Faculty of Medicine, Health, and Life Science

Investigating the response of *Lumbriculus variegatus* to cannabidiol and endocannabinoids

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Declaration and statements

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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Abstract

The endocannabinoid system (ECS) plays a crucial role in maintaining biological balance, encompassing G protein-coupled receptors, endogenous ligands, and catabolic enzymes. This system is targeted by endogenous cannabinoids such as anandamide (AEA), 2-arachidonoyglycerol (2-AG), and Δ9-tetrahydrocannabinol (THC), the psychoactive constituent of the cannabis plant. Cannabidiol (CBD), which isn't a component in the ECS, is a compound found in cannabis and has a proposed 56 molecular targets. CBD is non-psychoactive and complex in its mechanisms and only interacts with ECS receptors in the presence of THC.

Utilising the freshwater invertebrate *Lumbriculus variegatus* as a novel *in vivo* model, we investigate endogenous cannabinoids, along with AEA combined with the fatty acid amide hydrolase (FAAH) inhibitor URB 597, and CBD. Our objectives were to analyse cannabinoid-like receptor proteins in vertebrates and invertebrates, determining the lethal dose for 50% of the population, assessing behavioural effects post-CBD and endocannabinoid exposure, and developing novel methodologies for drug absorption analysis.

The conservation of endocannabinoid proteins in other animals suggest they have an essential role across different species. Our study reveals stereotypical movement is significantly reduced with exposure to ≥10 µM 2-AG, ≥10 µM AEA, and ≥10 µM AEA concentrations ± URB 597 (250 µM). Notably, combining AEA and URB 597 results in further movement reduction post-rescue period compared to AEA alone (≥5 µM vs. ≥10 µM). This study also shows CBD is toxic to *L. variegatus* at concentrations of ≥30 µM. Through GC-MS, we found CBD was undetectable at concentrations below 10 µM, which implies there is a minimum concentration threshold for CBD to be detected through GC-MS. Additionally saw CBD significantly alters stereotypical movement in *L. variegatus* at concentrations of ≥5 µM.

We show *L. variegatus* could potentially serve as an invaluable model for understanding cannabinoid interactions, facilitating drug design, and highlighting environmental impacts from cannabinoid exposure.

Table of Contents

List of Tables

List of Figures

Publications and Presentations

- Co-created *in vivo* pharmacology practical classes using the novel organism *Lumbriculus variegatus*

  Julanta Carriere, Nia A. Davies, Margaret R. Cunningham, Melisa J. Wallace, Aidan Seeley

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- *Lumbriculus variegatus* a novel in vivo model for studying drugs of abuse within educational settings

  Aidan Seeley, **Julanta Carriere**, Caitlin Bellamy, Lisa Wallace

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Abbreviations

2-AG	2- arachidonoylglycerol
AEA	Anandamide
AC	Adenyl cyclase
APW	Artificial pondwater
cAMP	Cyclic adenosine monophosphate
CB_1	Cannabinoid receptor Type 1
CB_2	Cannabinoid receptor Type 2
CBD	Cannabidiol
ECS	Endocannabinoid system
GC-MS	Gas chromatography–mass spectrometry
GPCR	G Protein-coupled receptor
FAAH	Fatty acid amide hydrolase
LC-MS	Liquid chromatography-mass spectrometry
MAPK	Mitogen-activated protein kinase
MAGL	Monoacylglycerol
NAM	Negative allosteric modulator
PAM	Positive allosteric modulator
PPARγ	Peroxisome Proliferator-Activated Receptor γ
3Rs	Replacement Reduction and Refinement
THC	Δ^9-tetrahydrocannabinol
TRPV	Transient receptor potential vanilloid

1. Introduction

1.1. The Endocannabinoid System

The endocannabinoid system (ECS) is found in essential biological systems and provides homeostatic balance (Silver, 2019). This system includes G-protein coupled receptors (GPCRs), endogenous ligands and catabolic enzymes (Silver, 2019) and shown in Figure 1.1.

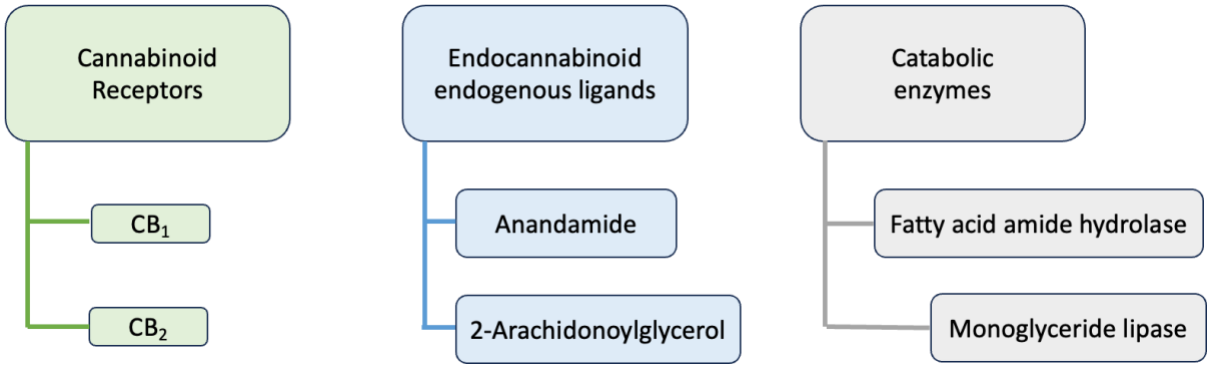

Figure 1.1. An illustration of how the different components of the endocannabinoid system is grouped into GPCR receptors, endogenous ligands, and catabolic enzymes. Image adapted from Bie et al. (2018).

Cannabinoids interact with the cell membrane by influencing the functional properties of integral proteins and ion channels of a lipid bilayer, and are able to directly bind to the transmembrane domains of ion channels which are embedded in the cell membrane (Makriyannis et al., 1990; Rabino et al., 2021; Reggio & Traore, 2000). Cannabinoids are lipophilic molecules which can enter into a lipid bilayer and alter the biophysical properties of a cell membrane (Oz et al., 2022). These changes take place by reducing membrane electrical resistance (Bach et al., 1976), increasing membrane fluidity (Dainese et al., 2012), increasing the stiffness of the cell membrane (Ghovanloo & Ruben, 2022) and elasticity (James et al., 2022), and by changing the brain's synaptic plasma membrane order (Bloom et al., 1997). Once the uptake of cannabinoids into the cells has taken place, these molecules are then transported through intracellular carrier-mediated transport, to effector molecules, such as cannabinoid receptors and catabolic enzymes (Fowler, 2013).

1.1.1. Cannabinoid Receptors

Cannabinoid Type 1 (CB_1) and cannabinoid Type 2 (CB_2) receptors, are seven GPCR-like rhodopsin (Rho) transmembrane receptors (Matsuda et al., 1990), the largest class of receptors in the GPCR family (Alhosaini et al., 2021). They are both coupled negatively to adenylate cyclase and positively to mitogen-activated protein kinase (MAP kinase or MAPK), through $G_{i/o}$ proteins, and receptor binding takes place via the cell's lipid bilayer, through lateral insertion of a ligand (Pertwee, 2006). The different sequences involved in cannabinoid binding sit in the N-terminal extracellular loop of the receptor (Shahbazi et al., 2020).

The CB_1 receptor is the primary site of action for endogenous ligands, anandamide and 2-AG, as well as Δ9-tetrahydrocannabinol (THC), which is the major psychoactive component of the cannabis plant (An et al., 2020). CB_1 receptor activation produces psychotropic effects and has risks associated with psychosis-type side effects (An et al., 2020). However, targeting both CB_1 and CB_2 receptors has been shown to provide therapeutic opportunities for conditions such as pain, inflammation, cardiovascular regulation, metabolic and neurodegenerative disorders, and cancer (An et al., 2020).

Both CB_1 and CB_2 receptors reduce adenyl cyclase (AC) activity and down-regulate the cyclic adenosine monophosphate (cAMP) pathway, which alters intracellular signal transduction and regulation of ion channels (Howlett & Fleming, 1984). AC is found in most cells, and is necessary for important functions of the Central Nervous System (CNS) such as , learning, memory and movement (Devasani & Yao, 2022). Reduction in AC activity is seen in patients with neurological disorders such as Alzheimer's disease (Yamamoto et al., 2000) and over expression is associated with bipolar disorder (P. Zhang et al., 2010). The cAMP signalling pathway has many cellular functions including gene and protein expression, and cell growth (YAN et al., 2016). Reduced levels of cAMP in the hippocampus is associated with sleep deprivation, which has been found to facilitate a reduction in memory in sleep-deprived animals (Walsh et al., 2023).

Cannabinoid receptors have some genetic conservation in invertebrates (Salzet & Stefano, 2002) but are highly conserved in vertebrates (Silver, 2019). GPCRs activate signalling effectors through different molecular mechanisms which are triggered by conserved amino acid residues (Leo et al., 2023). The presence of cannabinoid receptors in invertebrates is unclear. However, in the Elphick et al. (2003) study. They describe the identification of the CiCBR GPCR in *Ciona intestinalis*, a deuterostomia invertebrate. The CiCBR is orthologous to vertebrate cannabinoid receptors, but CiCBR cDNA has an encoded protein with the predicted length of 423 amino-acids, compared to the human CB_1receptor which has a predicted length of 472 amino-acids, and the CB_2 receptor whose predicted length 360 (Elphick et al., 2003). In addition to this, the protein-coding region of a vertebrate cannabinoid receptor gene is typically intronless. Whereas the *CiCBR gene* is interrupted by seven introns (Elphick et al., 2003).

1.1.1.1 Cannabinoid receptor type 1

It is believed CB_1 receptors are the highest expressed GPCR in the brain (Busquets-Garcia et al., 2018), and in humans, CB_1 receptors are primarily found in the central nervous system (CNS) and the peripheral nervous system (PNS). Low levels of CB_1 receptors have also been observed in the eyes, gastrointestinal tract, spleen, and immune cells (Spigelman, 2010).

In the CNS, CB_1 receptors are highly expressed by axons and the presynaptic terminals of the amygdala, cerebral cortex, basal ganglia, hippocampus, and cerebellum, and expression varies in these different locations (Shahbazi et al., 2020). For example, high expression levels of CB_1 receptor protein is found in cortical GABAergic interneurons compared to glutamatergic neurons, and expression is low in the hypothalamic area of the brain, compared to other regions (Busquets-Garcia et al., 2018). In addition, moderate to low levels of CB_1 receptor protein has been detected in noradrenergic, cholinergic, serotonergic, and dopaminergic cells (Marsicano & Kuner, 2008).

CB_1 agonists induce hypokinesia, catalepsy and analgesia (Shahbazi et al., 2020). These receptors are strongly linked with GABAergic and glutamergic cells, with the activation of CB_1

receptors inhibiting GABA and glutamate release (Shahbazi et al., 2020). The efficacy of G protein and CB_1 receptor coupling is linked to CB_1 protein levels (Marsicano & Kuner, 2008), and signalling patterns are dependent on which cannabinoid ligand stimulates the receptor (Busquets-Garcia et al., 2018). Endogenous or phytocannabinoids can direct their own specific signalling pathways, and these biased signalling patterns suggest CB_1 receptors can couple to different G-proteins (Busquets-Garcia et al., 2018). For example, THC is biased toward β-arrestin 1 recruitment at CB_1 , whereas endocannabinoids are biased towards G-protein signalling (Al-Zoubi et al., 2019).

$G_{i/o}$ proteins couple to CB_1 receptors and inhibit AC activity, which results in the inhibition of cAMP production (Zou & Kumar, 2018). cAMP is a second messenger which reduces immune functions such as inflammatory mediation and phagocytosis when cAMP is increased in cells, and clinical conditions related to infections are associated with cAMP increase (Serezani et al., 2008).

In addition to being coupled to AC. CB_1 receptors are coupled positively to A-type outward potassium channels, negatively to D-type outward potassium channels (Mu et al., 1999), and negatively to N-type and P/Q-type calcium channels (Twitchell et al., 1997). They are coupled through $G_{i/o}$ proteins, acting positively and negatively, inwardly and outwardly, rectifying these ion channels (Pertwee, 2006). CB_1 receptors are able to mediate the inhibition of neurotransmitter release (Howlett et al., 2002a), which they do by increasing calcium and potassium channel activity (Shahbazi et al., 2020). The release of neurotransmitters is associated with sleep regulation and activation of the CB_1 receptor induces sleep (Murillo-Rodríguez, 2008). Neurotransmitter suppression through the CB_1 receptor takes place by inhibiting voltage-gated Ca^{+} channels, which reduces the pre-synaptic Ca^{2+} influx or by inhibiting AC and the cAMP/PKA pathway (Zou & Kumar, 2018)

CB_1 receptor stimulation results in the phosphorylation and activation of the MAPK signalling pathways (Turu & Hunyady, 2010). The MAPK pathways initiate cell proliferation, regulate cell cycle processes, and stimulate cell death, and CB_1 receptors regulate these pathways

depending on the cell type or ligand (Zou & Kumar, 2018). Through the MAPK pathway, CB_1 receptors are phosphorylated which allows β-arrestins to be recruited (Flores-Otero et al., 2014). β-arrestins are key proteins which mediate ligand-gated GPCRs. Their functions include receptor desensitisation, receptor regulation, regulation sensitivity to agonists, and receptor Absorption regulation (Breivogel et al., 2008). β-arrestins mediate ligand gated- GPCR inactivation and desensitisation by binding clathrin and adaptor protein 2 which induces clathrin-mediated endocytosis, removing the receptor from the cell surface (Nogueras-Ortiz & Yudowski, 2016). The conformational changes in CB_1 receptors which result in β-arrestin coupling has not yet been fully elucidated (Morales et al., 2020), however studies have shown β-arrestin mediates signalling to ERK1/2, MEK1/2, and c-Src (Ahn et al., 2013).

1.1.1.2. Cannabinoid receptor type 2

CB_2 receptors are highly expressed in the immune system, but are also found in the spleen and tonsil cells, where they hold an immunomodulatory role (Turcotte et al., 2016). CB_2 receptors are expressed when there is inflammation (Bie et al., 2018) and modulate the release of cytokines resulting in an anti-inflammatory effect (Howlett et al., 2002b). Activation of CB_2 receptors in immune cells result in the inhibition of cell proliferation, and initiates apoptosis, as well as regulating cell adhesion and migration (Bie et al., 2018). Studies have found CB_2 receptor expression in leukocytes, and at different levels depending on the cell type (Turcotte et al., 2016). For example in peripheral blood immune cells, NK cells, B-lymphocytes, and monocytes express a higher level of CB_2 receptor than T-lymphocytes or neutrophils, and NK cells have the greatest variation in CB_2 expression levels (Graham et al., 2010)

Under normal physiological conditions, CB_2 receptors are found in the brain stem, cerebellum, cortex and hippocampus regions of the brain (Ferranti & Foster, 2022), and CB_2 expression is highly inducible on reactive microglia cells of the CNS in response to inflammation or injury (Bie et al., 2018). They also play a key role in the CNS by regulating neuronal function, and stimulation of CB_2 receptors inhibit dopaminergic activity and hippocampal pathways (Ferranti & Foster, 2022). Electrophysiological studies have observed CB_2 receptor expression in

dopaminergic and glutamatergic areas of the brain which is associated with schizophrenic disorders (Ferranti & Foster, 2022).

Like CB_1 receptors, CB_2 receptors are coupled with $G_{i/o}$ proteins (Shahbazi et al., 2020) and upon stimulation, these receptors inhibit AC activity, (Condie et al., 1996). The inhibition of cAMP levels through CB_2 stimulation results in MAPK cascade (Silver, 2019), leading to ERK1/2 and Akt phosphorylation (Bie et al., 2018), and activation of the p38-MK2 pathway (Shahbazi et al., 2020). Although CB_2 receptors has not been shown to affect potassium channels, CB_2 stimulation modulates intracellular calcium concentrations by releasing intracellular Ca^{+2} from the endoplasmic reticulum, and increases mitochondrial Ca^{+2} release (Turcotte et al., 2016)

1.1.2. Endocannabinoid ligands

The two main endocannabinoid ligands that have an affinity for CB_1 or CB_2 receptors include *N*-arachidonoyl-ethanolamine, more commonly known as anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Schurman et al., 2020). AEA and 2-AG are released from neurons through receptor-stimulated cleavage of lipid precursors (Basavarajappa, 2007). AEA and 2-AG are produced on demand, and their signalling function is terminated through enzyme hydrolysis. The enzymes responsible for the degradation of endocannabinoid ligands include fatty acid amide hydrolase (FAAH) and monoacylglycerol (MAGL) (Basavarajappa, 2007). Studies have shown that if FAAH or MAGL are inhibited, the level of endocannabinoid ligands increase, and inhibitors have been seen as potential new drugs for treatment of central nervous system disorders such as Alzheimer's disease or Parkinson's disease (Ren et al., 2020).

1.1.2.1 Anandamide

AEA is classified as a partial agonist at CB_1 receptors (Howlett et al., 2002a), and whilst AEA has a high affinity at CB_1 receptors, it has been found to be relatively inactive at CB_2 receptors (Mock et al., 2023). AEA is hydrolysed from N-arachidonoyl phosphatidylethanolamine (NAPE) by Phospholipase D (NAPE-PLD) (Liu et al., 2008) and degradation of AEA involves FAAH (Basavarajappa, 2007).

AEA is a lipid messenger and is part of the *N*-acylethanolamine (NAE) family involved in inflammation, anxiety, and energy metabolism (Mock et al., 2023). It behaves as a neuromodulator that continuously signals after being released through Ca^{2+} stimulation. AEA is commonly studied with the use of a FAAH inhibitor (Mock et al., 2023).

Physiologically, AEA acts as a homeostasis regulator and affects blood pressure and heart rate (Martín Giménez et al., 2018). It is involved in regulating food intake and lipid storage (Hansen & Diep, 2009), as well as regulating body-temperature (Wenger & Moldrich, 2002) and sleep (Vaughn et al., 2010). AEA is linked to both the male and female reproductive systems, and is present in in both male and female reproductive fluids (Di Marzo, 2008). AEA has also been found to act as an intracellular messenger and is linked to CB_1 receptor signalling in adipocytes (Di Marzo, 2008). In addition to cannabinoid receptors, AEA activates the transient receptor potential vanilloid 1 (TRPV1) ion channel and has also characterised as an endovanilloid (Stelt & Marzo, 2005).

1.1.2.2. 2-Arachidonoylglycerol

2-AG is synthesised on demand by hydrolysing diacylglycerol with diacylglycerol lipases (Reisenberg et al., 2012), and its levels are found to be ~170 times higher than AEA (Baggelaar et al., 2018). It acts a retrograde messenger that inhibits neurotransmitter release at the synapse, mediating short and long term plasticity (Kano, 2014), and its degradation is through Monoacylglycerol lipase (MAGL) (Gil-Ordóñez et al., 2018). 2-AG also binds to $GABA_A$ receptors, having a role in sedation and locomotion (Sigel et al., 2011), and it modulates interleukin (IL)2 expression by activating the Peroxisome Proliferator-Activated Receptor γ (PPARγ) (Rockwell et al., 2006). 2-AG also acts as a negative allosteric modulator of the human adenosine A_3 receptor, a GPCR which is involved in inflammation (Lane et al., 2009). It also acts at TRPV1 channels, where it plays a role in temperature and inflammation, as well as acting at G protein receptor 55 (GPR55), which is a potential drug target to treat cancers (Baggelaar et al., 2018).

The ECS has been shown to hold therapeutic value and targeting it has been successful in treating neurological and pathological conditions, as outlined in Table 1.1 (Barrales-Cureño et al., 2020). The ECS is complex, however, by targeting its different components and by stimulating its signalling pathways, we can trigger a range of therapeutic effects, such as pain relief, immune modulation, and neuroprotection (Zou & Kumar, 2018). Both AEA and 2-AG have been found to be non-toxic to animals; however, the therapeutic potential of these compounds has yet to be thoroughly studied (Pacher et al., 2020).

Table 1.1. List of the key biological systems which are regulated through the stimulus of the endocannabinoid system. Adapted from Barrales-Cureño et al., (2020)

Biological system influenced by ECS	Effect
Central nervous system	Impairs learning and memory Modulates emotions Inhibits neuronal excitability Increases locomotion Decreases motivation Reduces nociception Inhibits or disinhibits synaptic plasticity
Peripheral nervous system	Decreases neurogenic inflammation and nociception
Hypothalamus-hypophysis-adrenal axis	Corticosteroid release inhibits CB_1 expression
Skeletal system	Improves bone formation
Reproductive system	Inhibits sperm implantation and motility
Immune system	Reduces cytokine release

1.2. *Cannabis sativa*

1.2.1. The *Cannabis sativa* plant

The ECS was discovered because of the use of the Cannabis plant recreationally and medicinally. The Office of National Statistics (ONS) estimates cannabis is the most widely used drug since estimates began in 1995, with approximately 7.4% of UK adults aged between 16 to 59 years using cannabis in 2021/2 (ONS, 2023). The Cannabis plant is a dioecious plant from the *Cannabaceae* family and includes its sub-species *Cannabis sativa*, *Cannabis indica* and *Cannabis ruderalis* (McPartland, 2018).

1.2.2. The constituents of Cannabis sativa

The *C. sativa* plant, which is also known as hemp, has many constituents, all with a complex phytochemistry structure (Cox et al., 2019). To date, more than 550 organic chemical compounds have been isolated from the *C. sativa* plant (Barrales-Cureño et al., 2020). These compounds include mono and sesquiterpenes, sugars, hydrocarbons, steroids, nitrogenated complexes, and amino acids (Barrales-Cureño et al., 2020). These chemical compounds include 113 different types of plant-derived cannabinoids which are termed phytocannabinoids (Rock & Parker, 2021).

Phytocannabinoids are unique to the *Cannabis sp.* and include the phytocannabinoids Δ^9-tetrahydrocannabinol (THC), Δ^8-tetrahydrocannabinol (Δ^8-THC) cannabidiol (CBD), and cannabinol (CBN) (Barrales-Cureño et al., 2020). THC is the main psychoactive constituent of the *C. sativa* plant and has been historically used for its psychotropic and medicinal effects (Ng et al., 2023). Alongside THC, Δ^8-THC, CBD and CBN, the other phytocannabinoids present in the plant include, cannabichromene (CBC), cannabiciclol (CBL), cannabigerol (CBG), cannabigerol monomethyl ether (CBGM), cannabielsoin (CBE), cannabinodiol (CBND), cannabitriol (CBT), dehydrocannabifuran, and cannabicitran. These chemicals are measured in different levels depending on the *C. sativa* strain (Barrales-Cureño et al., 2020).

