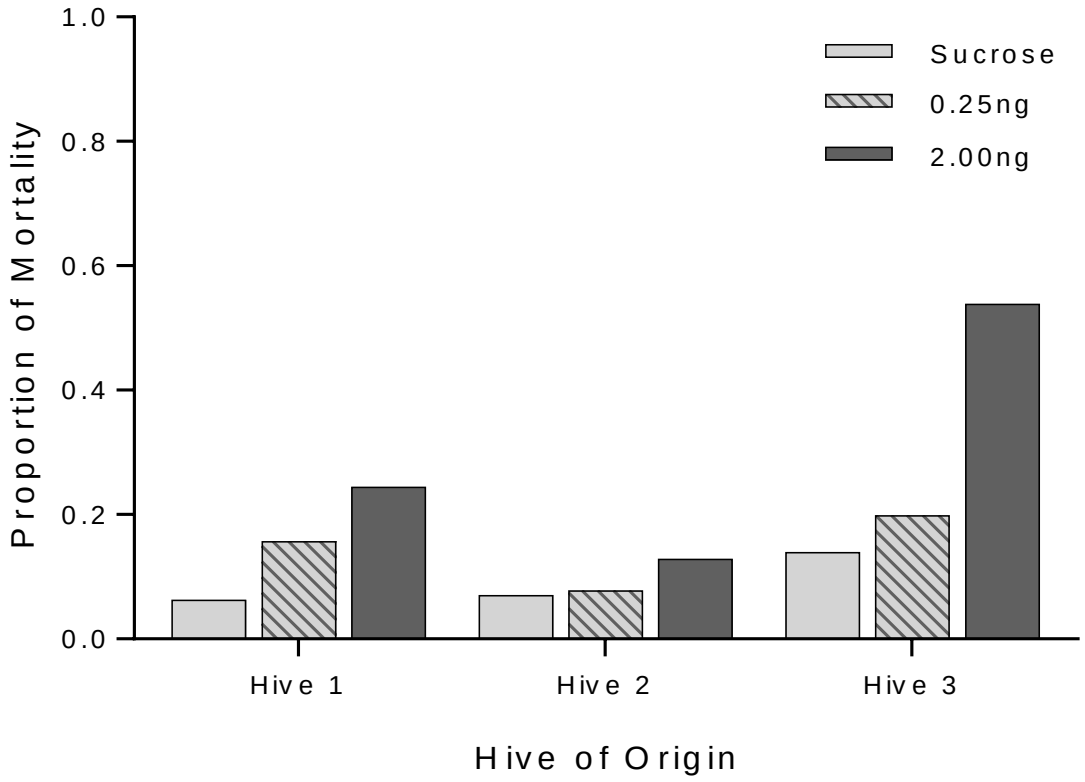

Figure 73. Effect of clothianidin treatment on mortality in the PER assay for bees from different hives. The exact proportion of mortality is plotted.

5.4.2.1.2 Gustatory responsiveness

There was no evidence of an effect of clothianidin on gustatory responsiveness (Fig. 74). For hive 1 gustatory responsiveness was highest, with 92.2% of bees responding after sucrose treatment, 93.0% of bees responding after a 0.25 ng/bee dose, and 83.3% of bees responding after a 2.00 ng/bee dose. Hive 2 had the lowest gustatory responsiveness, with 85.8% of bees responding after sucrose treatment, 81.0% responding after 0.25ng/bee treatment, and 83.4% responding after 2.00 ng/bee treatment. Hive 3 had an intermediate level of gustatory responsiveness with 87.3% of bees responding after sucrose treatment, 88.5% responding after 0.25ng/bee treatment, and 83.8% responding after 2.00 ng/bee treatment. Overall there was no *'Clothianidin' x 'Hive of origin*' interaction effect on gustatory responsiveness (GLM: $\chi^2 = 4.54$, d.f. = 4, P = 0.34) no a main effect of clothianidin on gustatory responsiveness. (GLM: $\chi^2 = 4.76$, d.f. = 2, P = 0.09). Bees had a slight inherent variation in gustatory responsiveness dependent on their hive of origin (GLM: $\chi^2 = 6.26$, d.f. = 2, P = 0.04).

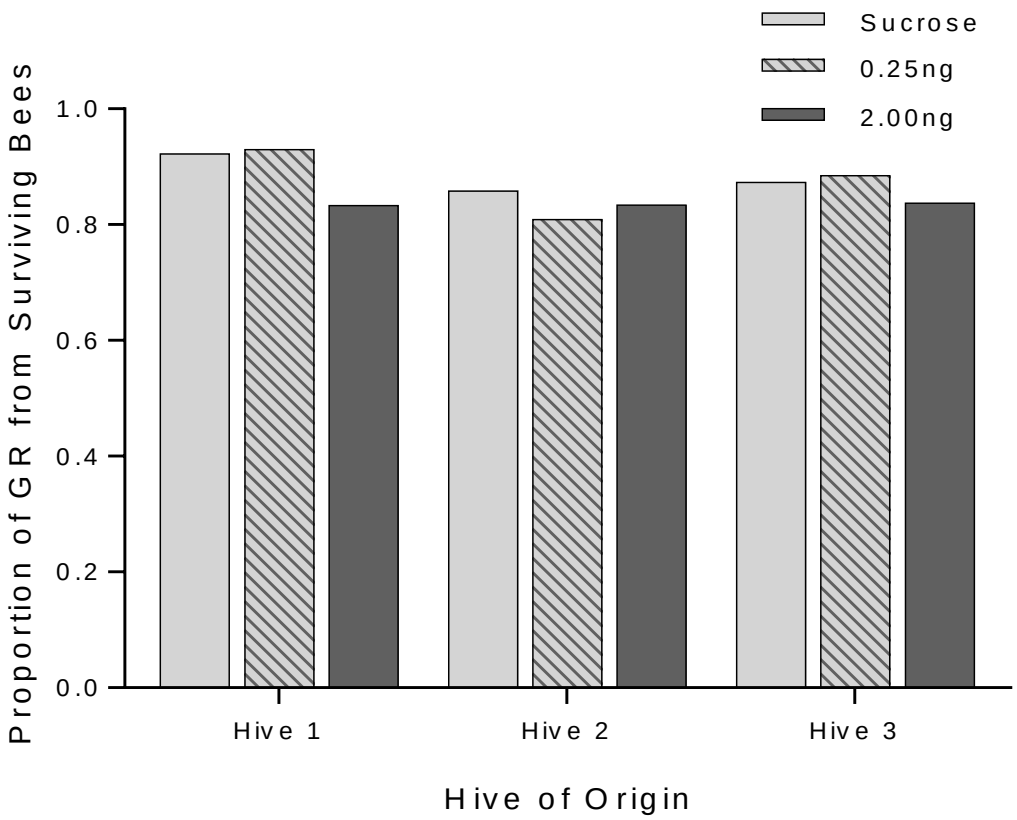

Figure 74. Effect of clothianidin on gustatory responsiveness in the PER assay for bees from different hives. Exact proportions are plotted

5.4.2.2 PER assay

To determine the combined impacts of ELF EMFs and clothianidin on learning, bees were exposed to twelve combination treatments: 1 of 3 clothianidin treatments (sucrose, 0.25ng, 2.00ng) 17 hrs prior to the PER assay, followed by 1 of 4 EMF treatments (control, 20 µT, 100 µT and 1000 µT) for 1 min after every conditioning trial during the PER assay. Control bees that were not exposed to clothianidin overnight and not exposed to EMFs during conditioning had the highest learning levels in the PER assay with 62% in only trial 2, and a peak of 87% learning.

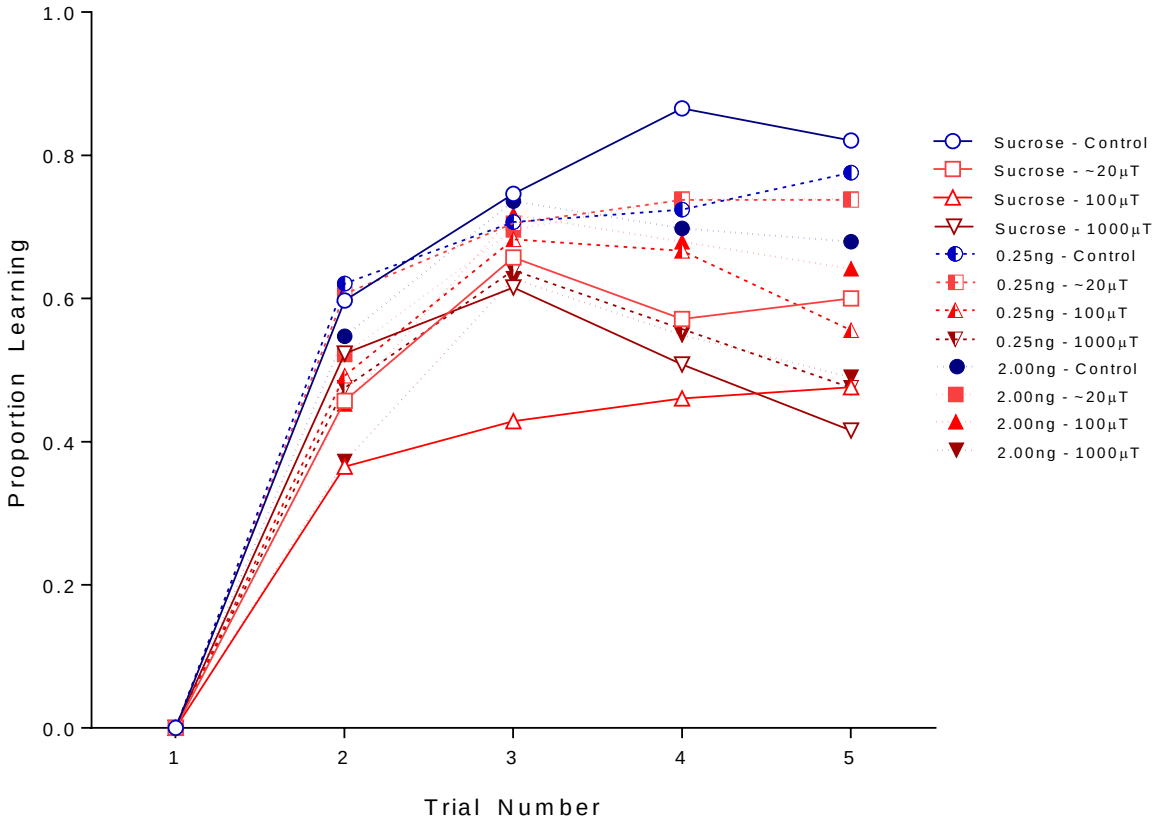

Figure 75. Effects of combined clothianidin and ELF EMF exposure on olfactory appetitive learning in the PER assay. Exact proportions of PER are plotted.

As a general trend, bees that were exposed to increasing intensities of EMFs in conditioning had reduced learning levels (Fig. 76, A-D), with bees exposed to no EMF during conditioning responding at 68-87 % (across all clothianidin treatments in trials 3-5) (Fig. 76, A), 20 µT EMF responding at 57-74 % (Fig. 76, B), 100 µT EMF responding at 43-72 % (Fig. 76, C), and 1000 µT EMF responding at 42-64 % (Fig. 76, D). Overnight exposure with clothianidin affected performance in the PER assay in a complex manner. Bees treated with clothianidin but not with EMFs exhibited slightly lower proportions of learning than control bees that were not treated with clothianidin (Fig. 76, A). For example, with no EMF exposure during conditioning and no clothianidin treatment, bees reached levels of 75-87 % learning (across trials 3-5), but a 0.25 ng treatment reduced proportions of PER to 71-78 %, and a 2.00 ng treatment reduced proportions of PER to 68-74 % learning.

A 4-way GLMM was used to consider the interactions of clothianidin and EMF with other factors including hive and trial number, on learning acquisition in the PER assay. There was no 4-way interaction effect of *'clothianidin' x 'EMF' x 'hive of origin' x 'trial'* (GLMM, F = 0.75, d.f. = 36, 2700, P = 0.86) on learning acquisition. There was a significant three-way *'clothianidin' x 'EMF' x 'hive of origin'* interaction effect on learning (GLMM, F =

2.70, d.f. = 12, 2700, P = 0.001). This appears to be because EMFs reduced learning levels, however clothianidin treatment reduced the impacts of ELF EMFs on cognitive behaviour. The reduction in ELF EMF impacts caused by clothianidin treatment was more prominent in hives that were more susceptible to clothianidin induced mortality, for example ELF EMF exposure reduced learning levels after 2.00 ng clothianidin treatment in Hive 2, but not after 2.00 ng clothianidin treatment in Hive 1 or Hive 3 (those which had significantly higher levels of clothianidin induced mortality at 2.00 ng exposure).

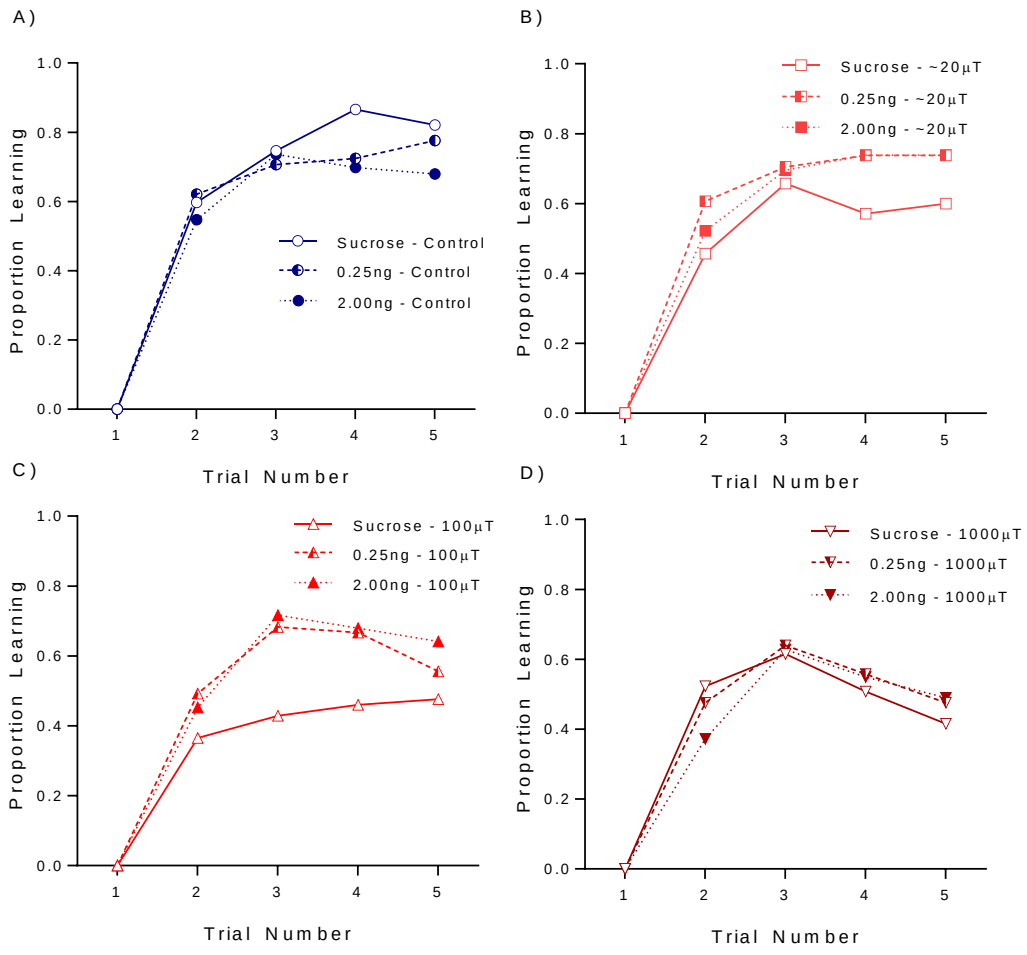

Figure 76. Effect of ELF EMF exposure with each combined clothianidin permutation on olfactory appetitive learning in the PER assay. Exact proportions of PER are plotted. A) control EMF. B) 20 µT. C) 100 µT. D) 1000 µT

EMF exposure reduced the proportions of PER, but with combined clothianidin exposure EMFs had less of an effect on learning (Fig. 77, A-C). For example, with no clothianidin treatment (Fig. 77, A), 75-87 % of control (EMF) bees exhibited learning (trials

3-5) but 20 µT EMF exposure reduced this by approximately 20% (to 57-66 %), while 100 µT EMF exposure reduced further by approximately 35% (to 43-48 %). When given 0.25 ng of clothianidin (Fig. 77, B), 20 µT EMF exposure did not reduce learning levels from control (control bees 71-78% PER; 20 µT bees 71-74% PER), and 100 µT EMF exposure reduced learning by only ~13% (to 56-68%). The reduced effect of EMF by clothianidin was even greater with the higher dose (Fig. 77, C) of 2.00 ng where 20 µT and 100 µT exposures did not reduce learning levels (control 68-74%; 20 µT 70-74%; 100 µT 64-70%). The strongest EMF exposure (1000 µT) was not affected by clothianidin exposure. This resulted in a significant *'clothianidin' x 'EMF'* interaction effect on learning (GLMM, $F = 3.97$, d.f. = 6, 2700, $P = 0.001$), as when bees were not exposed to clothianidin, EMF exposure at all magnetic field intensities reduced proportions of learning from controls (Bonferroni: $P < 0.001$ for all treatments 20 µT – 1000 µT). Only a 1000 µT EMF exposure was sufficient to reducing learning in bees treated with 0.25 ng clothianidin (Bonferroni: $P < 0.001$), and 2.00 ng clothianidin (Bonferroni: $P = 0.003$).