All phytocannabinoids have a 21-carbon carbocyclic structure in common, which is formed in three rings; cyclohexene, tetrahydropyran, and benzene, containing aromatic oxygenated hydrocarbons which lack nitrogen (Barrales-Cureño et al., 2020). CBD and THC have the same number of carbons, hydrocarbons and oxygens that are arranged differently, with the same molecular formula and chemical structure. However, having a different arrangement changes the phytocannabinoid effect. For example, THC and CBD both have the chemical structure $C_{21}H_{30}O_2$ with the atomic weight of 314.46 g/mol, but THC is psychotropic and CBD does not have the same effects (Barrales-Cureño et al., 2020). This is because, THC has a three-ring structure, making the molecule rigid, affecting how THC binds to receptors (Ng et al., 2023). On the other hand, CBD has a two-ring structure which is connected by a flexible bond. This results in CBD being adaptable, and allows CBD to fit into various receptors and interact with different pathways (de Almeida & Devi,. 2020).

1.3. Cannabidiol (CBD)

Cannabidiol (CBD) is a component of the *C. sativa* plant and is produced through chemical synthesis or extraction of the *C. sativa* plant. It is a non-psychoactive constituent; and has been shown to have a wide range of pharmacological activities with therapeutic potential, such as reducing seizures which are experienced by patients with tuberous sclerosis complex (Thiele et al., 2022). It accounts for 40% of the *C. sativa* plant's extract (Gaoni & Mechoulam, 1964) and binds to a wide range of targets (Castillo-Arellano et al., 2023).

The potential ecological impact of CBD, particularly through its unregulated release into the environment, is a growing concern. As a widely used compound in consumer products, CBD can enter natural ecosystems through various means, such as wastewater runoff or improper disposal. However, the full scope of its effects on non-target organisms, particularly aquatic invertebrates, remains unexplored. This study examines the behavioural and physiological responses of *L. variegatus*, an aquatic invertebrate, to CBD exposure. By investigating how CBD interacts with this species, the study provides valuable insights into the potential risks associated with the environmental presence of cannabinoids.

Aquatic invertebrates, such as *L. variegatus*, are essential components of ecosystems and often serve as bioindicators of environmental health (O'Gara et al., 2004). Studying their responses to CBD exposure can be crucial for environmental risk assessment, as changes in behaviour or locomotion can signal broader ecological disruptions, such as altered predator-prey dynamics or impacts on reproduction. This study contributes to the understanding of CBD's persistence and effects in aquatic environments, and could inform regulatory frameworks, helping to ensure that the use of cannabinoids does not pose unforeseen risks to ecosystems. Therefore, the study's findings are not only significant for pharmacological and toxicological assessments but also for evaluating the broader environmental consequences of CBD's widespread use.

1.3.1. The Pharmacokinetics of CBD

CBD and CBD-derived products, such as oils, waxes, or resins, can be inhaled, taken orally in the form of edibles, or used topically, with its absorption rate dependent on the route of administration. In humans, CBD is absorbed the fastest when inhaled and the absolute bioavailability of CBD after inhalation is ~30%, and 10 mg of CBD will have a maximum plasma concentration of ~3 μg/L at 2.8-hours (Castillo-Arellano et al., 2023). This bioavailability value is lower after oral consumption at around 7 to 13% and has a lower level of 4% bioavailability after administration by oral mucosal spray (Cox et al., 2019). This means that 70 to 96% of the CBD we consume is unavailable. CBD is highly lipophilic and is rapidly distributed to the lung, liver, heart, and brain (Lucas et al., 2018). Body size or disease can affect the distribution of CBD, and in long-term or chronic use, CBD can be found in adipose tissue for several weeks after use (Lucas et al., 2018). CBD is inconsistently absorbed through the gastrointestinal tract and undergoes liver metabolism, facilitated mainly by enzymes from the cytochrome CYP450 system (Gaston & Friedman, 2017). After absorption, rapid hydroxylation occurs, introducing a hydroxyl group and forming 7-OH-CBD (Nasrin et al., 2021). This hydroxylation process is primarily mediated by CYP2C19 and CYP3A4 enzymes, while other enzymes such as CYP1A1, CYP1A2, CYP2C9, and CYP2D6 may also play a role (Doohan et al., 2021). Subsequently, the liver further processes 7-OH-Cannabidiol, leading to the eventual excretion of metabolites in

faeces, with a small proportion being eliminated in urine (Doohan et al., 2021). The estimated half-life of CBD in humans ranges from 18 to 32-hours (Doohan et al., 2021).

1.3.2. The Pharmacodynamics of CBD

The mechanism of action of CBD is complex, and these mechanisms are debated and unclear (Castillo-Arellano et al., 2023). It is proposed that CBD has 56 different molecular targets, including enzymes, ion channels, and G protein-coupled receptors (GPCR), which are all involved in a multitude of neurological conditions. CBD can act as an agonist, inverse agonist, or antagonist as reviewed by Chayasirisobhon (2020). It can behave as a negative allosteric modulator (NAM) or positive allosteric modulator (PAM) at different sites, as well as affecting both neurological and hepatic enzymes (Castillo-Arellano et al., 2023).

Although CBD is a phytocannabinoid, it allegedly does not stimulate CB_1 or CB_2 receptors. Instead, CBD acts as an non-competitive NAM at these sites (Laprairie et al., 2015). For example, when administered together, CBD has been found to reduce THC's adverse cardiovascular or psychotropic effects (Lucas et al., 2018). CBD acts as an agonist at 5-HT_{1A} receptors which are coupled to $G_{i/o}$ proteins, and produce anxiolytic and antiepileptic effects (Martínez-Aguirre et al., 2020). CBD also serves as a NAM at 5-HT_3 receptors, and these receptors are found in areas of the brain associated with pain and mood (De Gregorio et al., 2019). In addition to NAM effects, CBD acts as a PAM of $GABA_A$ receptors, providing anti-anxiety and anticonvulsant effects (Wright et al., 2020). Moreover, CBD has also been found to act as an antagonist at the α7 nicotinic acetylcholine receptor (α7 nAChR) (Mahgoub et al., 2013). These receptors are calcium (Ca^{2+}) channels found in the nervous system, which are indicated in schizophrenia disorders, Alzheimer's disease, and seizures (Terry et al., 2023). CBD has also been shown to act as a full agonist at TRPV1 channels, and this mechanism of action is also associated with anti-anxiety, as well as pain relief and anti-inflammatory effects (Atalay et al., 2019). In addition, CBD has been shown to activate Transient receptor potential vanilloid type 2 (TRPV2) channels, which has been associated with chronic pain, however, transient receptors potential vanilloid type 3 (TRPV3), a temperature sensor expressed in the brain skin and tongue, has a low response to CBD (Castillo-Arellano et al., 2023). CBD is also believed to

act as a PAM on the delta-opioid receptor (δOPR) and accelerates agonist dissociation at the mu-opioid receptor (μOPR), reducing activity (Kathmann et al., 2006). The structural flexibility of CBD enables its binding to a wide range of therapeutic targets, increasing its potential for medical applications. However, this same flexibility also increases the risk of toxicity, particularly through drug-drug interactions and contraindications.

CBD is an antagonist of the novel cannabinoid receptor GPR55 which is expressed in the brain, PNS, and immune system (Castillo-Arellano et al., 2023). GPR55 receptors are associated with vascular function, motor coordination, bone physiology, pain, and cancer (Castillo-Arellano et al., 2023). CBD has also been found to act as an antagonist at G protein-coupled receptor 18 (GPR18) in lymphoid tissue, reproduction organs, and the brain and is associated with sperm physiology, metabolism, and diseases such as cancer and pain (Castillo-Arellano et al., 2023). CBD has also been shown to act as an inverse agonist at G protein-coupled receptor 3 (GPR3) associated with neuropathic pain and emotional disorders, GPR6, also expressed in the reproductive system, and GPR12, associated with memory, behaviour, and emotion (Castillo-Arellano et al., 2023). CBD has also been shown by Castillo-Arellano et al. (2023) to be an agonist at PPARγ, found in macrophages and adipose tissue. PPARγ is involved in glucose metabolism and lipid storage and has been found to possess anti-inflammatory activity that prevents neurodegeneration by reducing pro-inflammatory molecules (Castillo-Arellano et al., 2023). A summary of CBD targets can be found in figure 1.2.

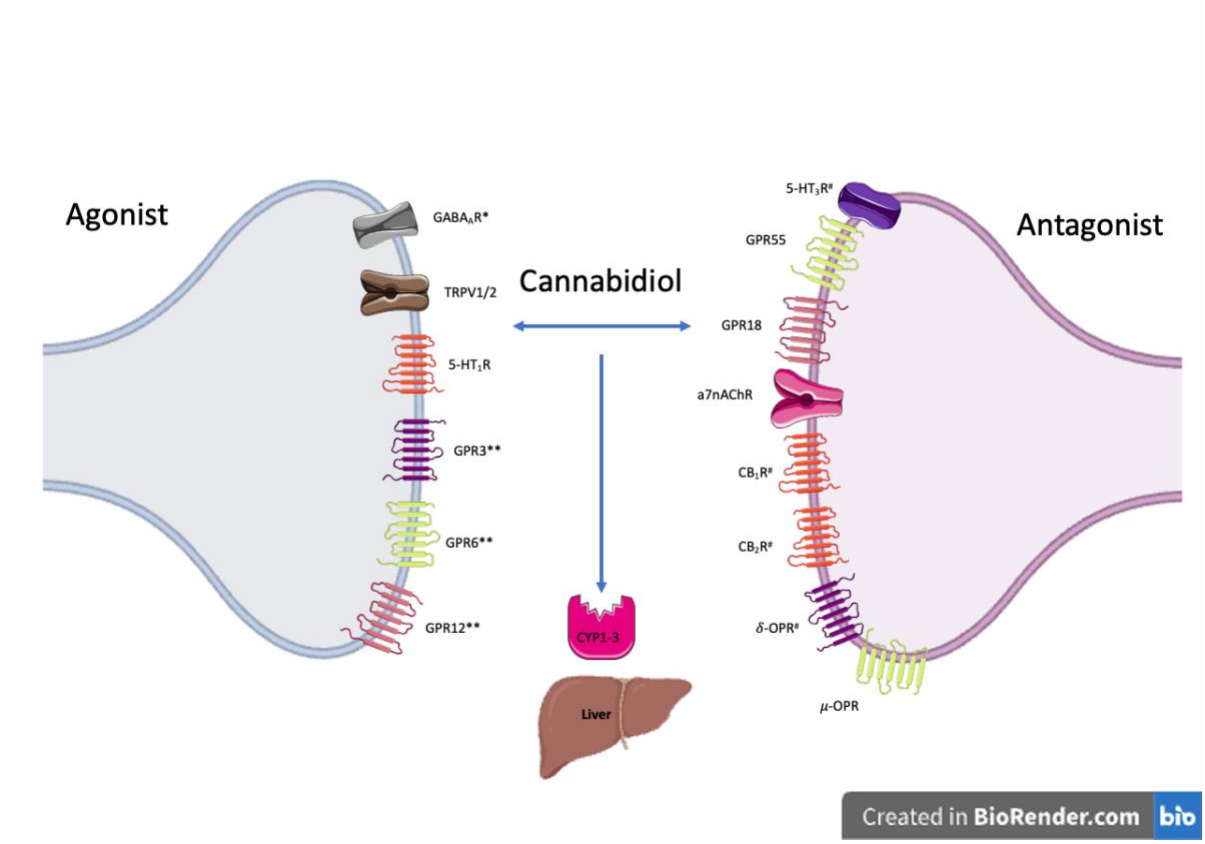

Figure 1.2. Diagram showing cannabidiol targets. Displaying the role of CBD as an agonist, *positive allosteric modulator (PAM), **inverse agonist, antagonist, and #negative allosteric modulator (NAM) on different target receptors and enzymes. Image adapted from Castillo-Arellano et al. (2023).

1.3.3. CBD-based products

In the UK, the medicinal use of CBD, in the form of Epidyolex®, is licenced to treat conditions such as Lennox-Gastaut syndrome (LGS). LGS is a rare form of epilepsy and mental-related impairment, usually seen in children under seven (Patel et al., 2021). Clinical trials have shown when using Epidyolex® as an add-on treatment for LGS, seizure frequency is reduced (Patel et al., 2021). Epidyolex® has also been shown to reduce the frequency of convulsive seizures in Dravet syndrome patients, an epilepsy disorder also seen in children (Koubeissi, 2017). CBD can also treat the Tuberous sclerosis complex (TSC), a genetic condition which causes a local malformation of cells in organ systems such as the brain, kidney, skin, lungs, heart, and eyes (Mizuguchi et al., 2021). Around 50% of those with TSC will also experience infantile spasms

with the onset of TSC seen in children under two years old (Cohen et al., 2021). Clinical trials have shown that CBD use can reduce the frequency of TSC seizures by ~40% with manageable adverse effects and is licenced to treat patients from 12-months old (Thiele et al., 2022). In the UK, cannabis-based products for medicinal use (CBPM) are prescribed under the guidance of the National Institute of Health and Care Excellence (NICE) Technology Assessments for the use of cannabidiol (Epidyolex®) for severe treatment-resistant epilepsies. CBD is also a constituent of Sativex, an oromucosal spray delivering 1:1 THC and CBD, and is used to treat muscle spasticity in patients with multiple sclerosis (Karschner et al., 2011).

In addition to medicinal use, unlicensed CBD products are sold as well-being products, available online and over the counter, including vaping products, food supplements, drinks, and cosmetics. Unlike THC, CBD is not a controlled substance in the UK, which poses problems when isolating CBD from controlled cannabinoids like THC during extraction processes. Therefore, in 2021, the Home Office proposed to work closely with the Food Standard Agency (FSA) to develop a legal framework for consumer CBD products, including agreeing on the legal level of THC in CBD products (ACMD, 2023). In October 2023, the FSA issued new precautionary advice which recommends adults should limit their daily consumption of CBD to 10 mg per day (ACNFP & COT, 2023)

1.4. Animal studies in pharmacology and drug discovery

Drug discovery is a long and expensive process, and can take around 12 to 15 years for a new product to get to market (Giunti et al., 2021). Animal models play a crucial role in identifying new drug targets and are necessary for evaluating the toxicity, pharmacokinetics, pharmacodynamics and efficacy of a novel therapeutic agent before it goes to clinal trials (Singh & Seed., 2021).

1.4.1. The use of vertebrate models

Animal testing is valuable in the discovery of novel drug treatments. It is also a regulatory requirement for any new medicine to be tested in two types of animal species before being

given to humans (Prior et al., 2018). Animals have many physiological similarities to humans and it is possible to predict animal-to-human pharmacokinetic similarities, for example, the absorption of a drug across the gastrointestinal tract (Lin, 1995). Mice and humans share physiological and genetic similarities, making mice invaluable for biomedical research (Bryda, 2013). Both have similar organ systems, metabolic pathways, and immune responses, with approximately 85% of mouse genes having human counterparts (Breschi et al., 2017).. Regulatory pathways and genes linked to diseases are also highly conserved (Ruberte et al., 2023). However, key differences exist. Mice are smaller, have shorter lifespans, faster heart rates, and higher metabolic rates, which can affect disease progression and drug metabolism (Ewing et al., 2019). Genetically, while their genomes are similar, some gene functions and immune responses differ, requiring careful interpretation when translating findings to humans (Breschi et al., 2017). After the sequencing of the human genome in 2001, the mouse followed in 2002, providing a wealth of genetic information (Chinwalla et al., 2002). This provided the ability to manipulate mouse genes to either have no expression (knocked-out), are expressed at specific development stages, or in selected cells (Bryda, 2013). Other animals commonly used for *in vivo* studies include rats, rabbits and other rodent models (Hickman et al., 2017). Zebra fish are also an important animal model and their use in biomedical research includes understanding the molecular mechanisms of human genetic diseases. For example, developmental disorders can be studied using zebra fish eggs which provide hundreds of developing embryos which can be imaged live (Choi et al., 2021).

However, although animal models are valuable in predicting human toxicity during drug discovery. There are limitations in reliably translating drug safety data to humans, and patients have been harmed during clinical trials with drugs which had been considered safe through animal studies (Van Norman, 2019). An example of this is Fialuridine, an antiviral agent used to treat the hepatitis B virus (Colacino et al., 1996). Preclinical trials for Fialuridine were conducted in mice, rats, rabbits, and monkeys, with toxic effects only observed at the maximum tolerated range, and no liver toxicity detected at low doses (Trials et al., 1995). However, during the phase II clinical trial, Fialuridine caused acute liver failure in seven patients and the death of five (McKenzie et al., 1995). In addition to this, conditions and

procedures conducted within laboratory environments influence animal behaviour, and there are variances between human and animal models of disease, as well as genetic and physiology differences in between species (Akhtar, 2015).

As well as the risks associated with using animal models, there are ethical reasons to consider, and researchers must be clear on the scientific purpose of using animals in their research project (Ghasemi & Dehpour, 2009). Animal testing is highly regulated through the Animals (Scientific Procedures) Act 1986 where there is a requirement to reduce the harm caused to animals through the Replacement Reduction and Refinement (3Rs) principles (Animals (Scientific Procedures) Act 1986).

1.4.2. The Replacement, Reduction, and Refinement (3Rs) Principles

The 3Rs framework was first proposed in 1959 and its aim is to reduce the harm and suffering of animals by providing guiding principles of conducting humane animal studies (Russell & Burch, 1960).

The 3R principles were defined as:

- Replacement, which is the substitution of conscious living animals with insentient material.
- Reduction in the number of animals used when obtaining information of a given amount using precision
- Refinement which decreases the severity of inhumane procedures applied to the animal used (Hubrecht & Carter, 2019).

To reduce the pain and distress of animals through research, the use of animal models has steadily declined (Home Office, 2022). The Home Office reports that in 2022, 2.76 million scientific animal procedures took place, the lowest number since 2002, and this number is a 10% decrease on the previous year (Home Office, 2022). However, without animal models, the safety and efficacy of novel drugs would be unknown, and animal models prevent the risk of serious harm during clinical trials. The use of *in vivo* models in an education setting is also

reducing and it is reported that less than 2% of students have *in vivo* opportunities during their degree (British Pharmacological Society and the Physiological Society, 2006). This reduction is due to the strict regulatory requirements through the Animals (Scientific Procedures) Act 1986, and the availability of alternative methods such as *in vitro* tissue or cell culture methods, *in silico* computer models, or the use of alternative organisms such as microorganisms or invertebrates (Doke & Dhawale, 2015). However the decline of animal use in pharmacology education loses students who are trained in *in vivo* pharmacology and confident within a laboratory setting (Seeley et al., 2021).

1.4.3. Invertebrate models

Invertebrate models serve as viable alternatives to vertebrate *in vivo* models, as they are not covered under the Animals (Scientific Procedures) Act 1986 and align with the reduction principle of the 3Rs framework. They are excluded from the Act due to their simpler nervous systems, which are thought to make them less capable of experiencing pain or suffering compared to vertebrates. (Smith et al., 2006). Invertebrates play a role in monitoring the health of an environment or ecosystem and are species which are cost-effective during the early stages of drug developing (Wilson-Sanders, 2011). Invertebrate species used for study include nematodes and insects, as well as freshwater and marine species, with the most popular species being *Drosophila melanogaster* and *Caenorhabditis elegans* (Wilson-Sanders, 2011). The *D. melanogaster,* also known as the fruit fly, has been used to study biological processes such as embryonic development and aging, as well as behavioural and learning studies (Tolwinski, 2017). *C. elegans*, a nematode, has been used to study the underlying molecular and cellular mechanisms of stroke and ischemia, and age-related neurodegenerative disorders such as, Alzheimer's and Parkinson's disease (Markaki & Tavernarakis, 2020).

The endocannabinoid system (ECS) is widely conserved throughout mammalian species; however, only some conservation has been seen in invertebrate species (Silver, 2019). Salzet and Stephano (2022) highlight that neither *Drosophila melanogaster* nor *Caenorhabditis elegans* hold cannabinoid receptors or FAAH orthologs and suggest that cannabinoid

receptors evolved after the separation of deuterostomes and protostomes (Salzet & Stefano, 2002). However, although *D. melanogaster* and *C. elegans* do not exhibit the presence of cannabinoid receptors, research shows that they display NPR-19, a functional ortholog of the mammalian CB_1 and CB_2 receptors (Clarke et al., 2021). Studies have shown that CBD is not toxic to *C elegans* when *C elegans* are given physiologically relevant CBD concentrations (Land et al., 2021). However, although the sea squirt, *Ciona intestinalis*, does not show cannabinoid receptors, FAAH is conserved in this organism (Clarke et al., 2021). Figure 1.3 displays cannabinoid receptor coding in 15 different species.

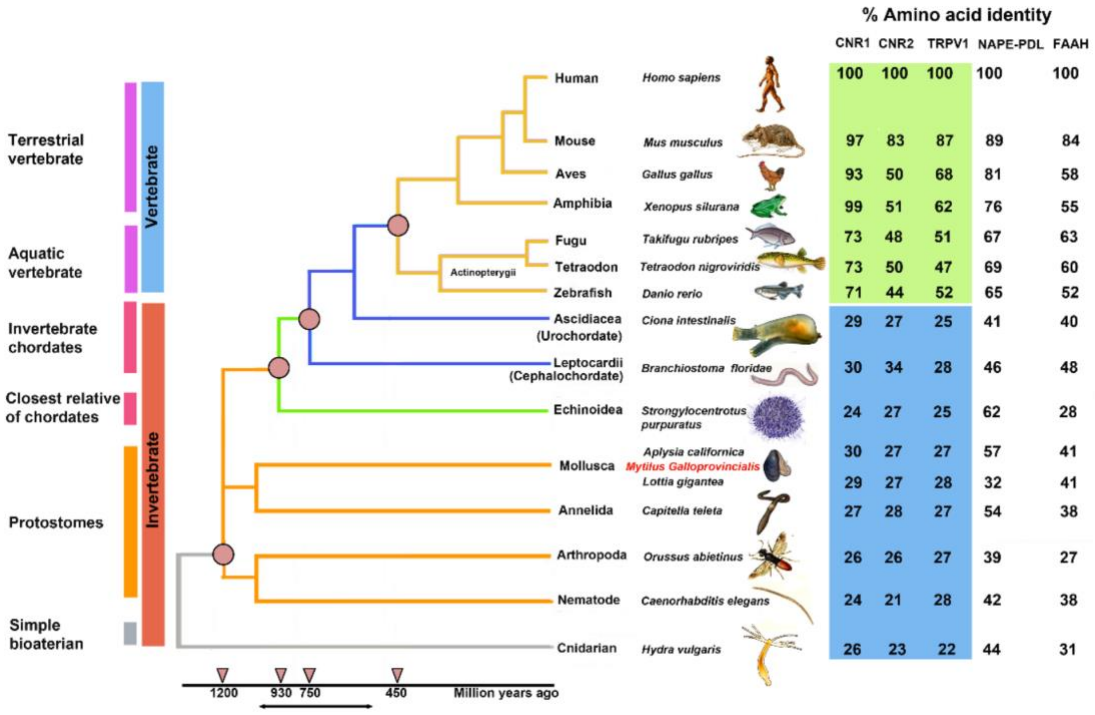

Figure 1.3. Diagram showing the endocannabinoid system phylogenetic tree. Displaying ECS receptor coding in 15 species with the ECS showing as being highly conserved in humans and other vertebrates. Taken from Mosca et al. (2021)

1.5. *Lumbriculus variegatus*

Lumbriculus variegatus (Figure 1.4) are more commonly known as the blackworm. They are small freshwater aquatic organisms naturally found in the sediment of ponds and marshes in North America and most parts of Europe (Drewes, 1999) and the presence of an ECS is

unknown in this organism. *L. variegatus* are sediment-dwelling organisms and have been extensively characterised as an ecotoxicology indicator model for metal or organic pollutants discharged into the environment (Kontchou et al., 2023; O'Gara et al., 2004).