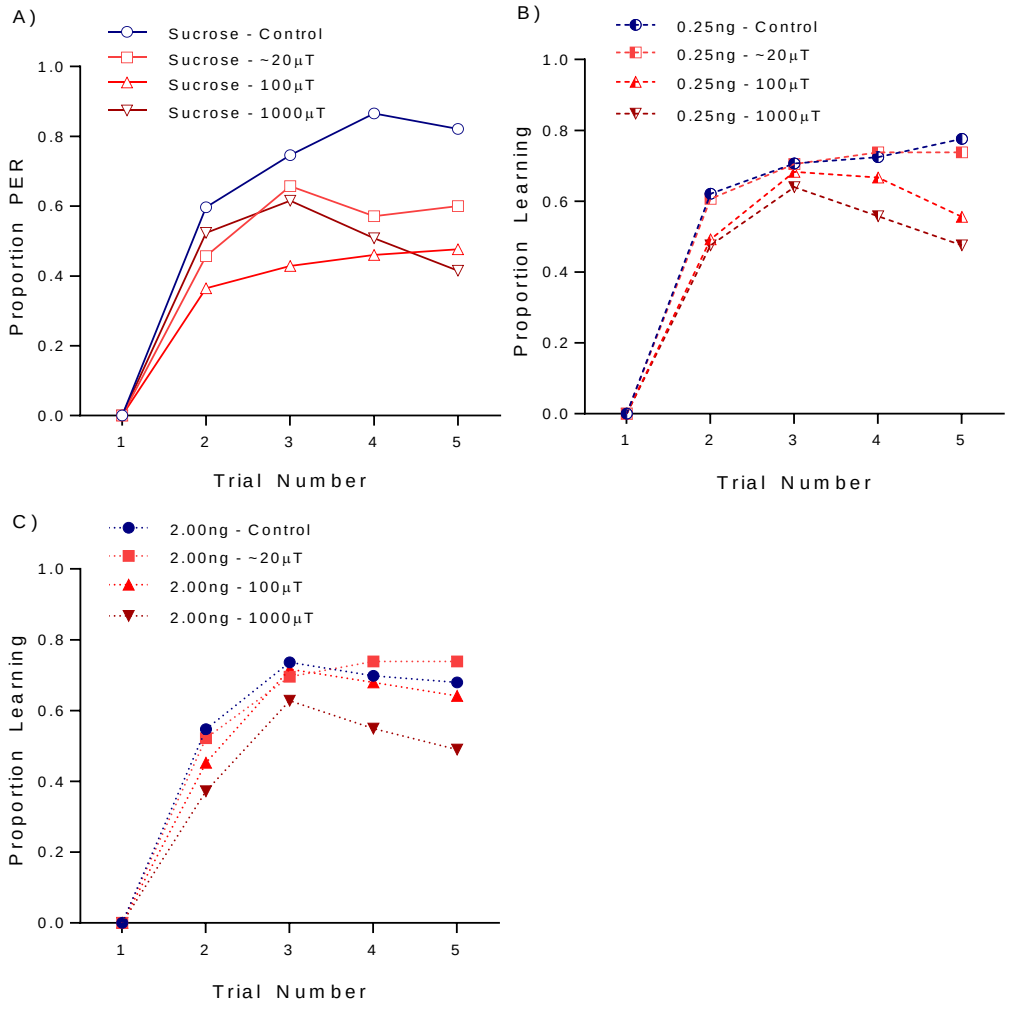

Figure 77. Effect of clothianidin exposure with each combined ELF EMF permutation on olfactory appetitive learning in the PER assay. Exact proportions of PER are plotted. A) Sucrose control. B) 0.25 ng/bee. C) 2.00 ng/bee.

Other model effects that were tested regarding the 'trial number' interactions with 'EMF' or 'clothianidin' effects, were non-significant. There was no three-way *'clothianidin' x 'EMF' x 'trial'* effect (GLMM, F = 0.63, d.f. = 18, 2700, P = 0.88), no three way *'clothianidin' x 'hive of origin' x 'trial'* effect (GLMM, F = 0.85, d.f. = 12, 2700, P = 0.60), and no *'EMF' x 'hive of origin' x 'trial'* effect (GLMM, F = 0.73, d.f. = 18, 2700, P = 0.79). There was no two-way *'EMF' x 'trial'* interaction effect on learning acquisition (GLMM, F = 1.34, d.f. = 9, 2700, P = 0.21), and there was no two-way *'clothianidin' x 'trial'* (GLMM, F = 0.33, d.f. = 6, 2700, P = 0.92) effect on learning. There was a general increase in the proportion of CS responses with trial number, generally plateauing after trial 3 (GLMM, F = 13.99, d.f. = 3, 2700, P < 0.001), as bees learned to respond to the CS in the PER assay.

In summary, ELF EMFs reduced learning levels with exposures as low as 20 µT. With clothianidin exposures of 0.25 ng and 2.00 ng the impact of ELF EMFs on cognitive behaviour was only prominent at 1000 µT exposure, and this clothianidin interaction with EMF impacts on learning was more prominent in hives that were more susceptible to clothianidin induced mortality.

5.4.3 Flight

5.4.3.1 Initial effect of clothianidin on wingbeat frequency

Tethered flight assays were conducted 24 hrs after clothianidin treatment, the same time point at which mortality was measured in the LD_{50} analysis and under exactly the same conditions (free feeding cage at 29ºC). After sucrose treatment there was 0% mortality at 24 hr in all hives. The lower 0.25 ng clothianidin treatment used in flight analyses caused 4% mortality in hive 1, 2% mortality in hive 2, and 7% mortality in hive 3 at 24 hrs. The higher 2.00 ng treatment caused 29% mortality in hive 1, 24% mortality in hive 2, and 33% mortality in hive 3.

Before the effects of acute ELF EMF exposure on flight were assessed, the effect of clothianidin on wingbeat frequency, as well as the variation from hive of origin, were assessed. With no clothianidin exposure, bees from hive 1 had the lowest wingbeat frequency at 104 ± 4 Hz, followed by hive 3 at 121 ± 5 Hz, and hive 3 with the highest wingbeat frequency at 125 ± 4 Hz (Fig. 78). Hive of origin significantly affected the initial wingbeat frequency of bees before EMFs were applied (Two-way ANOVA, $F_{2,360} = 20.84$, $P < 0.001$), with hive 1 having a significantly lower wingbeat frequency than hives 2 and 3 (Bonferroni: $P < 0.001$).

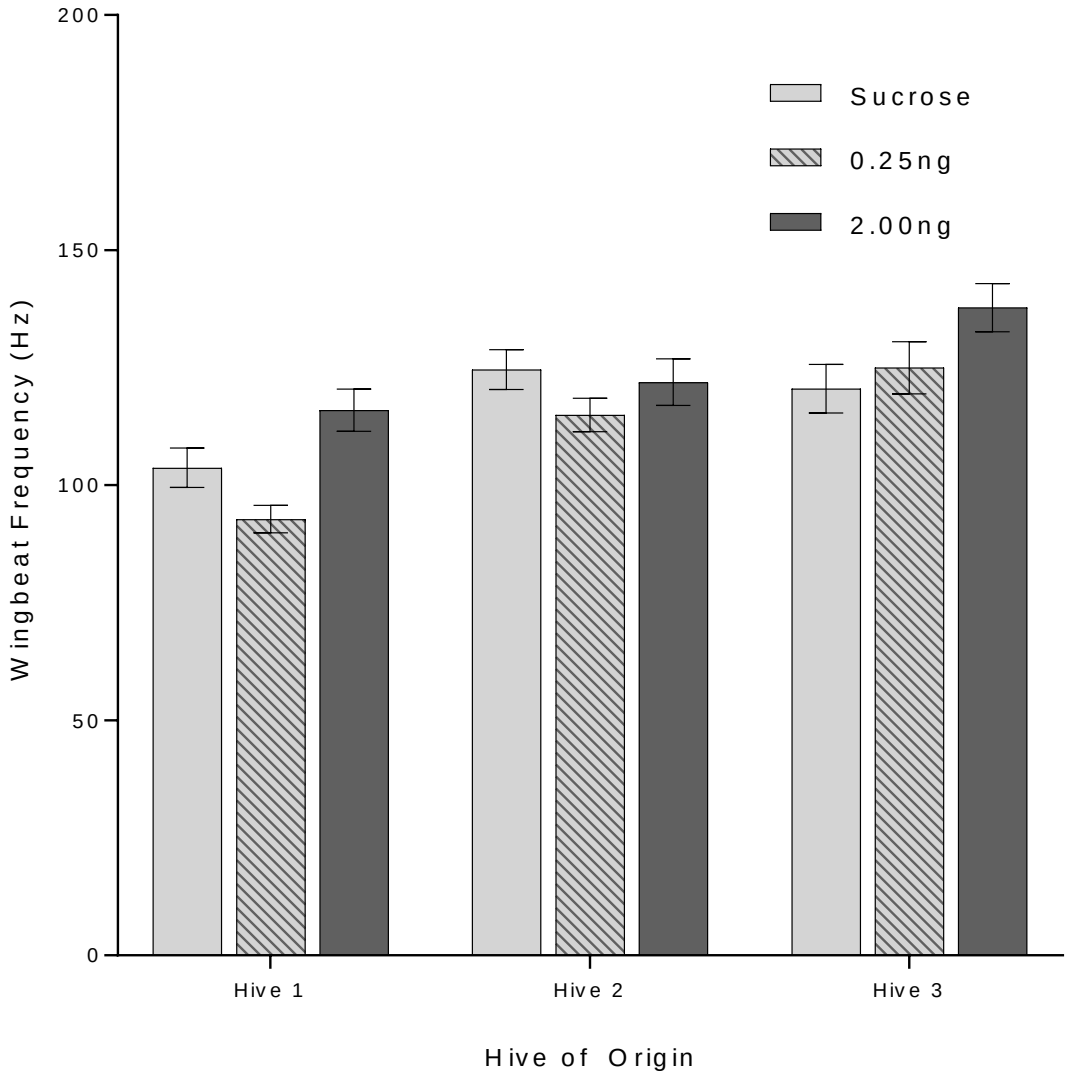

Figure 78. Effect of clothianidin exposure on wingbeat frequencies of bees in the tethered flight assay from different hives. Mean wingbeat frequency is plotted with SEM.

Across all hives with no clothianidin exposure, wingbeat frequency was 116 ± 3 Hz, whereas 0.25 ng exposed bees had slightly lower wingbeat frequencies at 111 ± 3 Hz, and 2.00 ng exposed bees had higher wingbeat frequencies at 126 ± 3 Hz (Fig. 79). Clothianidin had a significant effect on wingbeat frequency (Two-way ANOVA, $F_{2,360} = 7.54$, $P = 0.001$), as bees that survived 2.00 ng exposure had significantly higher wingbeat frequencies (Fig. 79) than both 0.25 ng (Bonferroni: $P < 0.001$) and sucrose (Bonferroni: $P = 0.026$) exposures. There was no significant difference between wingbeat frequencies of bees after sucrose and 0.25 ng treatments (Bonferroni: $P = 0.47$). There was no two-way *'clothianidin' x 'hive'* interaction effect on wingbeat frequency (Two-way ANOVA, $F_{4,360} = 1.93$, $P = 0.11$).

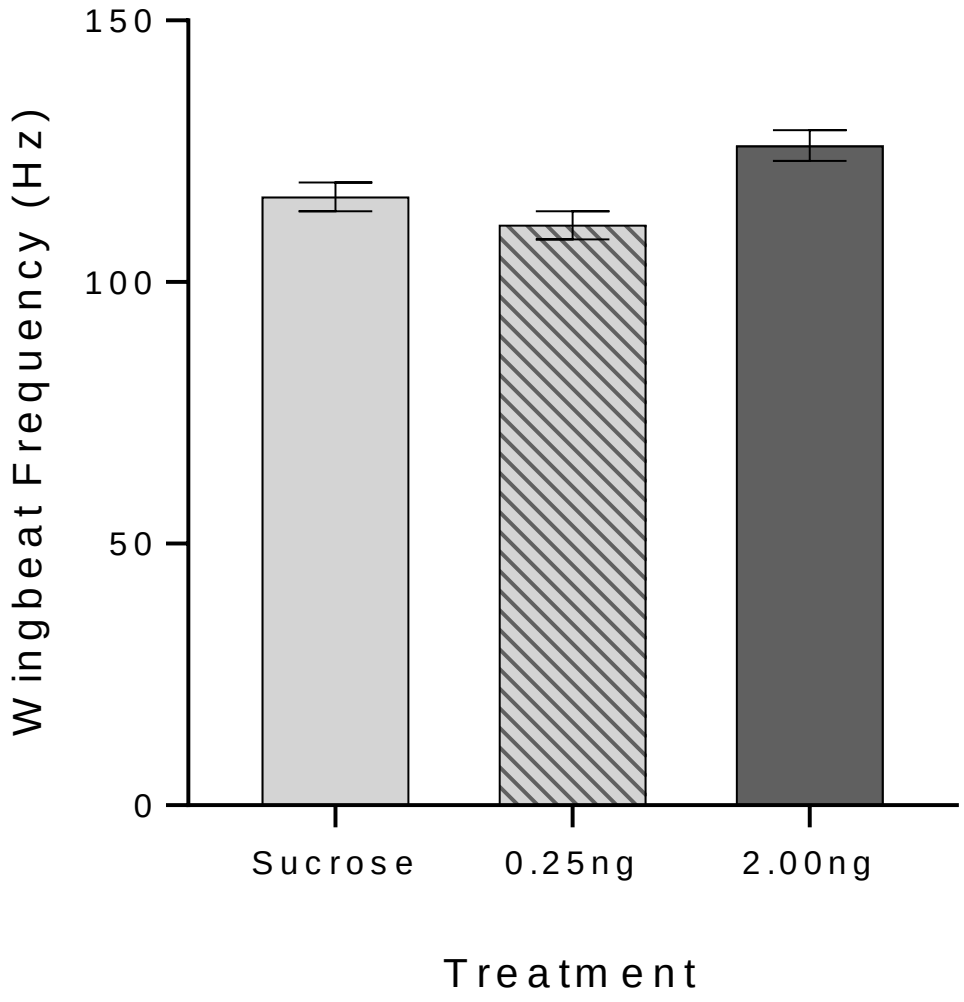

Figure 79. Main effect of clothianidin exposure on wingbeat frequencies of bees in the tethered flight assay with data from different hives pooled. Mean wingbeat frequency is plotted with SEM.

5.4.3.2 Combined effects of clothianidin and EMFs on flight

24 hr after clothianidin treatment, tethered flight assays were conducted in which bees were exposed to ELF EMF (100 µT, 1000 µT and 7000 µT) or control treatment. With no clothianidin treatment, and no EMF exposure, bees tended to increase wingbeat frequency by 2.4 ± 0.9 Hz, during the time period of the assay (Fig. 80). With each EMF exposure wingbeat frequency increased by a greater amount, as 100 µT increased wingbeat frequency by 3.0 ± 0.8 Hz, 1000 µT increased wingbeat frequency by 5.6 ± 0.9 Hz, and 7000 µT increased wingbeat frequency by 6.3 ± 1.1 Hz. With a 0.25 ng dose of clothianidin wingbeat frequency increased by 1.5 ± 0.9 Hz in control bees, and EMF exposure still increased wingbeat frequency. 100 µT increased wingbeat frequency by 1.7 ± 1.0 Hz, 1000 µT

increased wingbeat frequency by 2.8 ± 0.7 Hz and 7000 µT increased wingbeat frequency by 4.4 ± 0.9 Hz. After 2.00 ng clothianidin treatment wingbeat frequency increased by 0.6 ± 1.1 Hz for control bees, and EMF effects on this change were only apparent at the highest intensity. 100 µT increased wingbeat frequency by only 0.3 ± 1.3 Hz, 1000 µT exposure increased wingbeat frequency by 1.7 ± 0.9 Hz, and only 7000 µT exposure appeared to clearly increase wingbeat frequency more than other exposures with a 5.5 ± 1.3 Hz increase.

There was no three-way *'EMF' x 'hive of origin' x 'clothianidin'* interaction effect (ANOVA, $F_{12,333} = 0.95$, $P = 0.49$) on wingbeat frequency. There were also no two-way interaction effects of *'EMF' x 'clothianidin'* (ANOVA, $F_{6,333} = 0.41$, $P = 0.88$), *'EMF' x 'Hive'* (ANOVA, $F_{6,333} = 1.25$, $P = 0.28$), and *'Hive' x 'clothianidin'* (ANOVA, $F_{4,333} = 0.71$, $P = 0.59$) on wingbeat frequency.

ELF EMF significantly increased wingbeat frequency across all clothianidin exposures (ANOVA, $F_{3,333} = 9.19$, $P < 0.001$) with 7000 µT significantly increasing wingbeat frequency from both control (Bonferroni: $P < 0.001$) and 100 µT exposure (Bonferroni: $P < 0.001$). The difference between increased wingbeat frequency in control and 1000 µT exposed bees approached significance (Bonferroni: $P = 0.09$) and 100 µT EMF exposure caused no significant changes in wingbeat frequency from controls (Bonferroni: $P > 0.99$). Clothianidin exposure caused less change in wingbeat frequency across all treatments in the assay (ANOVA, $F_{2,333} = 6.82$, $P = 0.001$). 2.00 ng exposed bees only increased wingbeat frequency on average by 1.8 ± 0.5 Hz, which was significantly less than the average 4.3 ± 0.5 Hz increase after sucrose treatments (Bonferroni: $P < 0.001$). 0.25 ng clothianidin treated bees also exhibited significantly lower changes in wingbeat frequency, at 2.6 ± 0.5 Hz than sucrose controls (Bonferroni: $P = 0.046$). There was no significant differences between 0.25 ng and 2.00 ng clothianidin treatment effects on change in wingbeat frequency (Bonferroni: $P = 0.75$). Thus, clothianidin exposure reduced the increase in wingbeat frequency during the treatment time period for all treatments including control.

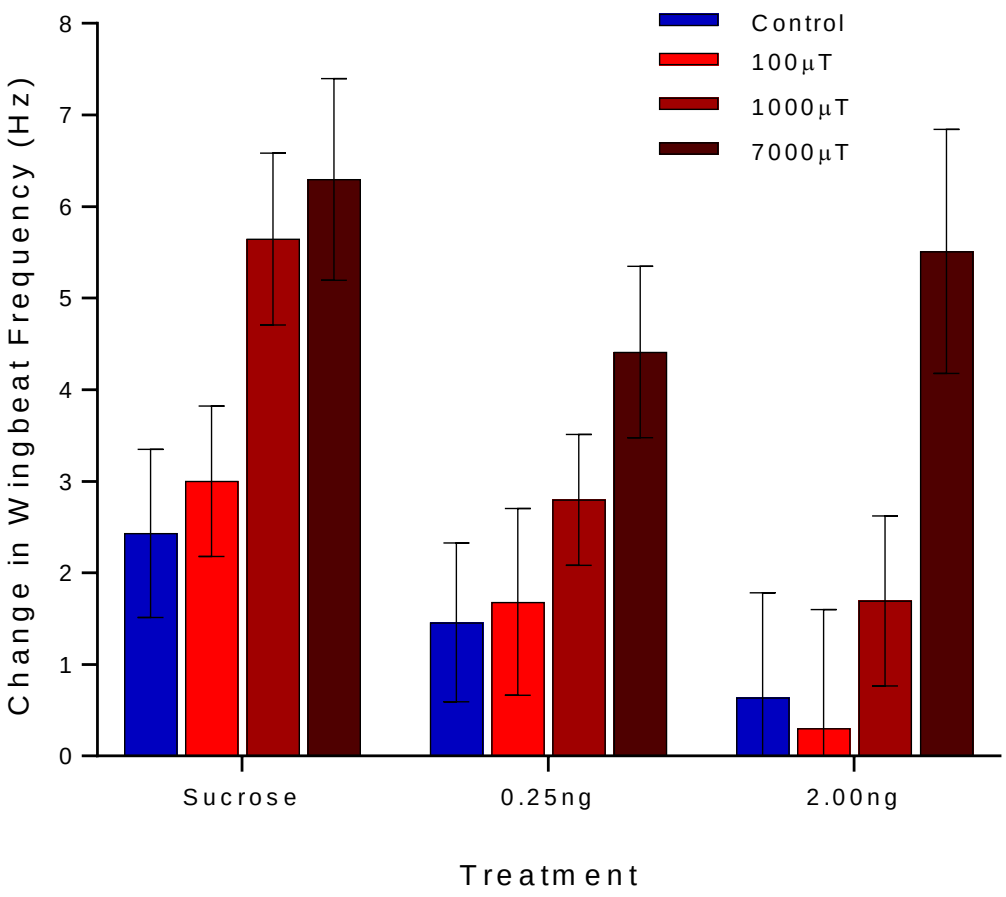

Figure 80. Effect of combined clothianidin and ELF EMF treatment on wingbeat frequency in the tethered flight assay. Mean and SEM changes in wingbeat frequency is plotted.