L. variegatus only reproduce asexually in laboratory conditions through fragmentation, but sexually and asexually reproduce in their natural environment (Drewes, 1999). A fertilised egg can develop into a worm 10 cm in length (N. Lesiuk, 2000), however, when these worms are reared artificially, they usually grow to between 1 to 10 cm (Ding et al., 2001). During asexual reproduction, and if any segments are injured or amputated, the surviving fragment will regenerate the head and/or tail segments into a new worm (N. Lesiuk, 2000). Regeneration starts with blastema and bud formation, and then the differentiation of new head or tail segments (Martinez Acosta et al., 2021).

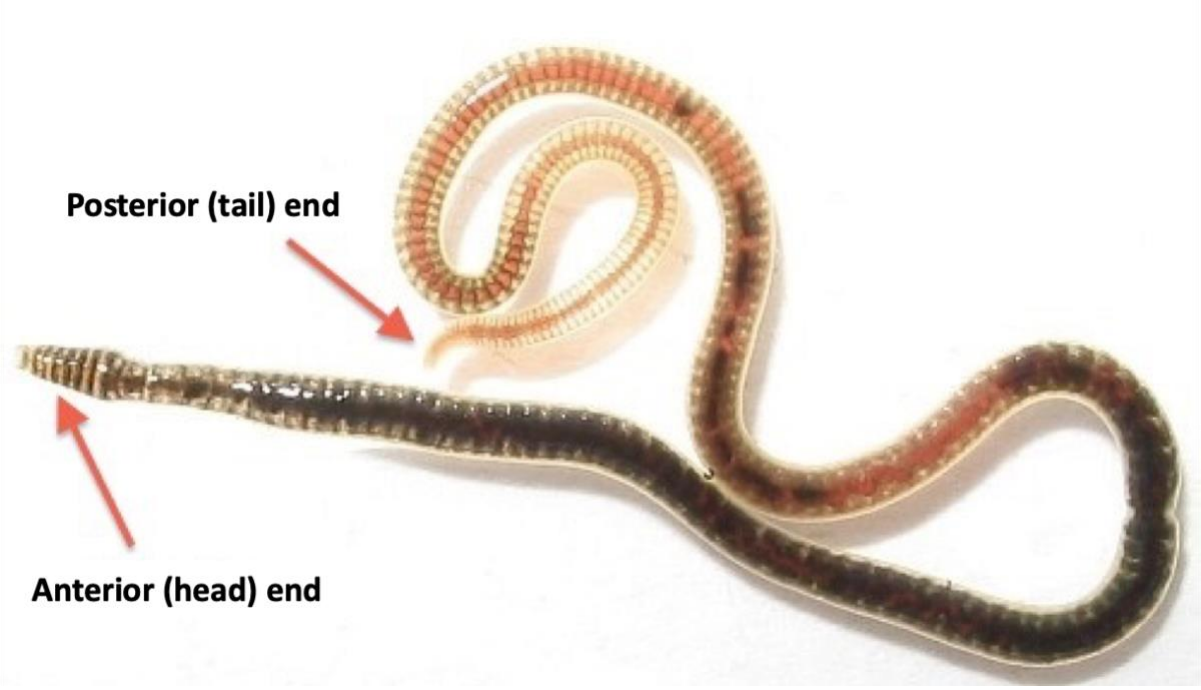

Figure 1.4. Image of *Lumbriculus variegatus*. Labels show the posterior (tail) end and anterior (head) end.

L. variegatus food source includes microorganisms and decaying vegetation, they also break down organic material, and provide a food source for larger organisms (Drewes, 1999). *L. variegatus* use its head end to forage for food in sediment, whilst its tail end is extended

towards the water surface and is used for gas exchange through cutaneous respiration (Ding et al., 2001.). *L. variegatus* are a prey species, which means they hold rapid response behaviours to stimuli. Upon anterior stimulation, this organism displays a body reversal movement, and at the touch of the posterior, they will exhibit a helical swimming response (O'Gara et al., 2004).

The *L. variegatus* nervous system contains a supra-oesophageal ganglion within its first segment and a ventral nerve extending along its body, which provide a rapid escape reflex (Lesiuk, 2000). This escape reflex is mediated by giant nerve fibres acting as giant interneurons, which are found in the ventral nerve cord (N. Lesiuk, 2000). Drugs can be easily diffused through the skin of *L. variegatus* and its pulse rate can be easily observed through their transparent skins (Bohrer, 2006.). Their circulatory system's major blood vessels include the dorsal blood vessel (DBV), the ventral blood vessel (VBV) and the lateral blood vessels (LBV). These blood vessels carry red blood containing erythrocruorin, a haemoglobin-like pigment, away from the tail via the DBV to the head through muscle contraction, and oxygenation takes place throughout the body (Drewes, 1999).

As this organism is an invertebrate, its use is not regulated through the Animals (Scientific Procedures) Act 1986 and using *L. variegatus* as an *in vivo* research or educational model meets the 3Rs guiding principles of the ethical use of animals in research. Using this organism as a model for educational purposes or drug discovery and development meets the requirements of the replacement of animals in research whilst being able to provide *in vivo* study opportunities.

L. variegatus has not been as extensively studied as *D. melanogaster* or *C. elegans*, with most *L. variegatus* studies being focussed on their ecophysiology and toxicology response (Zhou et al., 2023). Zhou et al. (2023) explored evidence of any genetic diversity in *L. variegatus* and studied its phytogeography. They did this by examining phylogeographic patterns of mitochondrial genes in specimens collected from different regions and discovered there are two *L. variegatus* species. However little genetic information is available about *L. variegatus*,

and unlike other invertebrates, there have been no previous studies exploring the conservation of any endocannabinoid proteins in *L. variegatus*.

L. variegatus is used in Swansea University Medical School as a novel *in vivo* pharmacological educational model within the Swansea Worm Integrative Research laboratory (SWIRL) (Carriere et al., 2023; Seeley et al., 2021). Previous work with *L. variegatus* has studied the antidepressant drug, fluoxetine (Nentwig, 2007), and the non-steroid anti-inflammatory drug diclofenac (Karlsson et al., 2016), and within the SWIRL group, channel-blockers such as dantrolene, lidocaine and quinine, as well as harmful drugs such as nicotine has investigated *L. variegatus* response to drugs by measuring stereotypical behaviour and free locomotion movement (Carriere et al., 2023; Seeley et al., 2021). Therefore *L. variegatus* could be a suitable model to study other drugs, including novel drugs or drugs that are misused.

1.6. Aims and objectives

The use of animal testing to understand the endocannabinoid system has opened the door to new treatments targeting pain, cancer, neurological disorders, stress and anxiety management, and inflammatory diseases. The conservation of the endocannabinoid system in *Lumbriculus variegatus is* unknown, and there is a lack of studies that focus *L. variegatus* response to CBD, or the ecotoxicology effects from CBD's release into the environment. In this project we will further characterise a model that could be valuable in both drug discovery and education and characterise the response to cannabinoid compounds in a novel organism that may indicate the presence or absence of the ECS in that species.

This project will have the following aims.

Aim 1: Analyse cannabinoid-like receptor proteins within vertebrate and invertebrate species using an endocannabinoid protein alignment analysis bioinformatics approach.
Aim 2: Characterise the behavioural responses of *L. variegatus* following exposure to human endocannabinoid compounds 2-AG and AEA, a FAAH inhibitor and the phytocannabinoid CBD

using stereotypical movement and free locomotion behavioural methods outlined by Seeley et al. (2021).

Aim 3: Develop novel methodology for determining drug absorption into *L. variegatus* via gas chromatography-mass spectrometry (GC-MS)

2. Methods and Materials

2.1 General

All inorganic material used in this project was of analytical grade. All drug solutions were prepared on the day and at the beginning of each experiment, and artificial pond water, 0.5% ethanol, or 0.5% dimethyl sulfoxide (DMSO) was used as vehicle control.

2.2. Safety

Formal risk assessments were conducted and recorded in line with the control of substances hazardous to health (COSHH) requirements. All waste was disposed of in accordance with the manufacturer's instructions.

2.3. *In vivo* model

One batch of *Lumbriculus variegatus* was purchased from Alpha Fish Foods, laboratory reared in aquariums in artificial pond water (O'Gara et al*.,* 2004), and fed with commercial fish food, as set out by Seeley et al. (2021). To climatise the worms and allow for asexual reproduction, *L. variegatus* were housed for three months before use in this study and sustained in a tropical aquarium containing artificial pond water using the following composition: 1 mM NaCl, 13 µM KCl 4 µM $Ca(NO_3)\cdot 4H_2O$, 17 µM $Mg(SO4)\cdot 7H_2O$, 71 µM HEPES buffer, and kept at room temperature (18-21 °C). Aquariums were lit using artificial fluorescent light on a 16:8 light dark cycle. Cultures were fed with TetraMin© flakes and 10 mg/L spirulina weekly. For experimentation, *L. variegatus* lacking any apparent abnormalities, such as fragmentation, were randomly selected. *L. variegatus* were removed and placed into six-well plates and left to acclimatise for 18-24 hours before experimentation took place. Following assay endpoints,

worms were euthanised through exposure to 70% ethanol before disposal. Ethical or university approval was not required for the use of blackworms in this project.

2.4. Products and chemicals

Table 2.1. List of chemicals and products. List of chemicals and products, including manufacturer details

Chemical	Supplier	Storage
2-Arachidonylglycerol (2-AG)	Tocris	-80°C
Anandamide (AEA)	Tocris	-20°C
Calcium nitrate tetrahydrate	Duchefa Biochemie	Room temp
(-)-Cannabidiol (CBD)	Tocris	-20°C
Chloroform	Sigma-Aldrich	Room temp
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Room temp
Ethanol	Sigma-Aldrich	Room temp
HEPES	Melford Laboratories	Room temp
Hexane	Sigma-Aldrich	Room temp
Magnesium sulphate heptahydrate	Duchefa Biochemie	Room temp
Methanol	Sigma-Aldrich	Room temp
N,O-Bis(trimethylsilyl)trifluoroacetamide	Sigma-Aldrich	Room temp
Potassium chloride	Melford Laboratories	Room temp
Protease Inhibitor Cocktail (PIC)	Calbiochem	-20°C
Pyridine	Thermo Fisher	Room temp
Sodium chloride	Melford Laboratories	Room temp
Sodium hydroxide	Sigma-Aldrich	Room temp
Trimethylchlorosilane (BSTFA)	Thermo Fisher	Room temp
URB 597	Tocris	4°C

2.5. Storage and preparation of drugs and solutions

2-Arachidonylglycerol (2-AG)

2-Arachidonylglycerol was dissolved in 100% DMSO to generate a 100 mM master stock and stored in aliquots at -80°C. For our 0-300 μM concentrations, this stock was diluted in artificial pond water and DMSO (100%), with a final DMSO concentration of 0.5% in artificial pondwater.

Anandamide (AEA)

Anandamide is supplied pre-dissolved in ethanol as 14.4 mM solution and stored at -20°C. For our 0-70 μM concentrations, this stock was diluted in artificial pond water, with a final ethanol concentration of 0.5%.

(-)-Cannabidiol (CBD)

(-)-Cannabidiol was dissolved in 100% DMSO to generate a 50 mM master stock solution, which was stored at -20°C in aliquots. CBD was then diluted into our 0-250 μM concentrations using artificial pond water, with a final DMSO of concentration 0.5% to provide wide range of CBD concentrations for the study.

URB 597

URB 597 was dissolved in 100% DMSO to generate a 50 mM master stock solution and stored in aliquots at -20°C. To generate our concentrations of 0-250 μM, this was diluted using 100% DMSO and artificial pondwater at a final concentration of 0.5% DMSO.

2.6. Species endocannabinoid protein analysis

An endocannabinoid protein alignment analysis was conducted using eleven species, including vertebrates and invertebrates, and their conservation of cannabinoid receptor type 1, cannabinoid receptor type 2, NPR-19, and FAAH proteins were compared to *Homo sapiens*. This analysis was conducted to examine the conservation of ECS proteins across different species, comparing their presence in vertebrates and invertebrates with that in humans. This comparison provides insight into the potential conservation of these proteins in *L. variegatus*.

Reference sequences were gathered from the National Centre for Biotechnology Information (NCBI), and conservation alignment was collated using CGL workbench 22.0. Analysis of species endocannabinoid protein conservation is displayed as a percentage (%).

2.7. Lethal dose (LD_{50}) concentrations in *Lumbriculus variegatus*

18-24-hours before conducting the assay, five worms per well were collected in Corning™ Costar™ six-well plates and housed in artificial pond water. After 18-24-hours, the artificial pond water was removed, any debris was then washed from the wells using 2 ml of artificial pondwater. *L. variegatus* were then exposed to CBD (0-250 μM), CBD (0-50 μM), 2-AG (0-300 μM), AEA (0-70 μM), URB 597 (0-250 μM), or AEA (0-70 μM) in combination with URB 597 (250 μM) for 24-hours. Chronic exposure was used in this assay as these are lipophilic compounds which can affect tissue absorption and are difficult to dissolve in water. DMSO 0.5% in artificial pond water as vehicle control for CBD, 2-AG and URB 597, 0.5% ethanol for drug AEA, and 0.5% DMSO and 0.5% ethanol for combination experiments with AEA and URB 597. DMSO 0.5% v/v and ethanol 0.5% v/v has previously been shown to have no effect (data not shown). After 24-hours, as per previous studies that have used *L. variegatus*, decomposition was determined by visible tissue degeneration and whole-organism tissue pallor at assay endpoints and was the main indicator of lethal toxicity (O'Gara et al., 2004).

2.8. Cannabidiol (CBD) absorbance analysis through gas chromatography-mass spectrometry (GC-MS)

2.8.1. Sample preparation

L. variegatus were collected and housed in artificial pond water as described in 2.7. After 18-24-hours, the artificial pond water was removed, and *L. variegatus* were exposed to 0-250 μM CBD for 10-minutes. This timeframe is consistent with the planned exposure time for the behavioural assays. After CBD exposure, *L. variegatus* were rinsed with artificial pond water and frozen at -20°C until analysis. When preparing samples, *L. variegatus* were resuspended in ddH_2O and vortexed. 1:2 (v/v) chloroform: methanol solution was added to each sample, followed by chloroform and ddH_2O, using a vortex each time a solution was added to the

sample. Samples were centrifuged at 1000 rpm for 5-minutes, separating the sample and forming an Inter-Spacial Fluff; a line which is formed through sample separation.

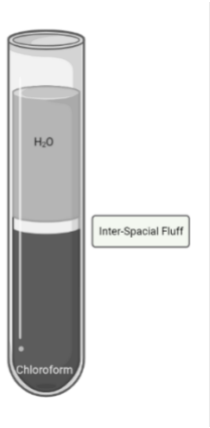

Figure 2.1 Test tube of sample preparation. Line shows separation in the sample.

2.8.2. Extraction of samples

Chloroform was extracted from the under the Inter-Spacial Fluff and transferred into gas chromatography vials. Each vial was transferred into a test tube and samples dried for 1-hour at 40°C using a SpeedyVac© vacuum centrifuge at 1000 rpm.

2.8.3. Derivatisation of sample for gas chromatography-mass spectrometry (GC-MS)

Following the protocol from Warrilow et al. (2016), we transferred anhydrous pyridine into the sample using a metal Leur-Lock syringe and bevelled tip SGE needle. Using a pipette, 250 µl of N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA) was added to the sample and vortexed before placing samples into a hot block for 30-minutes at 100°C. Once removed, we allowed the samples to cool. We then added 500 µL of hexane and vortexed the sample before transferring 500 µL of the sample into gas chromatography vials for gas chromatography-mass spectrometry.

2.8.4. Gas Chromatography

To conduct gas chromatography, we used GC column DB 624-UI and set our temperatures with a solvent delay of 1.8-minutes to prevent reading solvent activity at the earlier time points.

Table 2.2. Gas chromatography programming table

Rate (°C/min)	Temperature (°C)	Hold time (mins)
	70	3
10	180	2
10	250	20

Our Gas chromatography-Mass spectrometry analysis was completed using the Thermo Xcallibur™ mass spectrometry data system.

2.9. Stereotypical movement assay

As shown in Figure 2.1. These assays were conducted as outlined in Seeley et al. (2021) and Carriere et al. (2023). Briefly, individual worms were placed into Corning™ Costar™ six-well plates 18-24-hours before experimentation. A baseline test was conducted using 4 mL of artificial pond water for comparison. Body reversal and helical swimming behaviour of each worm were examined by stimulating the worm by touching the anterior and posterior five times of each worm. Stimulation was conducted with a clean 20-200 µL pipette tip, allowing for ~10-second intervals between stimuli. Baseline stereotypical movements from the worms in all the wells were measured and recorded on a recording sheet using the following scores, 1 = No movement, 2 = Partial stereotypical movement, 3 = Full stereotypical movement, as shown in Figure 2.1 Once baseline measurements were collected, the artificial pond water was removed. Whilst preventing prolonged periods out of the water, *L. variegatus* were dosed with vehicle (DMSO 0.5% or ethanol 0.5% in artificial pond water) or drug. Following the methods as set out by Drewes (1999). After a 10-minute drug incubation period, the same process was completed, and worms were assessed for their ability to perform stereotypical movements compared to the baseline. Once these movements were recorded, drug concentrations were removed, and wells were rinsed with 1 mL of artificial pond water to remove residual traces of the drug. This was aspirated and 4 mL of artificial pond water was placed into the wells to allow stereotypical behaviour to be measured at 10- minute and 24-hour rescue points.

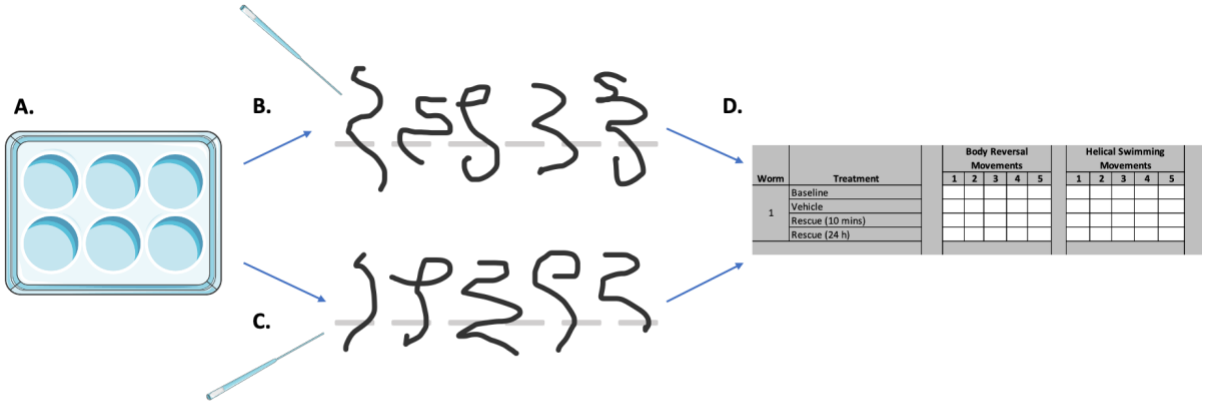

Figure 2.2. Measuring stereotypical movement of *L. variegatus*.(A) *L. variegatus* are plated in 6-well plates 18-24-hours before beginning of experiment. *L. variegatus* alternatively stimulated with 20-200 µL pipette tip at **(B)** anterior to stimulate body reversal and **(C)** posterior region to stimulate helical swimming. *L. variegatus* are stimulated 5 times/end, with 5-10-second intervals between stimuli. **(D)** movements are then scored and recorded 1 = no movement, 2 = incomplete movement, 3 = full stereotypical movement as set out by Drewes (1999). **(A-D)** is repeated for each worm before drug exposure to provide baseline ability to perform these movements. *L. variegatus* are tested again 10-minutes after drug incubation, and 10-minutes and 24-hours after the drug has been removed and in artificial pondwater. Data expressed as a ratio of movement score post drug exposure relative to baseline movement score. Figure adapted from Seeley et al. (2021).

2.10. Free locomotion assay

These assays were conducted as outlined in Seeley et al. (2021). Briefly, *L. variegatus* were transferred to Corning™ Costar™ six-well plates 18-24-hours before experimentation and the stereotypical movement assay's dosing, exposure, and incubation process was applied to the free locomotion assay. However, for the free locomotion assay, wells were filled with 2 mL of artificial pond water or drug solutions to limit vertical movement. Plates were placed under a 13-megapixel camera to capture free locomotion movement. Collected images were analysed using ImageJ software. These images were compiled into a z-stack image, this being a compilation of photographs taken at 1-second intervals over 50-seconds. An area of known distance within each z-stack image was measured and ImageJ calibrated to pixels per centimetre (pixels/cm) within each image. To determine the area covered by each worm, the foreground and background were separated using the thresholding functionality of ImageJ to separate the pixels activated by *L. variegatus*. The total area covered by the L. variegatus at baseline, drug exposure, rescue 10 min, and rescue 24 h was then determined based on the

calibration of pixels/cm within ImageJ. Data are expressed as a percentage of the area covered by *L. variegatus* in baseline conditions. The data collection method for this assay is shown in Figure 2.2.