5.5 Discussion

Exposure to EMFs alone reduced learning in a dose-dependent manner. Similarly, exposure to clothianidin alone also led to a reduction in learning. When applied in combination however clothianidin reduced the impacts of ELF EMFs on learning. Clothianidin treatment increased the initial wingbeat frequencies of bees. Results also showed that the effects of EMF and clothianidin exposure were dependent on the hive of origin of the bees with some hives more sensitive to the effects of clothianidin than others. For weak hives (hives that lost more bees through mortality to clothianidin), were more susceptible to clothianidin impacts on learning. For the stronger hive (which lost less bees to clothianidin exposure) clothianidin exposure did not reduce learning, and did not reduce the impacts of ELF EMFs on learning. In this case there appears to be a relationship between

susceptibility to clothianidin, and interactive impacts of ELF EMFs and clothianidin on cognitive behaviour.

ELF EMFs caused an increase in wingbeat frequency, however clothianidin exposure reduced changes in wingbeat frequency across all treatments (EMFs and controls). Flight is energetically costly (Harrison and Fewell, 2002), with increased wingbeat frequency strongly associated with greater energy expenditure (Harrison and Roberts, 2000). For bees that were already flying at a faster rate during the assay after clothianidin exposure, any further increases in wingbeat frequency may have been beyond their physiological limits, or too energetically costly under those environmental conditions.

5.5.1 Potential clothianidin and ELF EMF interactions with biological systems

At the sub lethal level it is extremely likely that the biological interactions of clothianidin with surviving bees has brought about some of the effects observed, as well as some potential ELF EMF interactions. The effects of neonicotinoids on locomotion have been described in a variety of studies including locomotory deficits, but also increased locomotory activity (Lambin et al., 2001 Nauen et al., 2001; Suchail et al., 2001; Medrzycki et al., 2003; Colin et al., 2004; Williamson et al., 2014; Charreton et al., 2015; Alkassab and Kirchner, 2018). The binding of neonicotinoids to nAChRs, which have an essential role in fast excitatory synaptic transmission in the CNS, causes neural stimulation, which is likely cause to the locomotory effects observed here (Matsuda et al., 2001; Jeschke and Nauen, 2008). At lower levels the impacts of neonicotinoids are excitatory, but at higher levels they can lead to overstimulation, and mortality. As a result, there was the possibility here that ELF EMFs, known to have excitatory effects, could have synergistic excitatory impacts with neonicotinoids. However, there was no interactive effect clothianidin and acute ELF EMF effect exposure during flight. Instead, where wingbeat frequency had increased from initial levels for all EMF exposures during the assay (Control, 100 µT, 1000 µT, or 7000 µT), the magnitude of this change was reduced for all exposures (Control and EMF) by clothianidin treatment. A possible explanation for this effect is that for bees that survived clothianidin exposure during the flight assay, sub lethal neural effects of clothianidin may have brought about the observed increased wingbeat frequency (at 2.00 ng clothianidin treatment). As a result bees treated with clothianidin in the flight assay had higher wingbeat frequencies before any exposure (Control, 100 µT, 1000 µT, or 7000 µT) and consequentially may have already been flying close to physiological limits under the experimental conditions.

For cognitive behaviour clear effects of neonicotinoids on neural systems are likely, and may provide a potential route for interactive EMF and clothianidin effects. For example clothianidin has been shown by Palmer et al (2013) to cause a depolarization-block of neural firing in Kenyon cells, which have critical functions in honey bee learning and memory. This kind of neurobiological effect may underpin the reductions in learning caused by clothianidin found here, as well as the other numerous impacts of neonicotinoids on learning and memory in bees that be been recorded to date (Decourtye et al., 2003, 2004a, 2004b; Han et al., 2010; Williamson and Wright, 2013; Stanley et al., 2015; Tan et al., 2015; Piiroinen and Goulson, 2016). There is also the possibility that through neural interactions clothianidin causes impacts that may otherwise have been caused by ELF EMFs. The mode of action of clothianidin is through hyperexcitation in the insect CNS (Matsuda et al., 2001; Jeschke and Nauen, 2008), and ELF EMFs are known to have excitatory effects on cellular systems (Dimbylow, 1998; Jacobson, 2005). If reductions in learning and memory caused by ELF EMFs are related to their neural effects, then pre-treatment of bees with clothianidin may reduce the ability of ELF EMFs to cause these same impacts of cognitive behaviour.

There are a variety of other mechanisms through which ELF EMFs and clothianidin may interact. Due to the range of neural effects of neonicotinoids caused by nAChR binding (Matsuda et al., 2001; Jeschke and Nauen, 2008; Jeschke et al., 2010), sensory effects from neonicotinoids are likely. Jin et al. (2015) found that clothianidin exposure of 0.76 ng/bee reduces sensory responses of solitary bees (*Osmia cornuta*) to a visual environment.

While there is much to learn regarding magnetosensory behaviour, bees are well known to show to magnetosensory abilities (Collett and Baron, 1994; Kirschvink et al., 1997, 2001; Gegear et al., 2008; Bazalova et al., 2016; Liang et al., 2016), and if magnetosensory abilities have a critical role in the effects of acute ELF EMF exposure on honey bees as found here, then clothianidin-related effects on sensory abilities (for example, reductions in sensory ability caused by nAChR binding) could cause the reduction in ELF EMF effects with increased clothianidin exposure that was observed here. This could be important, as if neonicotinoids can affect magnetosensory abilities, they would be likely to affect geomagnetic orientation by honeybees, which alongside effects on learning and flight observed here, could contribute to the well-documented impact of neonicotinoids on foraging/homing flights and orientation e.g. longer foraging flights (Schneider et al., 2012), reduced proportions of successful homing flights (Matsumoto, 2013), and impacts on components of navigation, including causing longer homing flights (Fischer et al., 2014).

Other interaction mechanisms between ELF EMFs and clothianidin may occur, for example clothianidin has been shown to modify transcription of a range of critical genes related to stress, health and immunity (Christen et al., 2016) including heat shock responses (Esther et al., 2015) which ELF EMFs have been shown to activate (Chapter 2 and 3) however ELF EMF induced heat shock responses occur after much longer exposures than were tested here. It seems unlikely with the acute ELF EMF exposures applied here that a molecular interaction in stress pathways would occur, however the impacts of acute repeated 1 minute ELF EMF exposures on stress pathways has not been tested, and it may be valuable in determining how these ELF EMFs impact upon learning to investigate the underlying biological effects of ELF EMFs of these acute durations.

5.5.2 Mortality

The overall median lethal dose found here was 3.16 ng/bee which is concerning given the high levels of clothianidin encountered in the environment. EFSA (2013) calculated a worst case scenario through clothianidin residues in oilseed rape foraging of 4.3 - 13.7 ng per day. While some studies have been critical (Carreck and Ratnieks, 2014) that a dose applied all at once (as was done here) is dramatically different from one applied over a week or even over the course of day, under the 'worst case scenario' described by the EFSA (2013) honeybees here would have to detoxify 1.4 - 4.3 times the LD_{50} a day, or 7.8 - 24.5 times the dose required to kill 10% of bees. In experiments where bees were allowed to freely feed clothianidin treatments at 25 ppb, the same concentration as the low clothianidin treatment here (0.25 ng in 10 µl), they consumed the equivalent of 2.99 ng/bee/24 hr (Williamson et al., 2014) resulting in 45% mortality. There does not appear to be a major difference here in mortality of a 2.99 ng dose (45%) received over 24 hr *versus* what was found here regarding mortality of a 3.16 ng dose (50%) received at once. Acetylcholinesterase, which would normally break down agonists of nAChRs, cannot break down neonicotinoids, making their binding irreversible (Matsuda et al., 2001). As a result detoxification would have to be very efficient for bees not be affected by clothianidin exposure, given the effects found here. Even under the circumstances where bees can detoxify neonicotinoids to survive, this process is known to modify gene expression, activate multiple stress pathways, and require intensive energetic investment (Esther et al., 2015; Gong and Diao, 2017). The LD_{50}'s tested here were in 10 µl of sucrose, and therefore the overall LD_{50} for the assay (3.16 ng/bee) is equivalent to 366 ppb. With clothianidin consistently found above 10,000 ppb in guttation fluid of treated plants (Girolami et al., 2009; Tapparo et al., 2011), bees from this assay would only need to consume 0.316 µl of guttation fluid of clothianidin treated crops to receive the

median lethal dose, and for extremes such as 717,000 ppb (EFSA, 2013) would require only 0.0044 µl to receive the median lethal dose.

There were clear variations in clothianidin toxicity between hives here. The median lethal dose for the strongest hive at 4.22 ng/bee was 63% higher than that of the weakest hive, 2.59 ng/bee. This kind of variation in the field is common, for example different 24 hr oral LD_{50}'s for clothianidin have been reported at different doses including 2.18 ng/bee (Matsumoto, 2013), 3.79 ng/ bee (EFSA, 2013) and 2.8 - 3.7 ng/bee (Laurino et al., 2011). It is well understood that neonicotinoid toxicity is dependent on a variety of factors including underlying health, for example from substandard protein feeding (Wehling et al., 2009), or infection (Alaux et al., 2010 ; Vidau et al., 2011), and other factors such as age (Suchail et al., 2000, 2001; Nauen et al., 2001; Guez et al., 2003) and genetics (Rinkevich, 2015). It is likely that underlying factors within each hive had a part to play in the variation in clothianidin toxicity that was observed. This is supported by the finding of consistent susceptibility of the weaker hives to clothianidin through various stages of the study.

The underlying health of bee hives may have had some part to play in the interactions found here between clothianidin exposures and the responses of bees to ELF EMFs, particularly if ELF EMFs are more likely to impact bees that are unhealthy, aged or stressed. For example, in olfactory conditioning in the two weak hives when clothianidin killed more bees, there was no evidence of ELF EMF effects, but in the strong hive, when clothianidin killed less bees, ELF EMFs still reduced learning performance. In this case unhealthy, aged, or stressed bees that may have been more susceptible to ELF EMF exposure may have already been eliminated from the assay through clothianidin induced mortality. No factors other than the initial susceptibility of hives to clothianidin were measured to give an indication of the health of different hives in this study, however if these interactions are assessed in the future in longer term field studies, it will be valuable to determine factors such as seasonal weight gain, brood levels, colony viral load and parasitism levels as further indication of how pre-exisitng hive health determines interactions with environmental stressors. Varied pre-treatment conditions (e.g. health) of bees from different hives that may have led to variation in clothianidin effects may also have caused variations in sub-lethal interactions of stressors. For example, clothianidin reduced learning in the weaker hives, but not in the stronger hive. Here, for unhealthy, aged, or stressed bees that may have been susceptible to ELF EMF impacts, learning may have already been limited by clothianidin exposure, thus reducing the impacts of ELF EMFs on learning in comparison to bees not treated with clothianidin. These findings could further be expanded to the flight assay, where

clothianidin increased the pre-treatment wingbeat frequency of all bees. Increased mortality of bees in this assay from clothianidin exposure may have left healthier faster flying bees in the assay.

5.5.3 Summary and conclusion

Ultimately ELF EMFs have been shown to impact honey bee learning and memory and flight, even when bees are treated with clothianidin, although the magnitude of the effect is reduced. Clothianidin on the other hand had dramatic impacts upon honey bee behaviour and health, which are likely to cause large scale ecological damage. The scale of potential neonicotinoid impacts on pollinators is enormous. At least 1 of 5 neonicotinoids (acetamiprid, clothianidin, imidacloprid, thiacloprid, and thiamexotham) has been found in 75% of honey samples from every continent except Antarctica, showing the extreme level of global contamination of neonicotinoids, and coexistence of pollinators with these chemicals (Mitchell et al., 2017). Even though in some place e.g. Europe restrictions on the usage of neonicotinoids are beginning, these chemicals will likely persistent in the environment for extensive periods of time. For example the half-life of clothianidin in soil is between 148 - 6900 days (Rexrode et al., 2003). Further to this, if the impacts of ELF EMFs on important cognitive and locomotory behaviours in pollinators transduce to field scenarios, then where impacts of neonicotinoids are reduced, ELF EMFs may become a greater factor as an environmental stressor of pollinators.

Chapter 6 General Discussion

6.1 Study findings

ELF EMFs have been shown to consistently affect critical biological processes in insects, both in bees and locusts. ELF EMF exposure at high levels (7000 μT), similar to those found close to HVTL conductors, for short-term periods induced stress-protein expression in both locusts (24 hr: Chapter 2) and bees (17 hr: Chapter 3). The EMF threshold level for this molecular effect in bees was between 1000 μT and 7000 μT. As mentioned in both Chapter 2 and Chapter 3, EMREs in the promoter regions of stress-related genes have been discussed as direct sites for ELF EMF induced gene expression, as well as indirect inducement of stress protein expression via molecular changes from ELF EMF exposure (Chapter 2 and Chapter 3).

In locusts, short-term ELF EMF exposure was found to cause neuromuscular effects including increased action potential duration and latency in an identified motor neuron, and reduced force generated by ETi muscle (Chapter 2), providing evidence under a priority research need from the WHO ELF EMF research agenda (WHO, 2007b) for more studies of ELF EMFs on intact neural networks. It is well known that ELF EMFs can induce electric fields and currents within organisms, causing excitability of neurons (Dimbylow, 1998; Jacobson, 2005; WHO, 2007a; Halgamuge et al., 2009), which in Chapter 2 was discussed as a potential mechanism for neuromuscular strain leading the observed neurophysiological effects. Different mechanisms of ELF EMF effects on neurons are described in the literature, including forced vibration of charged ions (Halgamuge et al., 2009) and reduced activity of the Na^+/K^+ ATPase disrupting the process by which critical ion gradient are maintained in neuronal signalling (Serpersu and Tsong, 1983; Liu et al., 1990; Blank, 1992; 2005). Due to the range of impacts ELF EMFs can have on charged particles, and the critical function of charged particles in physiological processes, a variety of other mechanisms may bring about impacts on neural circuits observed here. For example, ELF EMF exposure has been show to affect intracellular Ca^{2+} levels, as well as affecting action potential dynamics (Barbier et al., 1996; Ye et al., 2004).

As well as detecting ELF EMF induced effects at the molecular and physiological levels, clear effects were detected at the behavioural level. Short-term ELF EMF exposure at both 4000 and 7000 μT for 24 hr affected walking behaviour in locusts (Chapter 2), and

at 100 and 1000 µT affected cognitive behaviour in bees in the form of reduced aversive learning capabilities (Chapter 3). In addition, acute ELF EMF exposure affected flight behaviour, with 100 µT exposure and above in locusts causing fluctuations in wingbeat frequency and potential synchrony with the electromagnetic field (Chapter 2). In bees changes in flight were elicited at higher EMF exposures than with locusts, with increased wingbeat frequencies in bees exposed to 1000 µT and above (Chapter 5; Chapter 6). Acute ELF EMF exposure of 1 min during conditioning also reduced learning performance in bees for all EMF exposures, including 1000 µT, 100 µT, and as low as 20 µT (Chapter 3; Chapter 4; Chapter 6). These effects on flight and learning in bees appear to be reduced when in interaction with the neonicotinoid pesticide clothianidin, potentially due to a combination of clothianidin-induced mortality eliminating bees that would otherwise be affected by ELF EMFs, and clothianidin interactions with the biological processes that underpin ELF EMF effects (Chapter 6). Finally, in a semi-field realistic scenario the effects of ELF EMFs on flight and learning may give rise to the observed changes in foraging following exposure to ELF EMFs (Chapter 5).

Short-term exposure to high level EMFs that can be encountered in proximity to a HVTL caused detectable effects at a molecular level in terms of indicators of stress, at a physiological level in terms of neuromuscular control, and at a behavioural level in terms of locomotory behaviour. Short-term and acute EMF exposure a variety of cognitive behaviours and locomotory behaviours in honey bees, which are a critical pollinator species and have essential functions in broad ecological processes. These findings provide an initial indication that ELF EMFs that may be encountered in the environment can affect critical behaviours and biological processes in important insects, supporting the need for larger field studies to determine environmental ELF EMF impacts, as well as providing suggestions for further investigation to elucidate the mechanisms of biological effects of ELF EMFs.

6.2 Interactions with biological processes and research needs

ELF EMFs may affect biological processes in insects in a variety of ways to cause the effects found here. Many of these potential impacts have been discussed in detail in respective chapters, however there are some interactions that must still be highlighted as well as research needs to improve the understanding of the biological impacts of ELF EMFs. For example, understanding of the impacts of ELF EMF exposure on molecular biology is improving (Blank, 1995a; Blank 1995b; Lin et al., 2001; Goodman and Blank, 2002; Blank and Goodman, 2004; Li et al., 2013), but there is much to learn about how these pathways

are induced. It would be valuable to explore this heat-shock function with environmental ELF EMF stimuli, including the duration and intensity of ELF EMF exposure required to elicit response. Whilst thresholds for bees and locusts here appear to be between 1000 and 7000 μT after overnight exposure, it remains to be seen how thresholds may vary with lesser/greater duration ELF EMF exposures, as well as the persistence of increased stress levels after exposure, at what point these stress response mechanisms may be exhausted, and how varied these responses may be between insect taxa.