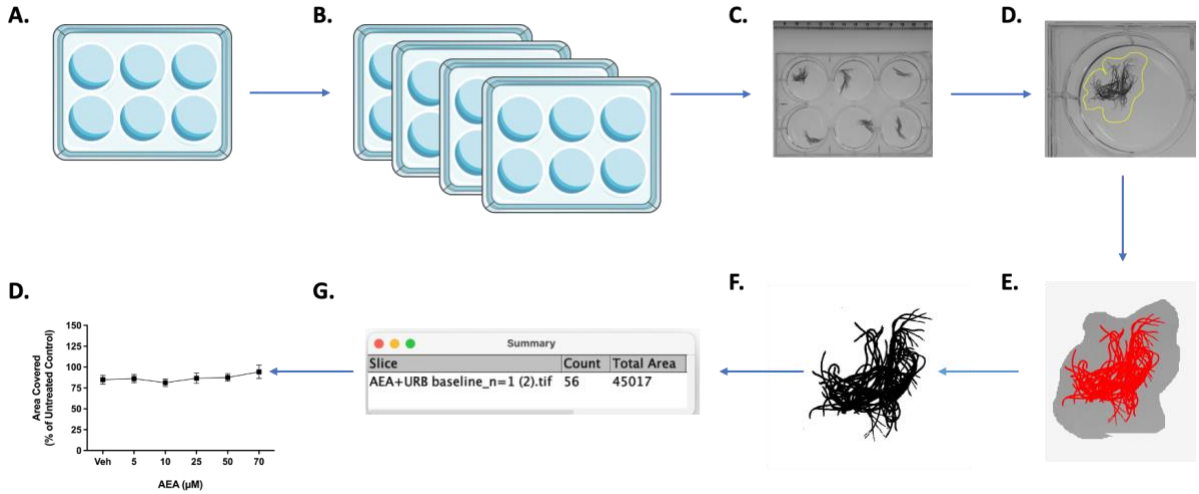

Figure 2.3. Measuring free locomotion movement of *L. variegatus*. (A) *L. variegatus* are plated in 6-well plates 18–24-hours before the experiment **(B)** 50 images are then collected at 1-second intervals at baseline, drug exposure, and rescue points. **(C)** Images are then arranged into a z-stack and the scale is set to an area of known distance within the z-stack. **(D)** Each *L. variegatus* is isolated using freehand selection and **(E)** thresholds are adjusted to select *L. variegatus* only before **(F)** background is then removed. **(G)** The total area covered by each *L. variegatus* can then be calculated using the set scale and **(H)** graphed for presentation and analysis. A-F is repeated for each worm to give the baseline movement before exposure to drug compounds, 10-minutes after incubation with drugs and 10-minutes and 24-hours in artificial pondwater only. Data is expressed as a percentage of baseline controls. Adapted from Seeley et al. (2021).

2.11. Statistical analysis

All data is reported as the mean and standard error of the mean (SEM). Statistical results were generated using GraphPad Prism 9, with stereotypical movement results analysed using a Wilcoxon *t*-Test and free locomotion and lethal dose assay results analysed using a Paired *t*-Test. These results compare drug concentrations against a vehicle control. In all the behavioural assays, the 10-minute and 24-hour rescue points are compared to the baseline with a two-way ANOVA, using Dunnett's multiple comparisons test. For statistical significance, the threshold was set at $p<.05$.

3. Results

3.1. Analysis of species endocannabinoid proteins

Due to the level of ECS protein expression being unknown in *L. variegatus*, we sought to investigate the presence of CB_1 receptor proteins, CB_2 receptor proteins, and FAAH across multiple species. Therefore, before investigating *L. variegatus* behavioural response to endo- and phytocannabinoids, an endocannabinoid protein alignment analysis was conducted using nine species, including both vertebrates and invertebrates, and their conservation was compared to *Homo sapiens*.

This analysis examines the conservation CB_1 receptor proteins exhibited in various organisms, including *H. sapiens, Gorilla gorilla, Rattus rattus, Mus musculus, Gallus gallus, Xenopus laevis, Danio rerio, Ciona intestinalis*, and *C. elegans*, as illustrated in Figure 3.1(A). Furthermore, we analysed the conservation of CB_2 receptor proteins in *Homo sapiens, G. gorilla, R. norvegicus, M. musculus, G. gallus, X. laevis*, and *D. rerio*, as seen in Figure 3.1(B). Additionally, the investigation included FAAH across species such as *Homo sapiens, R. norvegicus, M. musculus, G. gallus, D. rerio, C. intestinalis*, and *C. elegans*, as presented in Figure 3.1(C). Alignment was conducted using the Basic local alignment search tool (BLAST) protein alignment sequencing tool. BLAST is a fast online tool that can compare protein and DNA sequences, as well as identify members of gene families.

When comparing the percentage of conservation of endocannabinoid proteins against *H. sapiens*, vertebrates have the highest percentage for CB_1 and CB_2 receptor proteins. We observed *G. gorilla* holds a 99.79% conservation of CB_1 receptors and 99.44% conservation of CB_2 receptors (Table 3.1.). Additionally, a conservation of 97.04% was observed for CB_1 receptors in *R. rattus* (Table 3.1.), whereas the conservation of CB_2 receptor proteins in *R. norvegicus* stood at 81.11% (Table 3.1.), and FAAH exhibited a conservation rate of 82.90%, as depicted in Table 3.1. However, when comparing invertebrates, we saw *C. elegans* conserve 22.68% of the cannabinoid-like receptor, NPR-19 (Table 3.1.) and both *C. intestinalis* and *C.*

elegans have no CB_2 receptor conservation (Figure 3.1. B). FAAH was not found in *G. gorilla or X. laevis* but seen in all other species sequenced as seen in Figure 3.1. A-C.

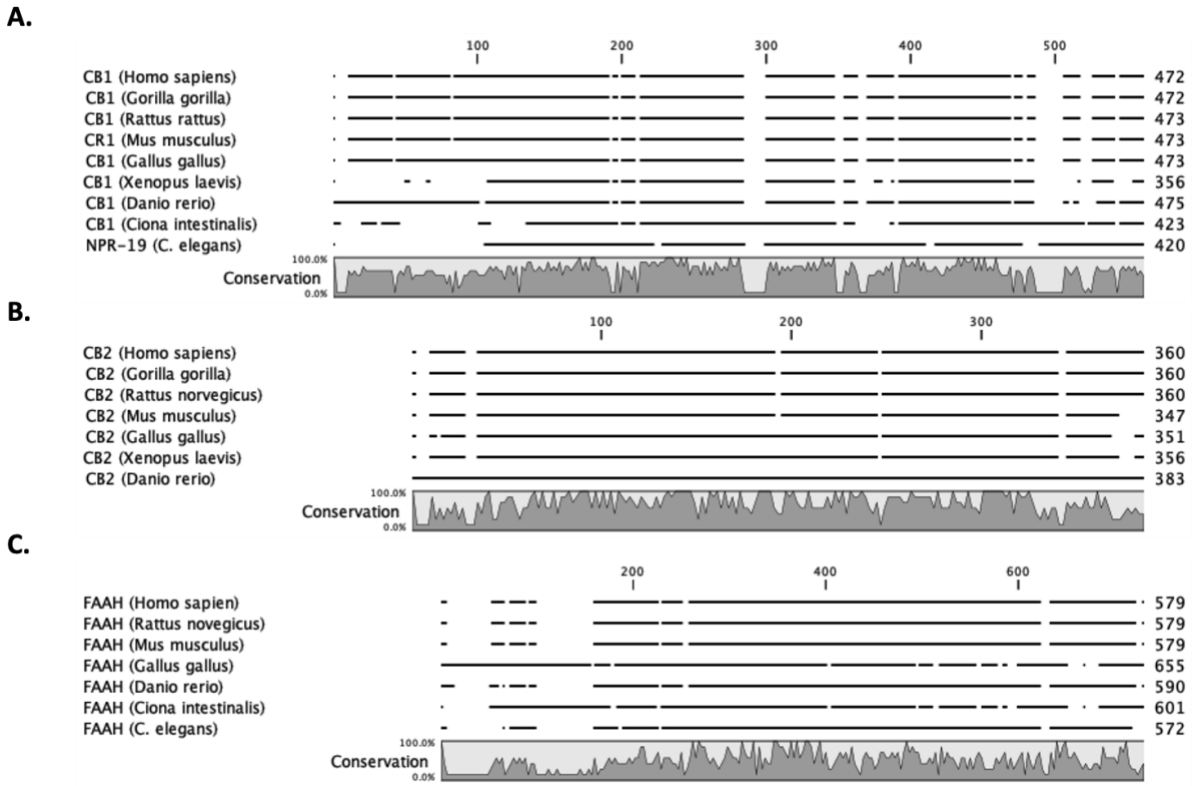

Figure 3.1 Protein alignment analysis across species for the endocannabinoid components cannabinoid receptor 1 (CB1), cannabinoids receptor 2 (CB2) and fatty acid amid hydrolase (FAAH). (A) Protein alignment for cannabinoid receptor 1 in Homo sapiens Primary Assembly – NCIB reference sequence: GenBank: KAI2543141.1, Gorilla gorilla Primary Assembly – NCIB reference sequence: XP_018885657.1, Rattus rattus Primary Assembly – NCIB reference sequence: XP_032760595.1, Mus musculus Primary Assembly – NCIB reference sequence: NP_001352810.1, Gallus gallus Primary Assembly – NCIB reference sequence: NP_001033741.1. Xenopus laevis Primary Assembly – NCIB reference sequence: XP_041439878.1, Danio rerio Primary Assembly – NCIB reference sequence: NP_997985.1, Ciona intestinalis Primary Assembly – NCIB reference sequence: NP_001027653.1, Caenorhabditis elegans NPR 19 Primary Assembly – NCIB reference sequence: GenBank: CCD69018.2. **(B)** Protein alignment for cannabinoid receptor 2 in Homo sapiens Primary Assembly – NCIB reference sequence: NP_001832.1, Gorilla gorilla Primary Assembly – NCIB reference sequence: XP_004024947.1, Rattus norvegicus Primary Assembly – NCIB reference sequence: NP_065418.3, Mus musculus Primary Assembly – NCIB reference sequence: NP_001292207.1, Gallus gallus Primary Assembly – NCIB reference sequence: XP_024998919.2, Xenopus laevis Primary Assembly – NCIB reference sequence: XP_018101882.1, Danio rerio Primary Assembly – NCIB reference sequence: NP_998129.3. **(C)** Protein alignment for fatty acid amine hydrolase (FAAH) in Homo sapien Primary Assembly – NCIB reference sequence: GenBank: AAD13768.1, Rattus norvegicus Primary Assembly – NCIB reference sequence: NP_001356055.1, Mus musculus Primary Assembly – NCIB reference sequence: NP_034303.3, Gallus gallus Primary Assembly – NCIB reference sequence: XP_040534432.1, Danio rerio Primary Assembly – NCIB reference sequence: NP_001103295.1, Caenorhabditis elegans Primary Assembly – NCIB reference sequence: NP_501368.1. Sequences gathered from the National Centre for Biotechnology Information (NCBI). Conservation alignment produced in CLC genomic workbench 22.0 and percentage of conservation shown by the line graphs below each panel.

Table 3.1. Conservation of protein sequence across species for endocannabinoid components compared with Homo sapiens. Percentage conservation was determined using NCBI Basic local alignment search tool (BLAST) protein alignment sequencing tool.

Species	**Protein conservation compared to *Homo sapiens***			
	Cannabinoid receptor type 1 (CB_1)	**Cannabinoid receptor type 2 (CB_2)**	**Fatty acid amide hydrolase (FAAH)**	NPR-19
Gorilla gorilla	99.79%	99.44%	-	Not found
Rattus	97.04%	81.11%	82.90%	Not found
Mus musculus	97.04%	82.71%	84.11%	Not found
Gallus gallus	93.04%	49.70%	33.73%	Not found
Danio rerio	70.75%	43.75%	51.87%	Not found
Xenopus laevis	48.88%	52.28%	-	Not found
Ciona intestinalis	29.03%	Not found	35.00%	Not found
Caenorhabditis elegans	Not found	Not found	38.67%	22.68%
Drosophila melanogaster	Not found	Not found	Not found	Not found
Carcinoscorpius rotundicauda	Not found	Not found	Not found	Not found
Phylum onychophora	Not found	Not found	Not found	Not found

3.2. Determining the lethal dose (LD50) of cannabidiol (CBD) in *Lumbriculus variegatus*

To determine the effects of phyto and endocannabinoids on *Lumbriculus variegatus*, we first investigated *L. variegatus* response to 0–250 μM CBD exposure for 24-hours (Figure 3.2. A), and sought to find the lethal dose (LD_{50}) of CBD in the worms. By conducting an LD_{50}, we were able to establish which concentrations to use in our movement assays which did not cause acute toxicity.

We observed toxicity with a 100% fatality rate at concentrations of ≥30 μM (n=6). To calculate a more accurate LD_{50}, we then tested 0-50 μM CBD (Figure 3.2. B). After *L. variegatus* had been exposed to CBD (0-50 μM) for a 24-hour period, we observed there was no significant difference in *L. variegatus* survival rates at the CBD concentrations of ≤20 μM ($p>.05$, $n=9$) but significant toxicity was observed at ≥25μM ($p<.05$, Figure 3.2. B). These findings determined an LD_{50} of 23.00 μM (95% CI: 21.74-24.26 μM, $n=9$) for CBD in *L. variegatus*.

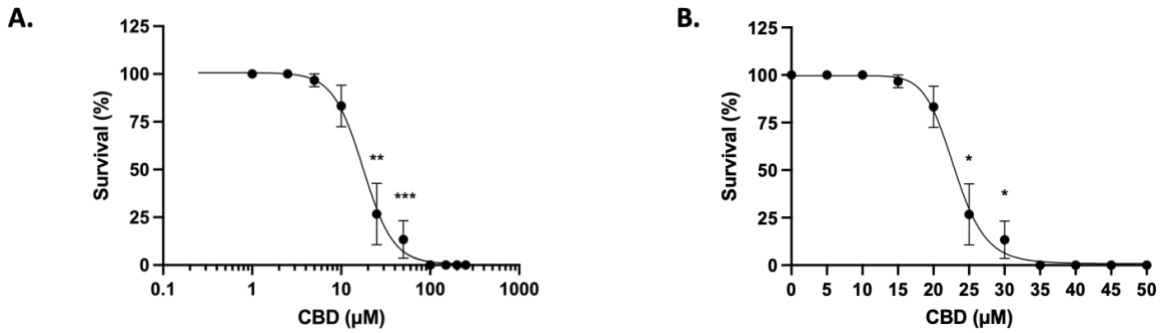

Figure 3.2. Dose response of CBD in *L. variegatus*. The effect of **(A)** CBD 0-250 μM (*n*=6) **(B)** CBD 0-50 μM *(n*=9, with five *L. variegatus*. per concentration per replicate) on the survival rate of 50% of population of *L. variegatus* after 24-hour CBD exposure. Survival expressed as a percentage of untreated control after exposure to CBD. Veh: 0.5% DMSO in artificial pondwater. Error bars represent the standard error of the mean. * *p* <. 05, ***p* <.01 or ***

3.3. Determination of cannabidiol (CBD) absorption in *Lumbriculus variegatus* through gas chromatography – mass spectrometry (GC-MS)

As we observed ≥30 µM CBD was having a toxic effect on *L. variegatus* (Figure 3.3. A), we sought to confirm if CBD was absorbed by *L. variegatus* by gas chromatography – mass spectrometry (GC-MS) analysis using CBD concentrations of 0-250 µM. The absorption of a drug determines its bioavailability, and absorption is an essential component of pharmacokinetics which effects the onset of a drug, its potency and its duration of action (Alagga et al., 2024).

For GC-MS analysis, a DB-624UI GC column was used, offering features specifically designed to enhance performance and enable rapid analysis of volatile compounds. This column is particularly well-suited for environmental and chemical sample applications, providing optimised efficiency and reliability. Through GC-MS, we saw palmitic acid and stearic acid in *L. variegatus* at all our concentrations but did not observe CBD at concentrations <10 µM (Figure 3.3 A-B). Palmitic acid and stearic acid are saturated fatty acids, found in animals, plants and microorganisms. However, we detected CBD within *L. variegatus* at concentrations of ≥25 µM (*n*=1, figure 3.3. C-D). This is represented by the mass fragmentation pattern of CBD (25 µM) at 536.88 mZ, which is shown in Figure 3.3. (C). The gas chromatograph of CBD (25 µM) is shown in Figure 3.3. (D) which highlights the distinct peak in the sample at the retention time of 32.38-minutes, and with a peak area of 246819891, as indicated in Table 3.2.

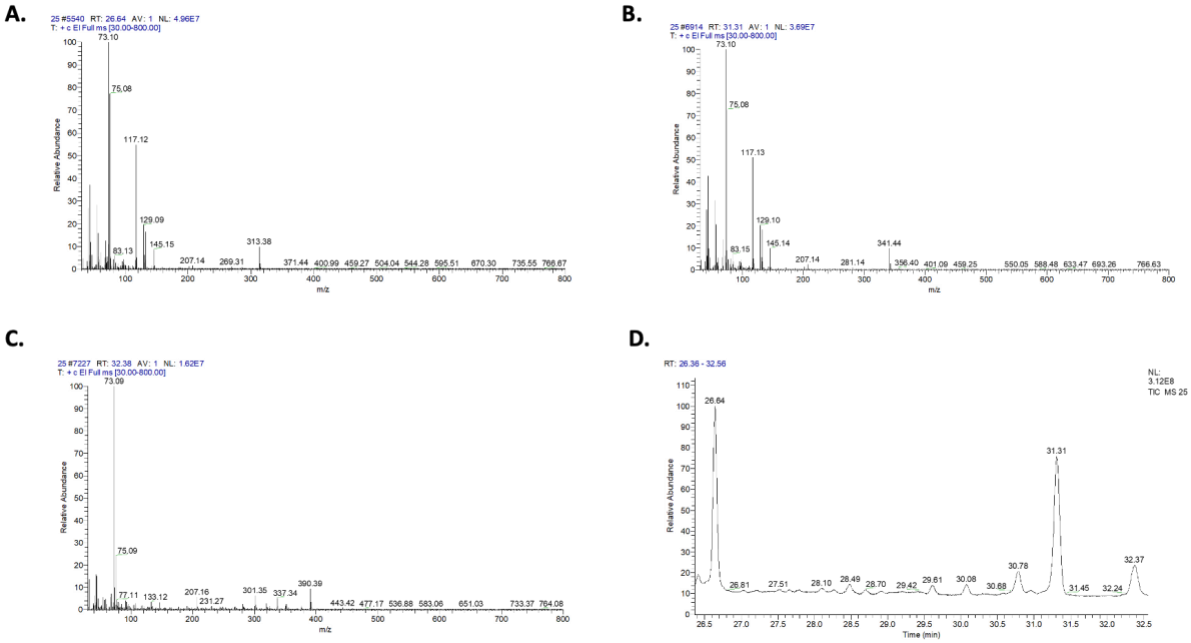

Figure 3.3. Gas chromatography-mass spectrometry base peaks. Showing **(A)** the mass fragmentation pattern of palmitic acid in mZ **(B)** the mass fragmentation pattern of stearic acid in mZ **(C)** the mass fragmentation pattern of CBD in mZ. (**D)** A representative gas chromatograph sample of *L. variegatus* (25 µM CBD) illustrating the peak seen in the sample.

Table 3.1. Mass spectrometry identification of fatty acids and cannabidiol (0–250 µM) in *L. variegatus*, showing retention time and gas chromatograph peak areas.

CONTROL			
Time (min)	**Compound**	**m/z[M-15]**	**Peak area**
26.63	Palmitic acid	313.39	927178296
31.29	Stearic acid	341.37	986771984
CBD [1 µM]			
Time (min)	**Compound**	**m/z[M-15]**	**Peak area**
26.64	Palmitic acid	313.37	1002471953
31.31	Stearic acid	341.41	1054922971
CBD [2.5 µM]			
Time (min)	**Compound**	**m/z[M-15]**	**Peak area**
26.64	Palmitic acid	313.38	958050490
31.31	Stearic acid	341.42	1020755661
CBD [5 µM]			
Time (min)	**Compound**	**m/z[M-15]**	**Peak area**
26.64	Palmitic acid	313.38	958050490
31.31	Stearic acid	341.42	1020755661
CBD [10 µM]			
Time (min)	**Compound**	**m/z[M-15]**	**Peak area**
26.65	Palmitic acid	313.35	10253922815
31.33	Stearic acid	341.41	10129108070
CBD [25 µM]			
Time (min)	**Compound**	**m/z[M-15]**	**Peak area**
26.64	Palmitic acid	313.38	958050490
31.32	Stearic acid	341.44	1020755661
32.38	CBD	536.88	246819891
CBD [50 µM]			
Time (min)	**Compound**	**m/z[M-15]**	**Peak area**

26.64	Palmitic acid	313.41	951005141
31.32	Stearic acid	341.44	968698375
32.41	CBD	532.27	584077335
CBD [100 µM]			
Time (min)	**Compound**	**m/z[M-15]**	**Peak area**
26.64	Palmitic acid	313.35	906455258
31.33	Stearic acid	341.41	984921577
32.39	CBD	535.11	1451154565
CBD [150 µM]			
Time (min)	**Compound**	**m/z[M-15]**	**Peak area**
26.65	Palmitic acid	313.38	958050490
31.32	Stearic acid	341.43	1021755661
32.40	CBD	594.26	2270874290
CBD [200 µM]			
Time (min)	**Compound**	**m/z[M-15]**	**Peak area**
26.66	Palmitic acid	313.37	903872927
31.32	Stearic acid	231.41	936276387
32.41	CBD	473.21	3261892711
CBD [250 µM]			
Time (min)	**Compound**	**m/z[M-15]**	**Peak area**
26.65	Palmitic acid	313.37	903872927
31.33	Stearic acid	231.41	936276387
32.40	CBD	470.29	3746451317

3.4. Investigating the effects of cannabidiol (CBD) on *Lumbriculus variegatus* stereotypical movement

Having confirmed CBD absorption by GC-MS*,* we performed a stereotypical movement assay using CBD concentrations that did not display lethal toxicity (Figure 3.2).