There is a potential for molecular impacts to link with the well discussed excitatory effects that ELF EMFs have on neurons. In *C. elegans* regulation of heat-shock responses has been linked to thermosensory neurons (Prahlad et al., 2008). If sensory systems have a role in regulating heat-shock responses in insects then interactions of ELF EMFs with these physiological systems may also underpin the molecular effects detected here. Future studies will not only need to determine main effects of ELF EMFs on biological systems, but determine downstream biological impacts. Expression of a variety of genes may be upregulated or downregulated after ELF EMF exposure. Larger scale proteomic studies may help understand the range of impacts ELF EMFs can have on molecular biology. Changes on the molecular level may in turn give rise to other biological effects, particular with longer term/chronic exposures.

The sensitivity of insect physiology to ELF EMF exposure must provide the foundation for the impacts of short-term and acute exposures on insect behaviour. This sensitivity may be caused by magnetosensory processes that are responsive to ELF EMF stimuli, disruptive interactions of ELF EMFs with underlying physiological processes, or a combination of both. For example, the neural pathways control, and may give rise, to many of the key behavioural ELF EMF impacts that were detected here including learning (Menzel and Müller, 1996; Hammer, 1997), flight (Hasselrot, 1960; Bastian, 1972; Reye and Pearson, 1988; Esch and Goller; 1991; Marder and Bucher, 2001) and limb movement in locomotion (Heitler and Burrows, 1977; Burrows and Pflüger, 1988; Burrows, 1995; 1996; Norman, 1996). To further determine effects of ELF EMFs, electrophysiological parameters must be explored in further detail, including the short and long term impacts on neural circuits, and the extent to which these impacts may be related to behavioural change. As well as this, other important physiological characteristics such as changes in levels of important neurochemicals that regulate these behaviours, e.g. octopamine and dopamine, should be explored. Magnetosensory systems may also have a part to play in some of the behavioural effects observed throughout this study, particularly acute ELF EMF effects, and the precise

physiological mechanisms by which this sense may occur must be characterised first for these potential impacts to be understood.

6.3 Implications of ELF EMF effects on insects

There is already a range of evidence that insects may be able to detect and respond to magnetic fields (Bergh, 1979; Collett and Baron, 1994; Walker and Bitterman, 1985; Dovey et al., 2013; Wijenberg et al., 2013), that magnetic fields can cause varied biological effects in insects (Ramírez et al., 1983; Mirabolghasemi and Azarnia, 2002; Todorović et al., 2011; Li et al., 2013; Dimitrijević et al., 2014; Pantenković et al., 2015; Zmejkoski et al., 2017), and that in the environment major sources of ELF EMFs may reduce the success of beneficial insects (Wellenstein, 1973, Greenberg et al., 1981; Rogers et al. 1982; Morse and Hooper, 1985). Given the effects found here, there is a large potential for biological effects of ELF EMFs in the environment that may have ecological ramifications. However, there a no published large scale field studies that have directly measured ELF EMF levels around powerlines, and their impacts on pollinators. The potential impacts of a typical field realistic scenario are unknown, and the evidence from this study justifies further exploration of this topic. The large range of area where insects may be affected by HVTLs (e.g. over 22000 km over HVTLs in the UK) has been mentioned (Chapter 1), and broader ecological studies should now consider whether locomotory, cognitive and physiological/molecular stress can occur in different insect taxa in the environment.

An important aspect in assessing the risks of ELF EMFs to insects, is to consider in what scenarios insects may be exposed to short-term/long-term/chronic EMFs and in what scenarios they may be exposed to EMFs in an acute manner. In the case of bees, particularly managed bees, short-term exposures (Morse and Hooper, 1985) and long-term and chronic exposure to EMFs is possible for colonies moved/kept in close proximity to, or even under HVTLs (Wellenstein, 1973; Greenberg et al., 1981; Rogers et al., 1982; Lee, 1989), and in all insects, including managed bees and wild pollinators long-term and chronic exposure to ELF EMFs is possible where specific habitat types for foraging/nesting are only present or are more common under HVTLs (Russell et al., 2005; Wojcik and Buchmann, 2012; Wagner et al., 2014; Hill and Bartomeus., 2016). Exposures of this type are more likely to occur in the 20 – 100 μT range.

In contrast, acute exposure can occur for a wide range of insects that encounter ELF EMFs in the environment when executing a range of behaviours including dispersal,

foraging, migration and a range of behaviours that involve movement around their environment. For acute exposures non-flying insects are still only likely to be exposed to ELF EMFs in the 20 - 100 µT range, but for flying insects (of which many pollinating insects are) crossing an HVTL can mean encountering and EMF from 20 - > 7000 µT depending on the insect's proximity to the conductor when crossing the HVTL margin. The foraging ranges of bees ranging from 1-10 km (Greenleaf et al., 2007) have already been discussed as making them likely candidates for acute ELF EMF exposure, however bees are not the only beneficial insects likely to be exposed to ELF EMFs. Long distance dispersal over 1 km and up to 13 km is critical in butterflies (Baguette, 2003). Extreme examples include in pollinators the monarch butterfly, where Eastern North American populations migrate over 4000 km (Brower and Malcolm, 1991), and other flying insects such as the desert locust where migrations can cover distances as great as 5000 km (Rosenberg and Burt, 1999). For many insects foraging, dispersal and migration will consequentially lead to widespread acute ELF EMF exposure.

Both short-term and acute EMF exposures reduced cognitive abilities in honey bees, and acute EMF exposure affected locomotory flight behaviour. Whilst the importance of these behaviours and potential impacts on honey bees has been discussed in respective chapters, the potential for these impacts to have even wider effects on insect communities has not. Potential ecological impacts of ELF EMF exposure may not be exclusive to honey bees, as learning and responding to sensory cues in essential behaviours like foraging and navigation are critical elements of pollination biology in general (Menzel and Giurfa, 2001; Smith and Raine, 2014), and cognitive behaviour is essential for the different functions insects have in ecosystems. Just in terms of pollinators, for a variety of taxa cognitive behaviour is critical to their foraging and pollination abilities, including bumble bees (Brian, 1957; Leadbeater and Chittka, 2005; Kawaguchi et al., 2006), stingless bees (Slaa et al., 2003; Nieh, 2004), wasps (Parrish and Fowler, 1983; Reid et al., 1994; Richter and Tisch, 1999) and butterflies (Goulson and Cory, 1993; Weiss and Papaj, 2003). Factors which restrict movement affect dispersal and gene flow in pollinators (Dillon et al., 2006; Jha, 2015) as well as limiting food source availability and consequentially the ability to pollinate plants (Wratten et al., 2003). Availability of food sources is critical to pollinator health (Brodschneider and Crailsheim, 2010). The consequences that these impacts may have individually, or in combination with other environmental stressors (Goulson, 2015) certainly needs further investigation as pollinators (Potts et al., 2010a) and flying insects in general (Hallman et al., 2017) are currently in serious decline.

To understand the potential implications of ELF EMFs in the environment larger scale field studies must be conducted to assess risks of pollinators for both long-term and acute ELF EMF exposures. This will require monitoring how critical behaviours and processes essential for pollinator health are affected by environmental EMF exposure. Studies assessing bee performance in proximity to HVTLs have been conducted (Wellenstein, 1973; Greenberg et al., 1981; Rogers et al. 1982; Morse and Hooper, 1985) however direct measurements of ELF EMF levels has not been done in these studies, and better methods now exist to measure chronic stress in colonies. Furthermore, acute exposures have not been investigated to any extent, and further experimentation with bees would be beneficial to determining the environmental risk that ELF EMFs could pose. As has been mentioned, powerline strips have been suggested to be important refuges for insect pollinators as large areas of land with beneficial host plants for pollinators, leading to relatively good species richness including rare species of bees (Russell et al., 2005; Wojcik and Buchmann, 2012; Wagner et al., 2014; Hill and Bartomeus., 2016) as well as other important pollinators such as butterflies (Berg et al., 2016). Russell et al. (2005), for example, suggested that the habitat elements under powerlines provide additional nesting sites for bees, in a changing modern environment where bee floral resources and nesting sites are being depleted. Wagner et al. (2014) found rare bee species under powerline strips, including *Epeoloides pilosula*, which was thought to be locally extinct from the United States (now listed as endangered). The two sightings of *E. pilosula* in habitat provided under powerline strips in Wagner et al. (2014) are the only occasions since the 1950's this bee has been recorded in the United States, as well as all of North America except two males found in Canada in 2002 (Ascher, 2005). With powerline strips providing crucial habitat to bee species, there is a risk with increasing usage of anthropogenic technologies, these already threatened bees will be exposed to increasing levels of exposure to ELF EMFs. The potential for field realistic ELF EMF stimuli to affect important and declining pollinators in these scenarios should be investigated in assessing the environmental risk of ELF EMFs.

One point to address is that whilst studies which have assessed pollinator diversity under powerlines (Russell et al., 2005; Wojcik and Buchmann, 2012; Wagner et al., 2014; Hill and Bartomeus., 2016; Berg et al., 2016) the main focus of these studies has been vegetation and habitat creation provided under powerlines (which differs in the other habitats compared in each study, for example Russell et al (2005) compared un-mowed powerline strips with mowed grassland). This is because powerline strips are often managed without expensive consistent mowing regimes, but removal of large dominant species e.g. tress, that may grow too close to the conductors. As a result powerline strips often have high vegetative

(and floral) diversity, providing suitable habitat types for a diversity of insect pollinators. Given higher habitat quality of power line strips than mowed grassland Russell et al (2005) found bee diversity and abundance was greater under powerlines, but not as much as expected. The intensity of ELF EMF exposure experienced around a powerline is not constant, and varies dependent on the current load on the transmission line (WHO, 2007a). Therefore the impacts of ELF EMF pollution from powerlines may vary between lines with high loads and low loads, in a similar manner to varied effects of roads on the environment dependent on roads with high levels or low levels of traffic. It is therefore not possible to determine if ELF EMF exposure was present in these studies, or to determine if differences in diversity could be due to ELF EMF exposure, or more likely due the differences in host plant species. For future studies to determine the impacts of powerlines (and ELF EMF pollution) at the community/population level, ELF EMF levels will have to be measured, and control sites should be the same habitat and/or contain a similar distribution/condition of plant species as exposure sites (before/after analysis could be beneficial here). Similar research needs have also been stated in a review of right-of-way habitats (Wojcik and Buchmann, 2012), where it is indicated that as these habitats are seen as reserves for wild bees and as declines in productivity are reported in honeybees kept in proximity to HVTLs (Greenberg et al., 1981), HVTLs may cause parallel declines in productivity or other effects in native bee communities, of which there is no data. If this is the case management regimes to improve pollinator diversity may improve by focusing plantings and pollinator refuges away from HVTLs. If however environmental levels do not impact wild populations these right-of-way habitats may continue to provide valuable habitat/resources for pollinators.

Whilst the main consideration of this study has been ELF EMF radiation, with the most likely exposure routes through HVTLs, other types of electromagnetic radiation should be considered in terms of the impacts of electromagnetic pollution on pollinators. Radiofrequency EMFs in particular may affect pollinators in the environment, and some already reported impacts on insects. Radiofrequency field disrupt magnetic orientation in cockroaches (Vacha et al., 2009) and in foraging ants (Cammaerts et al., 2013), as well as increasing aversive responses in honey bees (Favre, 2011) and reportedly increasing mortality (Darney et al., 2015). One field-based study with radiofrequency EMFs has been conducted, where the EMF levels were measured and related to insect distributions. Lázaro et al. (2016) found that radiofrequency EMFs from telecommunication antennae had a positive association with only underground nesting bees and bee flies (generally parasitoids of nesting bees) whilst beetle, wasp, and hoverfly abundance were negatively affected, and butterflies were not affected. This is interesting and may relate to some findings of bees

distributions in proximity top powerlines. Hill and Bartomeus (2016) reported that *B. muscorum* and *B. humilis* (which are ground-nesting carder bees) were only found under power lines. Wagner et al (2014) reported the identification of *Colletes productus* and *Melitta melittoides* (ground-nesting bees) as well as the globally rare *Epeoloides pilosulus* (a cleptoparasitic bee of ground-nesting bees) under power lines. Russell et al (2005) found greater species richness of ground-nesting, cavity-nesting and parasitic bees under powerlines. It must be made clear that in these studies ELF EMF levels were not measured, and vegetation differed under powerlines which was likely the predominant factor which caused different insect distributions. Furthermore the biophysical properties that may cause radiofrequency EMFs to affect insect distributions vary dramatically from ELF EMFs. Despite this, Hill and Bartomeus (2016) did suggest that cavities may counter risks of ELF EMF pollution under powerlines. In future studies consideration of other types of EMF radiation should continue, and studies with ELF EMFs could follow similar approaches to Lázaro et al. (2016), by considering major features of insect ecology such as nesting characteristics that may determine the susceptibility of certain insect species to ELF EMF exposure.

6.4 Management

If ELF EMF pollution is found to have ecological effects the mitigation of these impacts would have to be complex, and scientifically informed. Practices and technologies which produce EMFs are essential in modern human societies, and management solutions would need to consider alternative practices/technologies, modified practices/technologies, modified environmental/health policies to provide effective mitigation (MEA, 2005; World Health Organization [WHO], 2007a; Intergovernmental Panel on Climate Change [IPCC], 2015). The WHO (2007a) states that with implementation of any policy reducing EMF exposure there are direct costs to society (i.e. the direct changes made by policy) and indirect costs (e.g. less than optimal technology usage due to EMF restrictions) that policy makers must consider. A major point when considering these costs is what limit to lower ELF EMF is levels to.

The main protections on the environment from ELF EMF exposure the currently exist are indirection provisions made by exposure limits to the public. For example the EU has 100 μT exposure limits for the public (Swanson, 2014). It should be noted that when

powerlines are measured for meeting these exposure limits this measurement is taken from 1 m above the ground, and therefore does not protect flying insects, and other animals, from higher ELF EMF exposures. In other examples ELF EMF exposure regulations vary from no standard recommendations at all, to strict legal protections (Swanson, 2014). In Switzerland legal limitations are applied for newly install powerlines near 'sensitive areas' where HVTL exposure must be < 1 μT, and in the Netherlands recommendations to stakeholders are a maximum exposure of 0.4 μT to dwellings and schools. If environmental impacts of ELF EMFs are found then ELF EMF exposure should be considered in the context of what is environmentally safe, and not simply what is safe for public exposure.

The direct costs of implementing policy to reduce ELF EMF exposure are large. Kelfkens et al. (2002) compared four different methods of reducing magnetic fields for homes in Holland to 0.4 μT: vector-sequence rearrangement, phase conductor splitting, line relocation and undergrounding which costed €18,000, €55,000, €128,000, and €655,000 per dwelling, respectively. These are large direct costs that are clearly incurred immediately for any government choosing to restrict EMF exposure to a certain level, and this is before considering the indirect costs such as inefficiencies of technology as a result of policy. Impacts which could reduce the efficiency of energy transmission could lead to greater energy wastage, fuel consumption, and contribution to greenhouse gas emissions. These considerations are particularly important to make with regards to protecting the environment from EMF exposure.

Most EMF reductions in the Netherlands are set by the differential 'maximum design loads' of powerlines, which are the highest allowable line load, determined by the cross-section, material (aluminium, copper), and surface treatment of the conductors, as well as natural conditions such as ambient temperature (Kelfkens and Pruppers, 2006). An exclusion zone around a powerline is set based on the maximum design load, where dwellings, schools and nurseys cannot be located. For these features to be located within this zone appropriate steps, e.g. vector-sequence rearrangement, phase conductor splitting, line relocation and undergrounding, must be made to mitigate the impacts. Some of these solutions may be applied in terms of environmental protections, for example simply providing policy decisions to not prohibit HVTLs, but restrict emission in proximity of areas persevered for nature, or 'sensitive' areas may be beneficial, if ELF EMFs have wide-ranging environmental impacts. Other options include more rigorous consideration/regulations for in planning and construction applications for new HVTLs. Management options that lower EMF emissions, but don't completely remove them could be beneficial in sensitive areas,

dependent on the thresholds for wide scale economic impacts. If the goal was to achieve no ELF EMF emission the most expensive option of undergrounding powerlines could be made (Kelfkens et al., 2002). If wide scale impacts of EMFs occur in the environment, methods of mitigation may not need to be universal, dependent on the kinds of effects. For example where roads have provided barriers to movement in ecosystems, habitat permeability has been restored by creating wildlife crossings (Bissonette and Adair, 2008). In a similar manner if ELF EMFs limit movement of insects between habitats, undergrounding a section of HVTL may provide a lower cost method to not completely remove EMF pollution, but improve permeability between habitats. Other simple methods such as relocating managed pollinators away from HVTLs, and making habitat provisions for at-risk wild pollinators away from HVTLs, could reduce ELF EMF impacts on these important insects, without requiring changes in current technology systems.

As loads on lines vary enormously with time of day/year, and human energy consumption, other advances in engineering (factors away from transmission) such as better energy storage/batteries could be beneficial for not only reducing line loads at peak times and EMF emissions, but also energy efficiency (Kelfkens and Pruppers, 2006; Qian et al., 2011). By far, the method that reduces ELF EMF emissions the most is undergrounding of transmission lines, which results in a negligible EMF at ground level (Kelfkens et al., 2002). Whilst this approach is expensive, there may be some economic gains that occur with undergrounding, that do not occur with other methods, including more available land for residents, commercial activity, industry, agriculture or even natural preservation. As well as this, this is the only method which may remove the publically perceived health impacts of ELF EMF exposure that reduces house prices by 30% that are within 300 m of a powerline in the UK (Sims and Dent, 2005). Another potential management technique could be using High-Voltage Direct Current (HVDC) transmission instead of the AC transmission systems that are in place today. HVDC transmission produces much weaker electromagnetic fields, and has benefits including lower costs for cables to transfer the same capacity (relative to AC transmission) and efficiency in terms of simplicity of requirements for construction (Koshcheev, 2003). It is currently expensive to convert current from HVDC systems at terminal stations back to AC for energy usage. As HVDC is cheaper than AC transmission, but conversion costs and terminuses are high, HVDC is currently more cost effective for longer distance power transmission, whilst AC transmission is preferred over shorter distances (Koshcheev, 2003). Future improvements to systems that make HVDC transmission more cost effective, or political/scientific consensus that HVDC systems are a required alternative to AC transmission, may reduce environmental ELF EMF exposure. In

this context however the properties of EMFs generated by HVDC systems and the impacts of these EMFs on the environment would also have to be considered. Electromagnetic stimuli from HVDC systems being more similar to Earth's geomagnetic field are much less likely to have impacts such as cell excitability, but this biologically relevant signal may have other environmental impacts. If it is determined in future the ELF EMF exposures on the environment must reduce, then complex interdisciplinary economic and ecological analyses must be conducted to determine the best methods to bring about these changes.

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