As seen in Figures 3.4 A and B, we observed that CBD significantly inhibits *L. variegatus* body reversal and helical swimming movements, when exposed to concentrations of ≥5 μM for 10-minutes (p<.05, Figure 3.4. A-B). After a 10-minute rescue period in drug-free artificial pondwater, we observed body reversal movement continues to be significantly reduced at CBD concentrations ≥5 μM (p<.05, Figure 3.4. C), however, 5 μM did not have prolonged effects on helical swimming when removed (p>.05, Figure 3.4. D). Helical swimming was significantly restricted at concentrations of ≥10 μM (p<.05, Figure 3.4. D). However, after a 24-hour rescue period in drug-free artificial pondwater, we observed no long-term effects at 5-10 μM on either reversal or helical swimming (p>.05, Figure 3.4. C-D) but both body reversal movement (p<.05, Figure 3.4 C), and helical swimming (p<.05, Figure 3.4. D) are significantly inhibited at the ≥15 μM concentrations, compared to baseline.

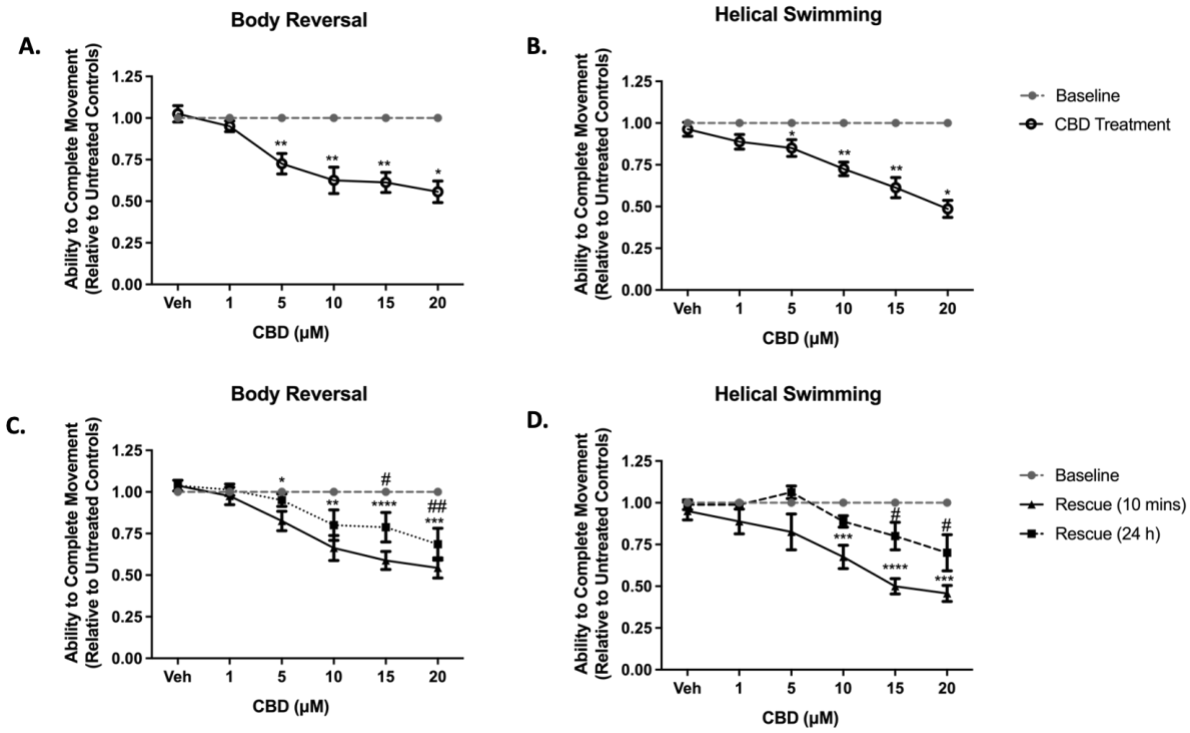

Figure 3.4. The effect of CBD on *Lumbriculus variegatus* stereotypical movement. *L. variegatus* were exposed to CBD (0-20 μM) for 10-minutes and the ability of *L. variegatus* to perform **(A)** body reversal or **(B)** helical swimming was measured. The ability to perform **(C)** body reversal or **(D)** helical swimming once CBD is removed and replaced with APW is then measured at 10-minutes (Rescue (10 mins)) and 24-hour points (Rescue (24 h)) after drug removal. All data is reported as the ratio of movement of the worm post-exposure, which is relative to movement at Baseline before CBD exposure. Error bars represent the standard error of the mean, with an n = 8 for each concentration. Veh: 0.5% DMSO in artificial pondwater */# p <. 05, **/## p <.01, *** p <.001**** p <.0001; where * refers to statistical significance between Baseline and CBD exposure or statistical significance between Baseline and Rescue (10 mins), # refers to statistical significance between Baseline and Rescue (24 h).

3.5. Investigating the effects of cannabidiol (CBD) on *Lumbriculus variegatus* free locomotion behaviour

Following the stereotypical movement assay, we assessed whether there were dose-dependent effects of CBD on unstimulated free locomotion behaviour. However, unlike stereotypical movement (Figure 3.4), CBD did not have a significant effect on unstimulated movement after being exposed to CBD concentrations (0-20 µM) for 10-minutes (p>0.05, Figure 3.5A-B). In contrast, after a 10-minute rescue period in drug-free artificial pondwater, we observed a significant increase in movement by 27±8.68% at 5 µM only (p=.0212, Figure 3.5C). After a 24-hour rescue period in drug-free artificial pondwater, we observed unstimulated behaviour is significantly reduced to 71±11.64% compared to baseline at 15 µM (p=.0188 Figure 5C) and 20 µM, where movement is reduced to 61±13.52% compared to the baseline (p=.0120, Figure 3.5C).

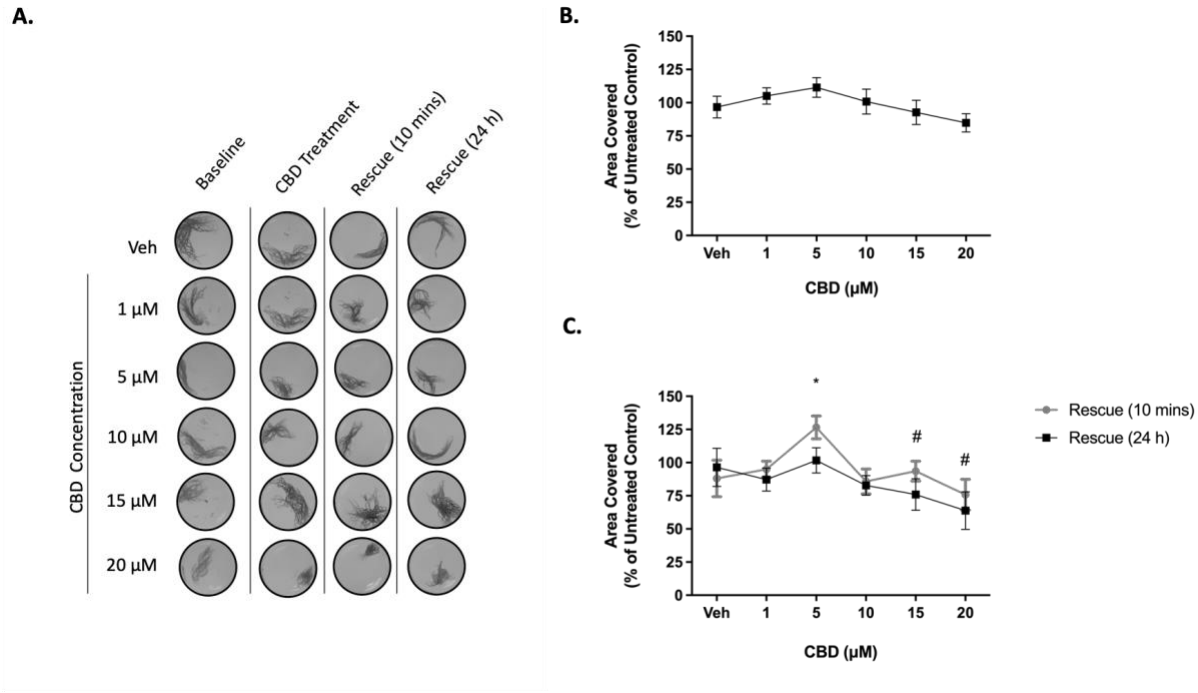

Figure 3.5. The effect of CBD on *L. variegatus* free locomotion behaviour. (A) Representative images of the effects of CBD measured at pre-exposure (Baseline), after 10-minute exposure to 0–20 µM CBD (CBD treatment), 10-minutes after CBD removal (Rescue (10 mins)) and 24-hours after removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* following **(B)** 10-minutes CBD exposure (0-20 µM) and **(C)** after removal of CBD for 10 minutes (Rescue (10 mins)) and for 24 hours (Rescue (24 h)). Area covered expressed as a percentage of movement relative to the Baseline. Error bars represent standard error of the mean, with an n = 8 for each concentration. Veh: 0.5% DMSO in artificial pondwater. */# p <.05; where * refers to statistical significance between Baseline and CBD exposure or statistical significance between Baseline and rescue (10 min), # refers to statistical significance between Baseline and Rescue (24 h).

3.6. Determining the lethal dose (LD_{50}) of 2-Arachidonoylglycerol (2-AG), URB 597, anandamide (AEA) ±URB 597 in *Lumbriculus variegatus*.

After observing a response to CBD in *L. variegatus*, we then investigated the response of *L. variegatus* to the endocannabinoids 2-AG and AEA ± the FAAH inhibitor, URB 597.

After a 24-hour exposure of 2-AG (0–300 µM) to *L. variegatus,* we observed that 200 µM resulted in mortality in 35±15.92% of the test population (Figure 3.6.A), though this was not significantly different from the vehicle control. However, a significant increase in mortality was observed at 225 µM (p=.0136, Figure 3.6.A). An LD50 of 199 µM (95% CI: 173.1–225.0 µM) was calculated, highlighting that while 200 µM approached the threshold of toxic effects, a significant response became evident only at slightly higher concentrations. This suggests that 200 µM is near the critical point for measurable toxicity in

To evaluate the potential toxicity of URB 597, a FAAH inhibitor, we exposed *L. variegatus* to concentrations ranging from 0 to 250 µM over a 24-hour period. The results showed no significant toxicity across all tested concentrations (p>.05, Figure 3.6.B). These findings suggest that URB 597 is well-tolerated by *L. variegatus* at these exposure levels and does not cause measurable adverse effects, even at the highest concentration tested. This lack of toxicity supports the compound's safety under the experimental conditions used in this study.

Next, we investigated the effects of AEA (0–70 µM) on *L. variegatus*. When exposed to AEA alone, we observed no significant toxicity across the tested concentrations (p.>.05, Figure 3.6.C). Similarly, no toxicity was observed when AEA (0–70 µM) was combined with 250 µM URB 597 (p.>.05, Figure 3.6.C). While 70 µM AEA + 250 µM URB 597 resulted in 80±20% survival, this effect was not statistically significant. Based on these findings and the rationale of selecting the highest dose that was safe, we proceeded to use AEA concentrations of 0–70 µM, both alone and in combination with 250 µM URB 597, for our subsequent paired behavioral assays.

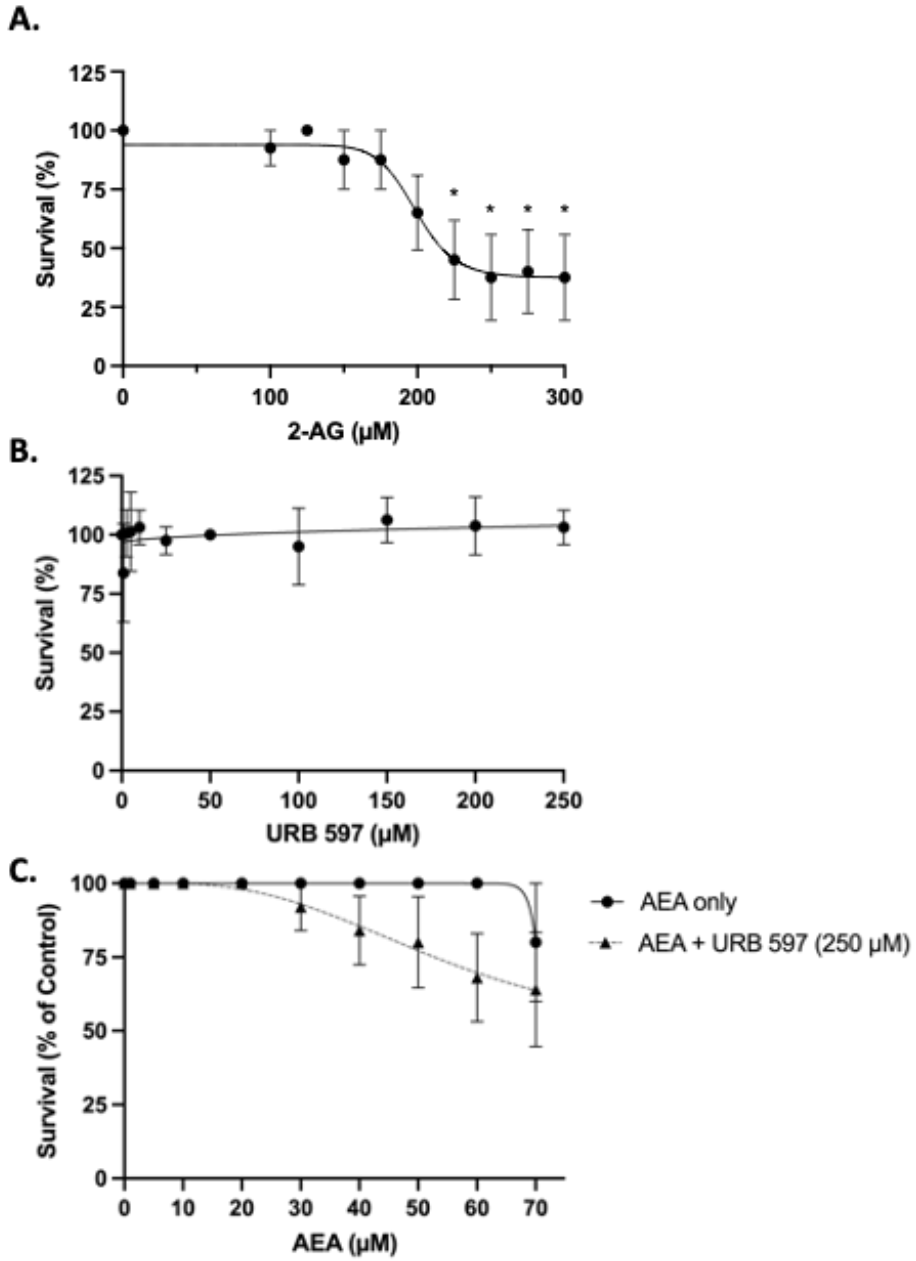

Figure 3.6. Dose response of 2-AG, URB 597 and AEA±URB 597. The effect of **(A)** 2-AG 0-300 µM (*n*=8) **(B)** URB 597 0-250 µM (*n*=8) **(C)** AEA 0-70 µM ± 250 µM URB 597 (*n*=5) on the survival rate of 50% of the population of *L. variegatus* after 24-h drug exposure. Survival expressed as a percentage after exposure to drug relative to the Baseline. Error bars represent the standard error of the mean. * p <. 05; where * refers to statistical significance of survival rates compared to untreated vehicle controls. Veh: 0.5% DMSO in artificial pondwater was used as vehicle control for 2-AG and URB 597, 0.5% ethanol in artificial pondwater for AEA experiments, and 0.5% DMSO and 0.5% ethanol in artificial pondwater for AEA+URB 597 combination experiments.

3.7. Investigating the effects of 2-Arachidonoylglycerol (2-AG) on *Lumbriculus variegatus* stereotypical movement

After conducting our lethal dose assays, we then sought to observe *L. variegatus* stereotypical behaviour after exposure to 2-AG for 10-minutes. 0-150 µM concentrations were used for our behavioural assays as these concentrations had exhibited no signs of toxicity (Figure 3.6. A).

We observed 2-AG significantly effects body reversal movement at ≥25 µM (p<.05, Figure 3.7. A) and helical swimming at the same concentrations of ≥25 µM (p<.05, Figure 3.7. B) when exposed for 10-minutes. After a 10-minute rescue period in drug-free artificial pondwater, we observed that body reversal movement was still reduced at concentrations ≥25 µM (p<.05, Figure 3.7. C) but not for helical swimming, where movement is significantly reduced from ≥50 µM (p<.05, Figure 3.7. D). After a 24-hour rescue period in drug-free artificial pondwater, body reversal movement continues to be significantly reduced from the concentration of ≥100 µM (p<.05, Figure 3.7. C), as well as helical swimming at the same concentrations of ≥100 µM (p<.05, Figure 3.7. D), compared to baseline.

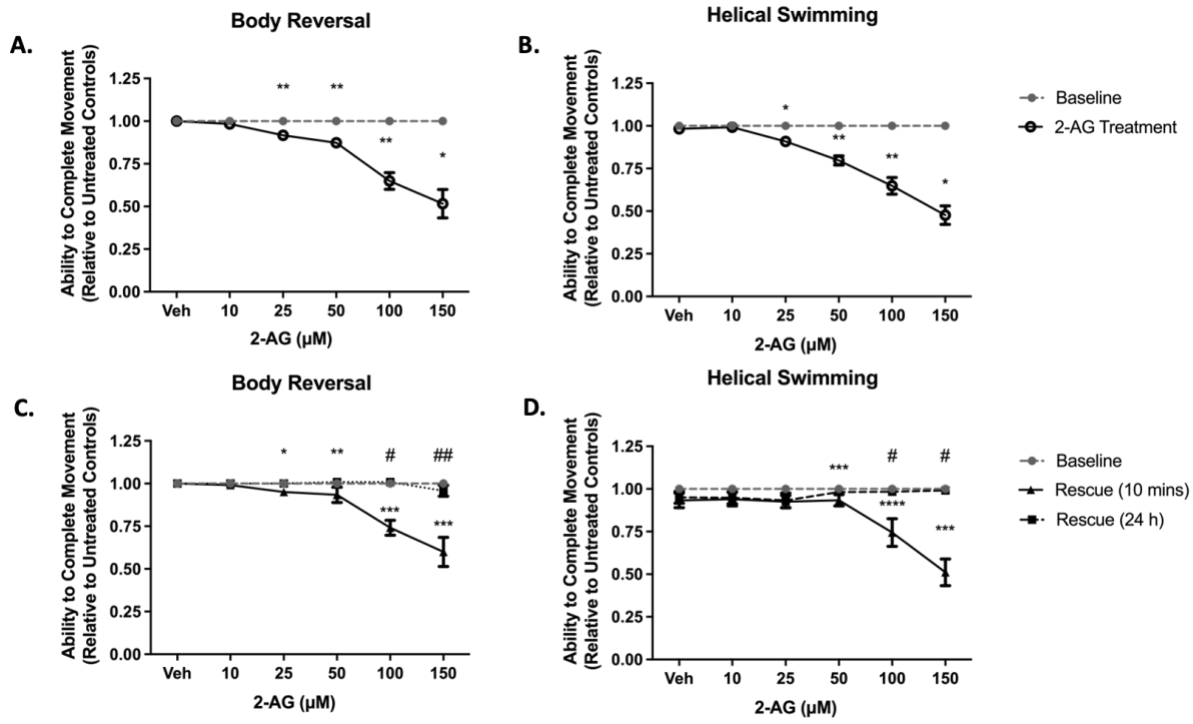

Figure 3.7. The effect of 2-AG on *L. variegatus* and stereotypical movement. *L. variegatus* were exposed to 2-AG (0-150 μM) for 10-minutes and the ability of *L. variegatus* to elicit **(A)** body reversal or **(B)** helical swimming was measured. The ability to perform **(C)** body reversal or **(D)** helical swimming once 2-AG is removed and replaced with APW then measured at 10-minutes (Rescue (10 mins)) and 24-hour points (Rescue (24 h)). All data is reported as the ratio of movement of the worm after exposure, which is relative to movement at Baseline before 2-AG exposure. Error bars represent the standard error of the mean, with an n = 8 for each concentration. Veh: 0.5% DMSO in artificial pondwater */# p <. 05, **/## p <.01, *** p <.001, **** p <.0001; where * refers to statistical significance between Baseline and 2-AG exposure or statistical significance between Baseline and Rescue (10 min), # refers to statistical significance between Baseline and Rescue (24 h).

3.8. Investigating the effects of 2-Arachidonoylglycerol (2-AG) on *Lumbriculus variegatus* free locomotion behaviour

Following the stereotypical movement assay, we went on to observe dose-dependent effects on free locomotion behaviour for 10-minutes. However, unlike stereotypical movement (Figure 3.8. A-B), 0-150 μM 2-AG had no significant effect on unstimulated movement for 10-minutes (p>0.05, Figure 3.8. B). This was also observed, after a 10-minute rescue period in drug-free artificial pondwater, (p>0.05, Figure 3.8. C), and after a 24-hour rescue period in drug-free artificial pondwater, (p>0.05, Figure 3.8. C).

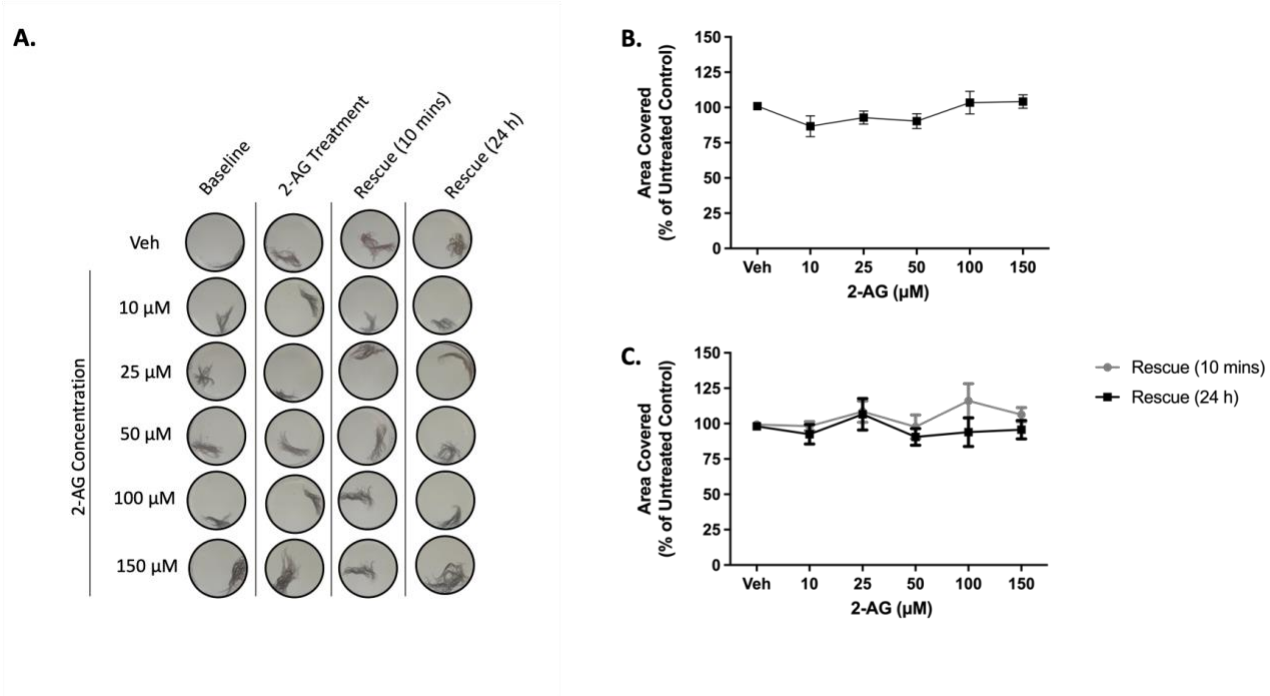

Figure 3.8. The effect of 2-AG on *L. variegatus* free locomotion behaviour. (A) Representative images at pre-exposure (Baseline), after 10-minute 2-AG treatment (0–150 µM), 10-minutes after 2-AG removal (Rescue (10 mins)) and 24-hours after removal (Rescue (24 h)). Quantification of the area covered by *L. variegatus* **(B)** 10-minutes 2-AG exposure (0-150 µM) and **(C)** after removal of 2-AG for 10 minutes (Rescue (10 mins)) and for 24 hours (Rescue (24 h)). Area covered expressed as a percentage of movement relative to the Baseline. Error bars represent standard error of the mean, with an n = 8 for each concentration. Veh: 0.5% DMSO in artificial pondwater.

3.9. Investigating the effects of anandamide (AEA) on *Lumbriculus variegatus* stereotypical movement behaviour

Having exposed *L. variegatus* to 2-AG, we sought to determine the effects of a different endocannabinoid ligand, AEA.

We observed that AEA significantly reduces both body reversal movement (p<.05) and helical swimming (p<.05) at concentrations of ≥10 μM after 10-minutes exposure, as seen in Figure 3.9. A-B. After a 10-minute rescue period in drug-free artificial pondwater, we observed 10 μM no longer influenced body reversal movement but was significantly reduced at concentrations of ≥25 μM AEA (p<.05, Figure 3.9. C). Whereas for helical swimming. After a 10-minute rescue period in artificial pondwater, helical swimming continues to be significantly reduced at the same concentrations as drug exposure (p<.05, Figure 3.9. D). After the 24-hour rescue period in drug-free artificial pondwater. Both body reversal and helical swimming movement returned to baseline levels at all concentrations tested (p>0.05, Figure 3.9. C-D).

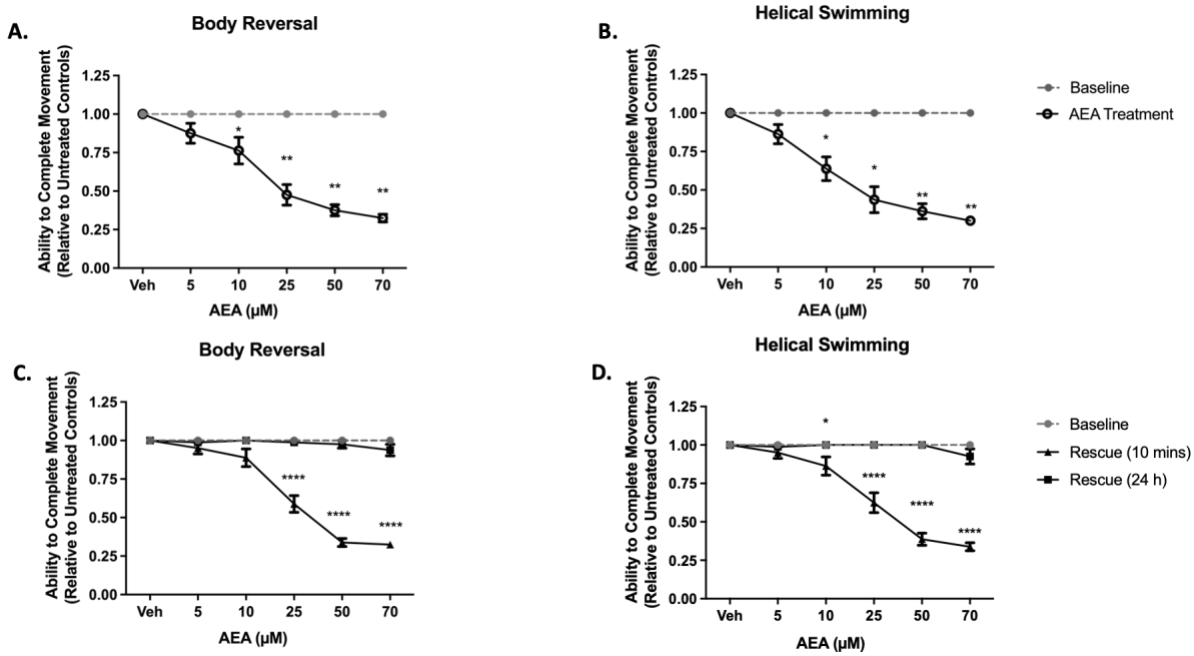

Figure 3.9. Effects of AEA treatment on *L. variegatus* stereotypical movement. *L. variegatus* were exposed to AEA (0-70 μM) for 10-minutes and the ability of *L. variegatus* to perform **(A)** body reversal or **(B)** helical swimming was measured. The ability to perform **(C)** body reversal or **(D)** helical swimming once AEA is removed and replaced with APW then measured at 10-minutes (Rescue (10 mins)) and 24-hour points (Rescue (24 h)). All data is reported as the ratio of movement of the worm post exposure, which is relative to movement at Baseline, and before AEA exposure. Error bars represent the standard error of the mean, with an n = 8 for each concentration. Veh: 0.5% ethanol in artificial pondwater * p <. 05, ** p <.01, **** p <.0001; where * refers to statistical significance between Baseline and AEA exposure or statistical significance between Baseline and Rescue (10 min).

3.10. Investigating the effects of anandamide (AEA) on *Lumbriculus variegatus* free locomotion behaviour

We then went on to observe dose-dependent effects of AEA (0-70 µM) on unstimulated free locomotion behaviour. Unlike stereotypical movement, we saw a significant reduction in movement by 24±6.69% at a concentration of 10 µM concentration only, after being exposed to 0-70 µM AEA for 10-minutes (p=.0074, Figure 3.10. A-B). After the 10-minute rescue period in drug-free artificial pondwater, we observed no significant difference in movement at any tested concentration (p>.05, Figure 3.10. C). However, after a 24-hour rescue period in drug-free artificial pondwater, we observed that unstimulated behaviour had decreased significantly by 26±7.53% at 25 µM (p=.0018, Figure 3.10. C), 53+5.5% at 50 µM (p=.0139, Figure 10C), and 70 µM AEA , where movement was reduced to 57±11.75% (p=.0139, Figure 3.10. C), compared to the baseline.

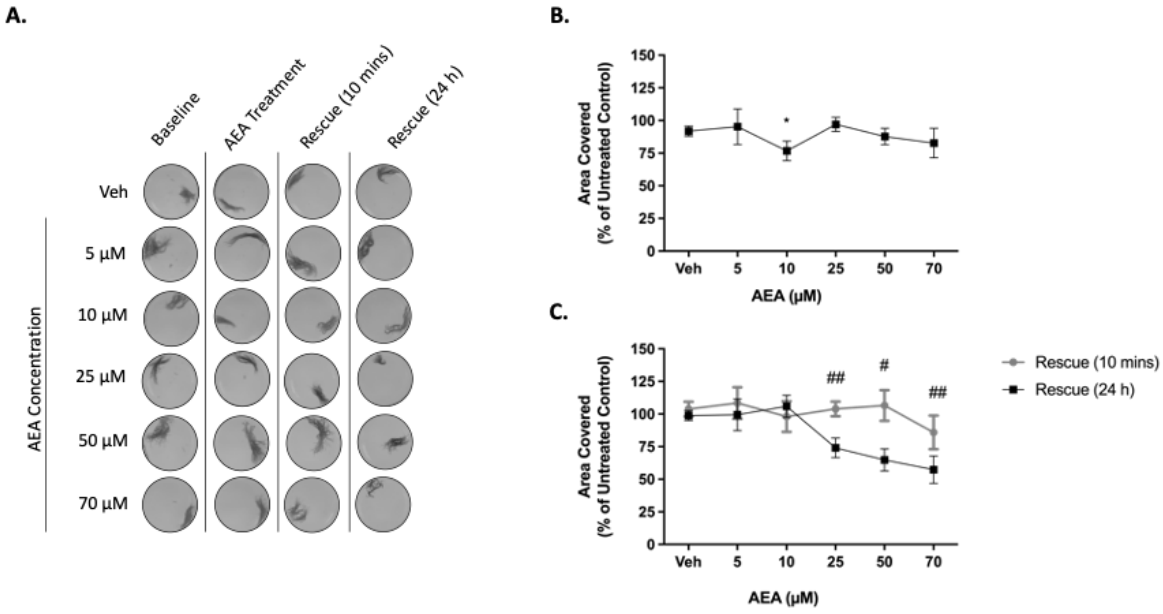

Figure 3.10. The effect of AEA on *L. variegatus* free locomotion behaviour. (A) Representative images of the effects of AEA measured at pre-exposure (Baseline), after 10-minute AEA treatment (0–70 µM), 10-minutes after AEA removal (Rescue (10 mins)) and 24-hours after removal (Rescue (24 h)). Quantification of the free locomotion by *L. variegatus* **(B)** when exposed for 10-minute to AEA (0-70 µM) and **(C)** after removal of AEA for 10 minutes (Rescue (10 mins)) and for 24 hours (Rescue (24 h)). Area covered expressed as a percentage of movement relative to the Baseline. Error bars represent standard error of the mean, with an n = 8 for each concentration. Veh: 0.5% ethanol in artificial pondwater. */# p <.05; where * refers to statistical significance between Baseline and AEA exposure, # refers to statistical significance between Baseline and Rescue (24 h).

3.11. Investigating the effects of anandamide (AEA)+URB 597 (250 μM) on *Lumbriculus variegatus* stereotypical movement behaviour

We then sought to investigate the impact of AEA alone and in combination with the FAHH inhibitor, URB 597 (250 μM), on *L. variegatus* movement behaviour.

Similar to AEA only, we saw that AEA in combination with URB 597 (250 μM) significantly reduces body reversal movement ≥10 μM (p<.05, Figure 3.11. A) after a 10-minute drug exposure, and for helical swimming, and movement is significantly reduced at the higher concentrations of ≥25 μM (p=.0078, Figure 3.11. B).

However, after a 10-minute rescue period in drug-free artificial pondwater, compared to AEA exposure, we saw body reversal movement is significantly reduced from the lower concentration of 5 μM (p=.0341, Figure 3.11. C). This was also observed for helical swimming at a lower concentration of 10 μM (p=.0049, Figure 3.11. D), compared to AEA alone. After the 24-hour rescue period in drug-free artificial pondwater, in comparison to AEA alone, where there was no significant difference in movement for body reversal or helical swimming at this time-point (Figure 3.9. C-D). We observed a significant reduction in movement at concentrations of ≥50 μM for body reversal (p<.05, Figure 3.11. C) and helical swimming (p<.05, Figure 3.11. D).

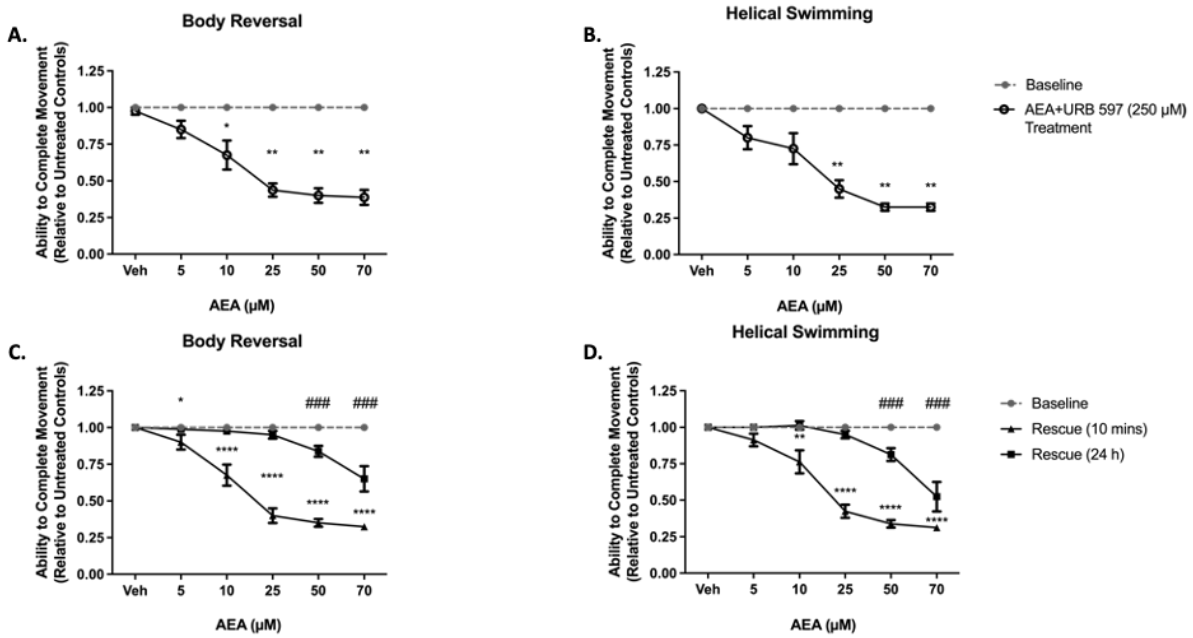

Figure 3.11. Effects of AEA+URB 597 (250 μM) treatment on *L. variegatus* stereotypical movement. *L. variegatus* were exposed to AEA (0-70 μM) and URB 597 (250 μM) for 10-minutes and the ability of *L. variegatus* to perform **(A)** body reversal or **(B)** helical swimming. The ability to perform **(C)** body reversal or **(D)** helical swimming once AEA+URB 597 (250 μM) is removed and replaced with APW then measured at 10-minutes (Rescue (10 mins)) and 24-hour points (Rescue (24 h)). All data is reported as the ratio of movement of the worm post exposure, which is relative to movement at Baseline, and before AEA+URB 597 exposure. Error bars represent the standard error of the mean, with an n = 8 for each concentration. Veh: 0.5% DMSO in artificial pondwater for URB 597 and 0.5% ethanol in artificial pondwater for AEA * p <. 05, ** p<.01, **** p<.0001 and ### p<.0001; where * refers to statistical significance between Baseline and AEA exposure or statistical significance between Baseline and Rescue (10 min), # refers to statistical significance between Baseline and Rescue (24 h).

3.12. Investigating the effects of anandamide (AEA) +URB 597 (250 µM) on *L. variegatus* free locomotion behaviour

Finally, we observed the effects of AEA (0-70 µM) in combination with URB 597 (250 µM) on unstimulated free locomotion. Unlike stereotypical movement, we saw no significant changes in free locomotion movement when *L. variegatus* was exposed to AEA (0-70 µM) and URB 597 (250 µM) for 10-minutes (p=>.05, Figure 3.12. B). After the 10-minute rescue period in drug-free artificial pondwater, we continue to see no significant changes in movement, as observed with AEA alone (p=>.05, Figure 3.12. C). However, after a 24-hour rescue period in drug-free artificial pondwater, we observed unstimulated movement was significantly decreased at all concentrations ≥10 µM (p<.05, Figure 3.12. C) with movement significantly reduced by 22±10.17% at 10 µM, 42±7.87% at 25 µM, 54±10.79% at 50 µM and 72±5.41% at 70 µM. Compared to AEA alone (Figure 3.10. C), we observed that when AEA is given in combination with URB 597, inhibitory effects on free locomotion were observed ≥10 µM whereas inhibition was only observed at ≥25 µM in AEA alone.

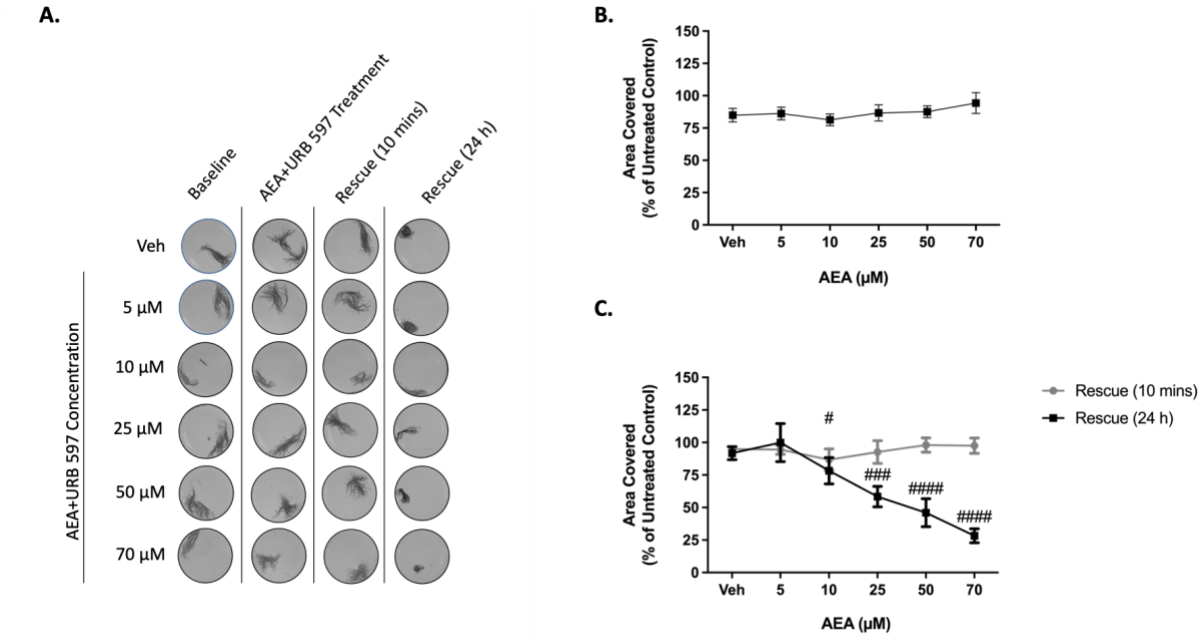

Figure 3.12. The effect of AEA+URB 597 on *L. variegatus* free locomotion behaviour. (A) An example of the effects of AEA+URB 597 measured at pre-exposure (Baseline), after 10-minute AEA+URB 597 treatment (0–70 µM), 10-minutes after AEA+URB 597 removal (Rescue (10 mins)) and 24-hours after removal (Rescue (24 h)). Quantification of the free locomotion by *L. variegatus* **(B)** when exposed for 10-minute to AEA (0-70 µM)+URB 597 (250 µM) and **(C)** after removal of AEA (0-70 µM)+URB 597 (250 µM)for 10 minutes (Rescue (10 mins)) and for 24 hours (Rescue (24 h)). Area covered expressed as a percentage of movement relative to the Baseline. Error bars represent standard error of the mean, with an n = 8 for each concentration. Veh: 0.5% DMSO in artificial pondwater for URB 597 and 0.5% ethanol in artificial pondwater for AEA. #p<.05; where # refers to statistical significance between Baseline and Rescue (24 h).

3.13. Chapter summary

In this section, we show that CBD is absorbed by *L. variegatus,* and that CBD affects stereotypical movement and free locomotion behaviour. We also demonstrate that when exposed to endocannabinoids, movement behaviour is also affected. Our results are summarised in Figure 3.13.

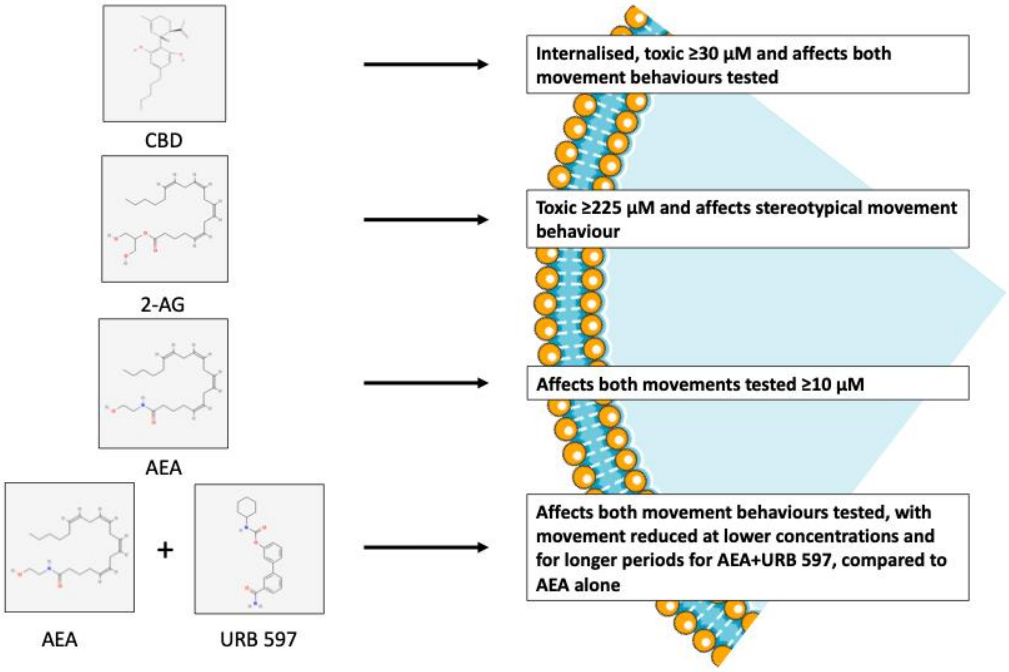

Figure 3.13. Representation of CBD and endocannabinoid effect on *L. variegatus*. CBD is internalised, toxic at concentrations ≥30 µM, and affects stereotypical movement and free locomotion behaviour. The endocannabinoid 2-AG is toxic at higher concentrations of ≥225 µM and only effects stereotypical movement, whereas, when AEA is used in combination with URB 597, movement is restricted for longer periods.

4. Discussion

We investigated the response of *Lumbriculus variegatus*, a freshwater oligochaete worm, to cannabinoids. The ECS is known for its role in various physiological processes, and this system has been evolutionarily conserved across vertebrate species. However, as Stephano (2022) highlighted, the ECS is lacking in conventional research invertebrates such as *C. elegans* and *Drosophila*. These invertebrates are commonly used to understand human biological mechanisms, used in drug discovery and can be used as educational models (Wilson-Sanders, 2011). *L variegatus* has a well-defined nervous system, and show sensitivity to environmental changes (Martinez et al., 2008). Other drugs such as channel blockers, nicotine, fluoxetine and histamine have been examined in this organism (Lesiuk & Drewes., 2001; Nentwig., 2007; Seeley et al., 2021; Carriere et al., 2023). Therefore, they can potentially be a valuable *in vivo* model for studying the effects of cannabinoids.

Our study sought to determine multiple objectives, including the analysis of cannabinoid-like receptor proteins in both vertebrate and invertebrate species, the characterisation of behavioural responses following exposure to endocannabinoids and phytocannabinoids, and the development of novel methodology for drug absorption analysis in this model. By employing a combination of bioinformatics approaches, behavioural assays, and gas chromatography–mass spectrometry (GC-MS) analysis, the study examines the interactions between cannabinoids and *L. variegatus*.

The findings presented here have implications for future drug design and environmental consequences, including understanding the levels of conservation of cannabinoid receptor proteins, toxicity concentrations, absorption mechanisms, and the behavioural responses to cannabinoid exposure.

4.1. Species conservation of endocannabinoid receptor protein

The comparison of the conservation percentages of endocannabinoid proteins across various species provides valuable insights into the evolutionary dynamics of these proteins. When

examining vertebrates, as shown in Figure 3.1, our findings indicate high conservation of CB_1 and CB_2 proteins, with *G. gorilla* displaying high conservation rates when compared to *H. sapiens*. This conservation in our analysis supports Silver (2019), who states the ECS is highly conserved in mammalian species. The observed 99.79% conservation of CB_1 receptors and 99.44% conservation of CB_2 receptors in *G. gorilla* highlight the evolutionary stability of these receptors in primates, which suggests these receptors have an essential role in biological functions.

Interestingly, while *R. rattus* exhibits a high conservation rate of 97.04% for CB_1 receptors, the conservation percentages for CB_2 receptors and fatty acid amide hydrolase in *R. norvegicus* are comparatively lower at 81.11% and 82.90%, respectively (Figure 3.1). These variations within the rodent species highlight potential species-specific evolutionary changes in the endocannabinoid system. Further investigations into these species' ecological or physiological variations may shed light on the factors influencing these differences.

Analysis of invertebrate results reveals a lack of conservation of endocannabinoid proteins in the fruit fly (*D. melanogaster*), velvet worm (*P. onychophora*), and horseshoe crab (*C. rotundicauda*). Similarly, no conservation of CB_1 receptors was detected in the nematode *C. elegans*. Instead, the cannabinoid-like receptor NPR-19, which has been associated with endocannabinoid signalling in *C. elegans* (Oakes et al., 2017), displayed only 22.68% conservation, indicating a much lower degree of similarity to vertebrate CB_1 receptors. Conversely, a 29.03% conservation of CB_1 receptors was observed in the marine organism *C. intestinalis*. These findings align with prior work by Stephano (2022), who reported an absence of an ECS in several invertebrates, including *C. elegans*. Collectively, these results suggest that ECS components, particularly CB_1 and CB_2 receptors, are poorly conserved across invertebrates, with variability likely driven by environmental and evolutionary factors.

The observed absence of FAAH in *G. gorilla* and *X. laevis*, despite its presence in other sequenced species (Figure 3.1), adds further complexity to understanding the evolution of the ECS. This variation in FAAH conservation may reflect species-specific adaptations or regulatory

mechanisms, underscoring the need for detailed functional studies to elucidate the roles of FAAH across different organisms. The conservation patterns observed across species highlight the potential for distinct evolutionary trajectories, particularly among invertebrates and non-mammalian vertebrates.

These interspecies variations in CB_1, CB_2, NPR-19, and FAAH conservation emphasise the importance of studying the functional implications of these differences. Examining species with lower conservation rates, such as *C. elegans*, could provide insights into unique adaptations or alternative signalling pathways related to the ECS. Exploring invertebrate models such as *L. variegatus* could further illuminate the evolutionary history of the ECS and its functional diversity.

The conservation of cannabinoid receptors in *L. variegatus* is currently unknown, though conservation in other invertebrates suggests that elements of the ECS may have been retained to some extent in this species. As indicated in Figure 3.1 and Table 3.1, varying conservation percentages in other invertebrate species imply evolutionary retention of ECS components in some taxa, potentially for functions distinct from those in vertebrates. Understanding whether *L. variegatus* possesses conserved ECS components is critical, as it would influence the interpretation of results in this species and their relevance to higher organisms.

If *L. variegatus* lacks conserved cannabinoid receptors, the observed effects in this species could be mediated through off-target mechanisms or alternative pathways unrelated to canonical ECS proteins. However, if any conservation exists, it suggests that findings in *L. variegatus* could have broader implications, particularly for understanding cannabinoid interactions and their evolutionary underpinnings. These findings are significant not only for expanding knowledge of ECS functionality across species but also for informing the development of cannabinoid-based therapeutics. By exploring conservation patterns in diverse taxa, including non-human and invertebrate species, researchers can better predict drug responses and toxicity, which are essential considerations for advancing cannabis-based drug development and therapeutic interventions.

4.2. The lethal dose (LD_{50}) of cannabidiol (CBD) in *Lumbriculus variegatus*

The evaluation of *L variegatus* response to varying concentrations of both phyto- and endocannabinoids provides a crucial insight into the potential impact of these compounds on this organism. Our findings reveal concentration-dependent toxicity, with a 100% fatality rate observed at concentrations of ≥30 µM CBD (Figure 3.2).

To accurately determine CBD's lethal dose (LD_{50}) in *L. variegatus*, we refined our concentration range to 0-50 µM (Figure 3.2.B). The 24-hour exposure period allowed us to observe the effects of CBD on survival rates, which was a longer period than Land et al. (2021) who used a shorter exposure time of 4-hours when exposing *C. elegans* to CBD in their toxicity studies. Notably, concentrations of 20 µM and below showed no significant difference in *L. variegatus* survival rates (Figure 3.2.B). However, at concentrations of ≥25 µM, we observed a significant increase in toxicity with a calculated LD_{50} of 23.00 µM (95% CI: 21.74-24.26 µM). This concentration-dependent response is, in contrast, inconsistent with Land et al. (2021) who showed CBD does not display any lethal toxicity in *C. elegans* when exposed to CBD concentrations of 0.4 to 4000 µM for 4-hours. These concentrations are far higher compared to our CBD concentrations of 30 µM and above for 24-hours, which caused a 100% fatality rate (Figure 3.2). However as highlighted by Matta et al. (2007), differences in the drug mediated results between species could be associated with specific *in vivo* responses. This could include CBD exposure through solid nematode growth medium plates or microfluidic chips (Land et al., 2021) or exposure to the same concentration in a liquid medium as used in this study (Turner et al., 2011). The lack of toxicity seen in *C. elegans* could relate to the transdermal permeability of CBD, the surface area in contact with the drug, the length of time the organism is exposed to a drug, or the organisms osmotic balance.

The observed toxicity of CBD in *L. variegatus* raises questions about the broader ecological consequences of CBD exposure in aquatic ecosystems. Cannabinoids are increasingly prevalent in wastewater and natural water environments (How & Gamal El-Din., 2021), and in a study conducted by How & Gamal El-Din., (2021), CBD was found in up to 80% of their sewage sludge samples, with dried weight CBD concentrations as high as 168 ng g^{-1}. This level

in sewage samples is significantly lower than the threshold for toxicity in *L. variegatus*. However, the actual risk depends on level of CBD discharged into *L. variegatus* environment, the length of CDB exposure, and any bioaccumulation of CBD in the worms. Therefore, understanding their impact on aquatic organisms is vital. The sensitivity of *L. variegatus* to CBD, as demonstrated in Figure 3.2, suggests that even relatively low CBD concentrations may threaten aquatic invertebrates. Future research could further investigate CBD toxicity in *L. variegatus* and explore potential sublethal effects on reproduction, regeneration, and behaviour. O'Gara et al. (2004) highlight that copper, regularly released into the environment, had behavioural and neurotoxic effects in *L. variegatus* after exposure, affecting their ability to evade predators. Future studies should consider any links between aquatic ecosystems and the impact of CBD exposure on these systems.

The mechanisms behind CBD's effects on *L. variegatus* can be linked to several factors that contribute to species-specific responses. Our bioinformatics analysis shows the ECS is not uniformly conserved across species. In invertebrates like *L. variegatus*, the ECS is likely to function differently than in vertebrates, given the distinct neurophysiological structures and biochemical pathways. The sensitivity of *L. variegatus* to CBD may be attributed to differences in cannabinoid receptor distribution, receptor affinity, and metabolic pathways for cannabinoid compounds. Furthermore, the exposure route, whether through waterborne uptake, sediment contact, or ingestion, could influence the bioavailability and subsequent effects of CBD in this species. As seen in the study by Land et al. (2021), the discrepancies between CBD toxicity in *L. variegatus* and *C. elegans* may also stem from variations in the organism's absorption mechanisms, membrane permeability, or detoxification processes, such as enzyme activity that helps metabolise cannabinoids.

The ecological significance of these findings is profound, particularly given the increasing risk of the contamination of aquatic ecosystems with cannabinoids like CBD. Studies, such as those by How & Gamal El-Din. (2021), have shown that CBD is already present in significant concentrations in wastewater and sewage sludge, which are key sources of environmental pollution. In ecosystems where aquatic invertebrates like *L. variegatus* play a crucial role in

nutrient cycling, sediment mixing, and as food sources for higher trophic levels (Aikins et al., 2023), the toxicological impact of CBD could have cascading effects. Even at relatively low concentrations, CBD exposure could impair vital functions such as locomotion, predation avoidance, and reproduction, potentially disrupting ecological balance. The potential for bioaccumulation of CBD in the worms could increase toxic effects over time, leading to long-term ecological consequences.

The sensitivity of *L. variegatus* to CBD highlights the need for further investigations into the sublethal effects of cannabinoids on aquatic organisms. These could include alterations in behaviour, predator-prey interactions, reproduction, and the broader ecosystem benefits provided by invertebrates. O'Gara et al. (2004) highlight *L. variegatus* as an important bioindicator species, but studies focussed on CDB in the environment are limited. Therefore, understanding its response to CBD exposure can help inform environmental risk assessments and guide regulatory decisions regarding the safe use and disposal of cannabinoid-containing products.

4.3. Determining cannabidiol (CBD) absorbance in *Lumbriculus variegatus* through gas chromatography-mass spectrometry (GC-MS)

Previous studies have quantified CBD in tissue and bodily fluids through GC-MS, and GC-MS is commonly used by toxicology laboratories for the analysis of cannabinoids in hair or oral fluid (Antunes et al., 2023). In this study we adapted the methodology as set out by Warrilow et al. (2016) for the use of GC-MS to determine the absorption CBD in *L. variegatus.*

The confirmation of CBD absorption by *L. variegatus* through GC-MS analysis provides evidence of the absorption of this cannabinoid within this organism. Our results reveal that CBD was not detected at concentrations below 10 µM in *L. variegatus* (Figure 3.3.D), suggesting a certain level of CBD is required for detection through GC-MS. Identifying palmitic acid and stearic acid in *L. variegatus* at all concentrations, as seen in Table 3.2, is noteworthy. These fatty acids are commonly found in biological tissues (Ulloth et al., 2003) and identification of these fatty acids through GC-MS confirm no flaws in the analysis, and serve

as controls. The absence of detectable CBD at concentrations <10 µM aligns with the survival data, indicating that the lack of detectable CBD correlates with non-fatal concentrations.

Absorption of CBD within *L. variegatus* was observed at concentrations ≥25 µM, corroborating the toxicological findings (Figure 3.2). The mass fragmentation pattern of CBD at 536.88 m/z further confirms the presence of CBD within the organism (Figure 3.3.C). The distinct peak in the gas chromatograph at a retention time of 32.38 minutes with a peak area of 246819891 provides quantitative data on the concentration of CBD within *L. variegatus* (Figure 3.3.D). The observed concentration for absorption aligns with the concentration-dependent toxicity, emphasising the importance of considering both exposure and internal concentrations. Confirming CBD absorption by *L. variegatus* through GC-MS analysis provides insights into drug absorption. This information is relevant for drug design and could assist researchers in understanding how compounds are absorbed within this organism.

Absorption of CBD in *L. variegatus* at concentrations associated with toxicity suggests that the adverse effects observed may be related to the presence of CBD within the organism rather than external exposure alone. Ding et al. (2000) state *L. variegatus* is suitable for study in aquatic or confined underwater spaces due to its natural habitat of muddy sediments or between decaying leaves. This finding highlights the importance of incorporating absorption effects into environmental studies for emerging contaminants like CBD. Future research could investigate the kinetics of CBD absorption in *L. variegatus* using liquid chromatography-mass spectrometry (LC-MS), exploring factors that may influence absorption rates and internal concentrations. Meng et al. (2018) highlight that LC-MS procedures are used in the forensic analysis of THC in blood, urine, and hair, and they use LC-MS methods in their study to quantify CBD levels in consumer products. Additionally, 7-hydroxy-CBD, the major CBD metabolite in humans, could be identified through GC-MS methods (Jeong et al., 2023.) Investigating the potential metabolism and transformation of CBD within *L. variegatus* could provide insights into the outcome of CBD in aquatic environments and support the future of cannabis-based drug development.

4.4. The effects of cannabidiol (CBD) on *Lumbriculus variegatus* stereotypical movement

The stereotypical movement assay was initially proposed for studying *L. variegatus* by Drewes (1999), with this project following the behavioural measurements by Seeley et al. (2021), who investigated *L. variegatus* response to drugs with diverse pharmacokinetics properties. Through these assays, we demonstrated specific movement responses of *L. variegatus* to cannabinoids, including endocannabinoids and phytocannabinoids. Understanding these responses provides a basis for evaluating the potential therapeutic or toxic effects of cannabinoids on behavioural patterns. The stereotypical movement assay conducted after confirming CBD Absorption by GC-MS offers valuable insights into the sublethal effects of CBD on *L. variegatus,* and our findings indicate that CBD significantly impacts the stereotypical movements of *L. variegatus,* even at concentrations that do not induce lethal toxicity.

In the stereotypical movement assay, we observed a significant and rapid inhibition of body reversal and helical swimming at concentrations ≥5 µM during a 10-minute exposure (Figure 3.4.A-B). This shows CBD influences movement, even at relatively low concentrations and highlights its effect on behaviour in *L. variegatus*. Following a 10-minute rescue period, inhibitory effects on body reversal movement continued at concentrations ≥5 µM, while helical swimming displayed no prolonged effects at 5 µM (Figure 3.4.C-D). This different response indicates that CBD exerts concentration-dependent and movement-specific effects and emphasises a complex nature of cannabinoid interactions with *L. variegatus* nervous system. After a 24-hour rescue period in drug-free artificial pond water, no lasting effects were observed at concentrations of 5 to 10 µM on either body reversal or helical swimming, but concentrations ≥15 µM continued to exhibit an inhibitory effect on both movements (Figure 3.4.C-D). These findings suggest that while lower concentrations may not induce persistent movement differences, higher concentrations of CBD have a lasting impact on *L. variegatus* stereotypical movements, even after an prolonged recovery period, providing valuable insights into the effects of CBD on the movement behaviour of aquatic organisms. Zhang et al. (2022) conducted a CBD locomotion study with *C. elegans* where they measured the effect of CBD on body bending and pharyngeal pump rates, after raising temperatures to cause

paralysis. Their results show an increase in delayed paralysis at the 100 µM CBD concentrations, compared to the higher concentration of 400 µM, and suggest higher concentrations of CBD causes stress to *C. elegans* (Y. Zhang et al., 2022). This study further emphasises the concentration response in *C. elegans* to CBD is much higher than the concentrations required to affect movement in *L. variegatus.* Other studies using loratadine show higher concentrations are required for study in *C. elegans* (Viering et al., 2023) compared to *L. variegatus* (Carriere et al., 2023). Viering et al. (2023) investigate loratadine ability to combat Methicillin-Resistant *Staphylococcus aureus* in *C. elegans* using concentrations of 40 µM, whereas Carriere et al. (2023) saw effects on movement from exposure to loratadine from 0.6 µM.

The work conducted by Drewes (1999) describes the two distinct body reversal and helical swimming patterns measured in this project. Through this assay, we observed the behavioural effects of CBD on these stereotypical movements in *L. variegatus*. Our results are consistent with Seeley et al. (2021), who demonstrate a movement response from drug exposure in this organism and show the use of *L. variegatus* as an effective *in vivo* pharmacological teaching model. Compared to *L. variegatus,* Land et al. (2021) show CBD exposure does not significantly impact movement in *C. elegans* except at a 4000 µM concentration. However, as previously mentioned, variations between species in the effects of drug concentration exposure could be related to species-specific *in vivo* responses such as whether the drug is in a solid plate or liquid culture (Matta et al., 2007).

4.5. The effects of cannabidiol (CBD) on *Lumbriculus variegatus* free locomotion

The assessment of unstimulated free locomotion behaviour following the stereotypical movement assay provides additional insights into the effects of CBD on *L. variegatus*. Unlike the stereotypical movements, our results show unstimulated movement does not exhibit immediate effects to CBD exposure but instead reveals dose-dependent effects during recovery periods.

In contrast to the stereotypical movements observed in Figure 3.4, unstimulated movement of *L. variegatus* did not show a significant response to CBD at concentrations of 0-20 µM after a 10-minute exposure, as shown in Figure 3.5.B. These findings imply that CBD may not rapidly influence free locomotion within the exposure time investigated. However, after a 10-minute rescue period, a noteworthy increase in movement was observed at 5 µM, indicating a potential stimulatory effect at this concentration (Figure 3.5.C). This stimulatory effect contradicts the inhibitory responses seen in stereotypical movements with CBD concentrations ≥5 µM (Figure 3.4.C-D). The conflicting results between stereotypical movements and free locomotion further highlight a complex nature of cannabinoid interactions with *L. variegatus.* After a 24-hour rescue period, a dose-dependent reduction in unstimulated behaviour was observed. Specifically, at concentrations of 15 µM and 20 µM, movement was significantly reduced as seen in Figure 3.5.C. This indicates a prolonged effect of CBD on free locomotion in *L. variegatus*, even after a substantial recovery period. This response could be attributed to several species-specific factors related to their physiology, metabolism, or the way CBD interacts with their biological systems. First, the uptake of CBD by *L. variegatus* may be influenced by their surface permeability, potentially affecting the rate at which the compound enters their system. Additionally, *L. variegatus* lack the complex enzyme systems found in vertebrates, which may result in slower processing and a gradual accumulation of CBD. Furthermore, as a lipophilic compound, CBD may accumulate in the fatty tissues of *L. variegatus*, leading to a prolonged effect. Environmental factors, such as water temperature and oxygen levels, could also impact CBD's solubility, uptake rate, and bioavailability, further contributing to delayed effects in this species.

This delayed response aligns with the concentration-dependent long-term effects observed in the stereotypical movement assay. Differences on the effect of different movement behaviours after drug exposure is also seen in the study conducted by Seeley et al. (2021). In this study their stereotypical and free locomotion movement results from dantrolene exposure also do not align. Their results show, dantrolene has a significant stimulatory effect on *L. variegatus* free locomotion after a 5 µM dantrolene exposure, compared to no effect on stereotypical movement after exposure, apart from a significant reduction in movement at 50

µM further highlighting the complexity of this organism. Our different results between short-term and long-term recovery periods further emphasise the importance of considering the ongoing effects of CBD on movement. Evaluating unstimulated free locomotion behaviour in *L. variegatus* following CBD exposure reveals a dose-dependent response during recovery. The lack of immediate effects during the short-term exposure suggests a delayed and concentration-dependent impact on free locomotion movement.

Acosta et al. (2021) identify movement in this organism as being generated by region-specific motor networks present in *L. variegatus*, including a medial and two giant lateral fibres. These motor networks in *L. variegatus* differ from *C. elegans*, which hold 302 different neuronal cells, of which 75 are motoneurons. These motoneurons stimulate muscles in the wall of the body, providing a thrust through locomotion (Gjorgjieva et al., 2014). CBD is a highly lipophilic molecule which crosses the blood brain barrier and has a T_{max} of 1-hour in mice after oral administration (Brookes et al., 2023), therefore it is also worth considering the CBD absorption rates in this organism and whether a 10-minute exposure time is sufficient for CBD to permeate, bind and take an effect. Effects of CBD were observed at concentrations of ≥25 µM at our 24-hour time point (Figure 3.2), contrasting with the absence of such effects during short-term exposure. This highlights the importance of investigating the impacts of longer-term CBD exposure in *L. variegatus* as these effects may not been seen at short term exposure. These findings contribute to an understanding of the sublethal effects of CBD on aquatic organisms and emphasise the need to assess the ecological consequences of CBD exposure. In addition, these behavioural assays can be valuable in drug development when understanding the impact of novel compounds on locomotor behaviour and potentially predicting their effects in more complex organisms.

4.6. The lethal dose (LD50) of 2-Arachidonoylglycerol (2-AG), URB 597, and anandamide (AEA) (±URB 597) in *Lumbriculus variegatus.*

The investigation into the response of *L. variegatus* to the endocannabinoids 2-Arachidonoylglycerol (2-AG), Anandamide (AEA), and the fatty acid amide hydrolase inhibitor (FAAH) URB 597, provides insights into the effects of these compounds in combination within

the organism. Our results show the behavioural effects and potential toxicological implications associated with these endocannabinoids and the effects of inhibiting the breakdown of AEA using a FAAH inhibitor.

Exposure of *L. variegatus* to 2-AG revealed concentration-dependent toxicity, with 200 µM resulting in a significant mortality rate of 35±15.92% and a calculated LD_{50} of 199 µM (95% CI: 173.1-225.0 µM) was calculated (Figure 3.6.A). These findings suggest that, like CBD, 2-AG can induce toxic effects in *L. variegatus* but at higher concentrations. Identifying an LD_{50} provides a quantitative measure of the impact on *L. variegatus* from 2-AG exposure, facilitating further investigation of the existence of an endocannabinoid system in *L. variegatus.* The observed toxic effects of 2-AG highlight the potential risks associated with endocannabinoids in drug design. Understanding the impacts of these compounds is crucial for assessing their safety and possible effects on body systems. Other studies have exposed *C. elegans* to a range of different concentrations of 2-AG. For example Galles et al. (2018) expose *C. elegans* to 50 µM of 2-AG added to NGM plates in their study investigating cholesterol transport, whereas Oakes et al. (2017) expose *C. elegans* to 320 µM of 2-AG spread in solid medium to investigate nociceptive behaviours in this organism. Our LD_{50} concentration sits between these exposure levels and our LD_{50} concentration of 199 µM (Figure 3.6.A) did not cause lethality in the Oakes et al. (2017) study.

In contrast to 2-AG, the FAAH inhibitor URB 597 and AEA did not exhibit toxicity at any concentrations tested (Figure 3.6.B-C). FAAH inhibition typically results in increased levels of endogenous cannabinoids, such as AEA (Fowler et al., 2001). The absence of observed toxicity in *L. variegatus* suggests that conserved proteins involved in the endocannabinoid system may not be present in this species. Furthermore, any elevation in endogenous cannabinoid levels resulting from FAAH inhibition at the tested concentrations appears to have no adverse effects on *L. variegatus*, highlighting potential differences in their endocannabinoid signalling pathways.. Pastuhov et al. (2012) expose *C. elegans* to the higher concentration of 290 µM of AEA in their study investigating neuron regeneration. These similar results suggest AEA is a non-toxic endocannabinoid when exposing both *C. elegans* and *L. variegatus*. In studies

conducted by Chen et al. (2019) *C. elegans* were exposed to the FAAH inhibitor URB 597, using up to 50 µM, which is slightly lower than the concentrations used in this study.

The investigation into the response of *L. variegatus* to 2-AG, URB 597, and AEA provides important toxicological insights. The concentration-dependent toxicity of 2-AG, the lack of toxicity with URB 597, and the non-toxic nature of AEA at tested concentrations contribute to our understanding of the effects of endocannabinoids on this aquatic organism.

4.7. Investigating the effects of 2-Arachidonoylglycerol (2-AG) on *Lumbriculus variegatus* movement

After identifying the lethal dose of endocannabinoid ligands in *L. variegatus*, we sought to investigate *L. variegatus* behavioural response after exposure to 2-AG.

Exposure to 2-AG for 10-minutes resulted in a significant concentration-dependent effect on stereotypical behaviours in *L. variegatus* at concentrations ≥25 µM, as seen in Figure 3.7.A-B. These findings indicate that, even at concentrations not linked to acute toxicity, 2-AG rapidly influences the stereotypical movement of this species. After a 10-minute rescue period, inhibitory effects on body reversal movement continued at concentrations ≥25 µM (Figure 3.7.C), but helical swimming showed a more temporary response, with no significant reduction in movement at concentrations <50 µM (Figure 3.7.D). The distinct recovery responses for both stereotypical behaviours highlight the need of investigating movement-specific reactions to AEA. Following a 24-hour rescue period, both body reversal movement and helical swimming remained significantly reduced at concentrations ≥100 µM (Figure 3.7.C-D) and this continued effect suggests a lasting impact of 2-AG on stereotypical movement, even after a 24-hour recovery period.

In contrast to stereotypical movement, unstimulated movement during a 10-minute drug exposure showed no significant response to 2-AG at concentrations up to 150 µM (Figure 3.8.B). These results indicate that the influence of 2-AG on locomotion is movement-specific, with particular systems being more sensitive or responsive to 2-AG. After a 10-minute rescue

period, the lack of significant effects on unstimulated movement continued (Figure 3.8.C). This finding contrasts with the sustained reduction in body reversal movement observed in the stereotypical movement assay after the same rescue period. The inconsistency in response between stereotypical movement and free locomotion highlights the complexity of the movement effects induced by 2-AG. The absence of significant effects on unstimulated movement after a 24-hour rescue period aligns with the short-term recovery observations (Figure 3.8.C). However, the recovery of unstimulated movement is inconsistent to the continued reduction in body reversal movement and helical swimming observed in the stereotypical movement assay after the same recovery period.

Oakes et al. (2017) show 2-AG can also inhibit forward locomotion in *C. elegans* when exposed to agar plates containing 2-AG concentrations of 320 µM, and they hypothesise these behaviours are exerted via the cannabinoid-like receptor NPR-1. Oakes et al. (2019) also suggest there is an endocannabinoid signalling pathway present in *C. elegans* via the transient receptor vanilloid 1 (TRPV1) channels. This is also seen in humans (Ryskamp et al., 2014), but there is no evidence in the literature of transient receptor channels present in our worms. These channels belong to the transient receptor vanilloid (TRPV) channel family which are expressed at the end of sensory nerve fibres and neuronal cells (Du et al., 2019), and have been shown to modulate neurotransmitter release at both pre-synaptic and post-synaptic terminals (Meza et al., 2022). They are associated with nociception and thermal hyperalgesia, and can also be activated by capsaicin (Meza et al., 2022) which is the compound found in chilli peppers (Fujiwake et al., 1980).

The movement assays following 2-AG exposure reveal concentration-dependent and continued effects on the stereotypical behaviour of *L. variegatus*. 10-minute exposure induces rapid responses, while the sustained impact on behaviour after recovery periods highlights the importance of considering both immediate and delayed effects. These findings contribute to our understanding of *L. variegatus* exposure to 2-AG. They also highlight the question of an endocannabinoid response through other pathways in this organism which shows the need

for further investigations into the molecular and physiological mechanisms underlying these behavioural changes.

The movement-specific effects observed in this project suggest that 2-AG may selectively modulate specific locomotor movements while leaving others unaffected. 2-AG biosynthesis controls the development of functional neuronal circuits, and 2-AG signalling is required for axon growth in a developing brain (Martella et al., 2016). Increased levels of 2-AG reduces neuroinflammation, reduces neuropathology, and improves synaptic and cognitive functions in animal models of neurodegenerative diseases, including Alzheimer's disease, multiple sclerosis, Parkinson's disease, and traumatic brain injury-induced neurodegenerative disease (C. Chen, 2023). Therefore, when developing novel drugs to treat neurological conditions. Movement responses to 2-AG, is important when understanding the underlying neurobiological mechanisms of a developing or aging brain .

4.8. Investigating the effects of anandamide (AEA) on *Lumbriculus variegatus* movement behaviour

After investigating *L. variegatus* behavioural response to 2-AG, we went on to investigate *L. variegatus* movement response to the other endocannabinoid, AEA. Comparing the effects of AEA with those of 2-AG reveals both similarities and differences. While both endocannabinoids induce concentration-dependent reductions in stereotypical movements, the recovery effects and concentration levels for significant effects differ.

Exposure to AEA for 10-minutes resulted in concentration-dependent reductions in body reversal and helical swimming at concentrations ≥10 µM, indicating a variation in specific locomotor behaviours in *L. variegatus* (Figure 3.9.A-B). This response provides insights into the organism's sensitivity to AEA exposure. After a 10-minute rescue, inhibitory effects on body reversal movement continued at concentrations ≥25 µM, while helical swimming significantly reduced at concentrations ≥10 µM (Figure 3.9.C-D). The varying recovery results suggest that the concentration of AEA exposure influences the recovery effects on stereotypical movements. Notably, a complete return to baseline levels for both movements

occurred after a 24-hour rescue period, indicating a reversible and temporary nature of the effects induced by AEA on stereotypical movements. The observed full recovery after 24-hours suggests the organism's ability to adapt and overcome the acute effects of AEA exposure.

Contrary to stereotypical movements, where significant reductions were observed at higher concentrations (≥10 µM) (Figure 3.9), unstimulated movement exhibited a significant decrease only at the concentration of 10 µM (Figure 3.10.B). These results indicate different effects on free locomotion from AEA compared to stereotypical movements. The concentration-dependent response highlights the importance of considering other locomotor behaviours when assessing the effects of endocannabinoids. After a 10-minute rescue period, no significant differences in unstimulated movement were observed at any tested concentration (Figure 3.10.C). This behaviour contrasts with the continued reduction in stereotypical movements observed in the 10-minute recovery after AEA exposure, highlighting the behaviour-specific nature of AEA's effects on free locomotion. The most notable findings are the significant reductions in unstimulated behaviour after a 24-hour rescue period at concentrations of 25 µM, 50 µM, and 70 µM (Figure 3.10.C). These results indicate AEA's delayed and sustained impact on free locomotion, even after a substantial recovery period. The concentration-dependent reduction in movement at higher concentrations raises questions about AEA's continual and potential cumulative effects on *L. variegatus* free locomotion behaviour.

The investigation into the effects of AEA on stereotypical movements of *L. variegatus* reveals concentration-dependent and reversible responses. The short-term and long-term recovery results provide essential insights into the organism's adaptive capacity to cope with acute exposure to this endocannabinoid. Further research into the underlying molecular and physiological mechanisms will contribute to a more comprehensive understanding of the impact of endocannabinoids on *L. variegatus*. The contrasting responses between stereotypical movements and unstimulated free locomotion highlight the complexity of AEA's effects on different locomotor behaviours. While stereotypical movements exhibited

concentration-dependent reductions during exposure and recovery, the unstimulated movement showed a different response, with a significant decrease at 10 µM during exposure and delayed effects at higher concentrations during recovery.

Levichev et al., (2022) show that exposing *C. elegans* to AEA, affects feeding behaviours in this organism by increasing appetite and food consumption of nutritionally superior foods. As *C. elegans* are bacterivores, this study uses bacteria to measure feeding rates after exposure to 100 µM of AEA (Levichev et al., 2022). This concentration is slightly higher than the maximum concentrations we used during our investigations, where we saw a behavioural response. Interestingly, this feeding behaviour highlighted in the Levichev et al. (2022) study is consistent with humans, who report in having an increase in appetite after using cannabis (Kirkham & Williams, 2001). However, feeding behaviours is beyond the scope of this thesis, where we focussed on movement behaviour assays.

The observation of dose-dependent effects of AEA on unstimulated free locomotion behaviour reveals a movement-specific response with concentration-dependent and delayed effects. Compared to stereotypical movements, the different effects of AEA on free locomotion emphasise the need for an understanding of the movement responses of *L. variegatus* to other endocannabinoids. Further research into the underlying mechanisms and long-term consequences of AEA exposure on movement behaviour could contribute to understanding endocannabinoids' pharmacological impacts in drug design.

4.9. The effects of anandamide (AEA)+URB 597 (250 µM) on *L. variegatus* movement

We then conducted paired movement assays using AEA concentrations ± URB 597 (250 µM) to explore the behavioural response of *L. variegatus* to these compounds in combination. This approach allows for a further understanding of the effects of AEA, and the potential modulatory role of FAAH. The investigation into the effects of AEA alone and combined with an FAAH inhibitor, URB 597, on *L. variegatus* movement behaviour provides an understanding of the interactive effects of endocannabinoids and FAAH inhibition. The results presented in Figure 3.11 reveal concentration-dependent responses and different recovery results.

Exposure to AEA alone and in combination with URB 597 (250 µM) resulted in significant reductions in both body reversal movement and helical swimming at concentrations ≥10 µM after a 10-minute exposure (Figure 3.11.A-B). This suggests an increase of the inhibitory effects on stereotypical movements with the combined exposure compared to AEA alone. Following a 10-minute rescue period, the combination of AEA and URB 597 showed a further reduction in body reversal movement at lower concentrations (5 µM) and helical swimming at 10 µM compared to AEA alone (Figure 3.11.C-D), indicating different effects on the recovery of stereotypical movements.

A notable finding was the delayed effect observed after a 24-hour rescue period, where the combination exhibited a significant reduction in both body reversal movement and helical swimming at concentrations ≥50 µM, unlike AEA alone where no significant differences were observed at this time-point (Figure 3.9.C-D and Figure 3.10.C-D). This suggests that the combination of AEA and FAAH inhibition may delay the effects on stereotypical movements compared to AEA alone.

In contrast to stereotypical movements, unstimulated free locomotion showed no significant changes when exposed to AEA (0–70 µM) and URB 597 (250 µM) for 10 minutes (Figure 3.12.B). This suggests that the effects observed in the stereotypical movement assay may not directly translate to free locomotion, highlighting a fundamental difference between these two types of assays. The stereotypical movement assay involves a response to a stimulus, requiring sensory detection and motor coordination, whereas the free locomotion assay measures natural, unstimulated movement in the absence of external cues. As a result, it is possible that the drugs interfere with the organism's ability to sense or respond to stimuli, rather than directly impairing movement itself. This distinction could explain why significant effects are observed in the stereotypical movement assay but not in unstimulated free locomotion within the same exposure period.

Comparing the effects of AEA alone and in combination with URB 597 (250 µM) reveals concentration-specific interactions. The combination of both compounds increases the

inhibitory effects on movement at lower concentrations during the recovery period, indicating a potential synergistic effect. This response was anticipated, as URB 597 inhibits FAAH, the enzyme responsible for AEA breakdown, meaning AEA remains active in the system for longer. These findings suggest that AEA metabolism in *L. variegatus* requires FAAH, leading to prolonged effects when FAAH is inhibited. Specifically, the enhanced and prolonged impact on movement behaviour highlights the potential for environmental or experimental exposure to compounds like URB 597 to amplify the effects of endogenous AEA.

Additionally, no significant changes were observed in unstimulated movement following a 10-minute rescue period, consistent with observations of AEA alone in Figure 3.10.C. However, a delayed effect became evident after a 24-hour rescue period, during which the combination of drugs caused significant reductions in free locomotion at all concentrations ≥10 µM (Figure 3.10.C and Figure 3.12.C). This delayed response may reflect a cumulative or secondary effect of the drugs, potentially related to longer-term disruptions in signalling or metabolic pathways. The observed interactions between AEA and FAAH inhibition have important implications for understanding the pharmacological consequences of altered endocannabinoid metabolism in these organisms.

For *L. variegatus*, these findings emphasise the need to explore how AEA is metabolised and how its effects are regulated, which could differ significantly from other invertebrate systems. If the prolonged effects observed in this study stem from unique metabolic processes in *L. variegatus*, this may provide insights into species-specific adaptations to endocannabinoid signalling. Furthermore, understanding these mechanisms is critical not only for drug design and environmental risk assessments but also for interpreting the broader implications of endocannabinoid signalling in invertebrate models.

Chen et al. (2019) discovered only FAAH-4 provides a metabolic function in *C. elegans,* and that this enzyme acts as a lifespan regulator in this organism. This study further highlights that parts of the ECS are not conserved in invertebrates such as *C. elegans,* but these invertebrates

hold significant ECS-type proteins such as NPR-19 (Oakes et al., 2017). The effects of FAAH on AEA regulation has previously been studied by Cravatt et al. (2001), with FAAH indicated as a key regulator of AEA signalling in mice. This study assessed nociception through locomotor activity after drug exposure and N-Acyl Ethanolamines in the brain were measured through LC-MS.

Rocha et al. (2022) evaluated the safety, tolerability, pharmacokinetics, and pharmacodynamics of the FAAH inhibitor BIA 10-2474 through the first-in-human study which investigated BIA 10-2474. This phase I clinical trial was conducted by Biotrial in France between July 2015 and March 2016, but had to be terminated at the Multiple Ascending Dose phase due to serious adverse events (Rocha et al., 2022). During this study, five out of the six participants who received multiple doses of BIA 10-2474, experienced serious CNS related adverse events requiring hospitalisation; with one of the participants dying, and four others developing irreversible brain damage (Kaur et al., 2016).

Prior to the phase I clinical trial, animal toxicology studies were conducted in mice, rats, dogs, and monkeys (Hayes et al., 2021). These studies resulted in few adverse effects being reported, apart from where at higher doses, BIA 10-2474 was found to damage the medulla oblongata in monkeys, which was not further investigated (Kaur et al., 2016). Other drugs such as the immunomodulatory drug TGN1412 also caused death during phase I trials (Attarwala, 2010). This drug had also been found to be safe during preclinical animal studies (Attarwala, 2010), highlighting If toxicity had been found in other species, human toxicity may have been predicted.

Studies in invertebrates using FAAH inhibition would be invaluable in drug development when understanding the role of novel drugs which increase our endogenous cannabinoids safely. By understanding these inhibitors, we could potentially develop drugs which use our own endocannabinoids to provide the medical benefits of cannabinoids such as THC, without the unwanted off-target effects.

5. Overall Summary

5.1. Aim 1

Firstly, we aimed to analyse cannabinoid-like receptor proteins within vertebrate and invertebrate species using an endocannabinoid protein alignment analysis bioinformatics approach.

Through our investigation into the presence and characteristics of cannabinoid-like receptor proteins across both vertebrate and some invertebrate species, we provide insights into these receptors' evolutionary conservation and divergence. This bioinformatics approach allowed for analysis across species and showed the conserved protein domains across a selection of vertebrates and invertebrates. Identifying these receptors in a group of diverse species opens avenues for understanding the broader roles of cannabinoid-like receptor signalling in biological systems.

5.2. Aim 2

Secondly, we sought to characterise the behavioural responses of *L. variegatus* following exposure to human endocannabinoid compounds 2-AG and AEA, a FAAH inhibitor, and the phytocannabinoid CBD, using stereotypical movement and free locomotion behavioural methods outlined by Seeley et al. (2021).

We were able to characterise behavioural responses to cannabinoids by conducting movement assays which assessed the response of *L. variegatus* to human endocannabinoid compounds, 2-AG and AEA, along with a FAAH inhibitor and the phytocannabinoid CBD. The behavioural methods outlined by Seeley et al. (2021) and Carriere et al. (2023) provided a framework for our investigations. The movement changes seen offer insights into the potential impacts of these compounds on the physiology of *L. variegatus*, contributing to our understanding of the interaction between cannabinoids and non-mammalian organisms.

5.3. Aim 3

Lastly, we aimed to develop a novel methodology for determining drug absorption into *L. variegatus* through gas chromatography-mass spectrometry (GC-MS).

We developed a novel methodology for determining drug absorption in *L. variegatus* using GC-MS. This methodology has established a reliable technique for assessing drug absorption in this organism, increasing our ability to investigate the pharmacokinetics of cannabinoids and related compounds, and lays a foundation for future studies aiming to elucidate absorption mechanisms.

6. Future Directions

The combination of these results across our aims highlights a diverse approach taken in this project. The connection between bioinformatics analyses, behavioural studies, absorption methodologies, and intracellular signalling investigations provides a wide-ranging view of the impact of cannabinoids on *L. variegatus*.

Future studies could further build upon these findings using abnormal CBD compounds and other endo or phytocannabinoids to examine the presence of an ECS in this organism. Western blotting assays could identify receptor proteins, or the expression of heat shock proteins associated with cannabinoid exposure. To investigate *L. variegatus* metabolism of cannabinoids further, an LC-MS spectrometry analysis could be conducted to determine the presence of 7-hydroxycannadidiol after exposure to CBD. 7-hydroxycannadidiol is a major active metabolite of CBD, metabolised by the CYP2C19 enzyme in humans (Beers et al., 2021).

In addition, studies could be conducted that delve into the specific ecological implications of CBD. Previous studies have used *L. variegatus* to investigate the effects of microplastics released into the environment and found in pond and lake sediments. Silva et al. (2021) investigated *L. variegatus*' physiological, biochemical, and reproductive response after the ingestion of microplastics after short-term and long-term exposure. They did this by measuring glutathione and glutathione-*S*-transferase activity in antioxidant and detoxification mechanisms and studied aerobic energy production. Through this project, we know *L. variegatus* absorbs CBD at concentrations of ≥25 µM. The methods used in this study could be translated into CBD studies measuring oxidative damage and reproduction and biomass levels after CBD exposure.

In addition to genome sequencing, work could be conducted to measure the regeneration effects of CBD and other cannabinoids. This work could be conducted by following the methods established by Tweeten., (2009), who measured growth through blastema formation.

7. Conclusion

We found that interactions between cannabinoids and *L. variegatus* are complex. Feeding behaviours in *D. melanogaster* have been studied after exposure to cannabinoids (He et al., 2021), but for the first-time, movement effects of cannabinoids were measured in this species. This provides insights for drug design and highlights potential environmental risks. This study reveals a high conservation of cannabinoid receptors in vertebrates and some invertebrates, emphasising the evolutionary significance of the ECS. Behavioural responses to cannabinoids, particularly CBD and endocannabinoids like AEA and 2-AG, are revealed through different assays, highlighting concentration-dependent effects on stereotypical movements and unstimulated free locomotion.

Additionally, this project examines the absorption of CBD, offering insights into the pharmacokinetics and drug distribution within *L. variegatus*. By investigating how CBD is taken up and potentially metabolised in this invertebrate model, the study provides an understanding of cannabinoid bioavailability in invertebrate organisms. This information not only sheds light on the capacity of *L. variegatus* to absorb and process CBD but also raises important questions about the potential accumulation of cannabinoids in invertebrate tissues and their subsequent impact on behaviour and physiology. Such findings are particularly significant for understanding environmental exposure risks, as invertebrates like *L. variegatus* often inhabit ecosystems where cannabinoids and other pharmaceutical pollutants may accumulate as toxins. Furthermore, insights into the absorption and distribution of CBD could inform future studies on its mechanism of action and efficacy in invertebrate models, while offering comparative perspectives on cannabinoid pharmacology across species.

Combining AEA with the FAAH inhibitor URB 597 notably demonstrates significant short-term and long-term recovery effects, suggesting potential combined properties. We also sought to analyse cannabinoid-like receptor proteins within vertebrate and invertebrate organisms, which was conducted using a bioinformatics approach.

Overall, these findings demonstrate *L. variegatus* is a model for understanding cannabinoid interactions, and offers a platform for drug design, whilst highlighting the environmental implications of cannabinoid exposure. In conclusion, this project contributes to pharmacology, ecology, and bioinformatics by examining cannabinoid-like compounds in *L. variegatus*. These methodologies have provided an understanding of the complex interactions between cannabinoids and non-mammalian organisms.

8. References

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9. Appendices

9.1 Investigating intracellular signalling effects of test compounds through protein concentrations within *L. variegatus*

Based on observations that CBD had been absorbed by *L. variegatus* (Figure 3.3.) and from observing a behavioural response to CBD (Figures 3.4-5), an investigation was conducted to determine if CBD affected protein function and cell signalling through phosphorylation. This was carried out using a human microarray immunoassay with a Human Phospho-Kinase Array Kit.

9.2. Additional Methods and Material

9.2.1 Product

Human Phospho-Kinase Array Kit: Supplier - Ahn, K. H., Mahmoud, M. M., Shim, J.-Y., & Kendall, D. A. (2013). Distinct Roles of β-Arrestin 1 and β-Arrestin 2 in ORG27569-induced Biased Signaling and Internalization of the Cannabinoid Receptor 1 (CB1)*. *Journal of Biological Chemistry*, *288*(14), 9790–9800. https://doi.org/10.1074/jbc.M112.438804

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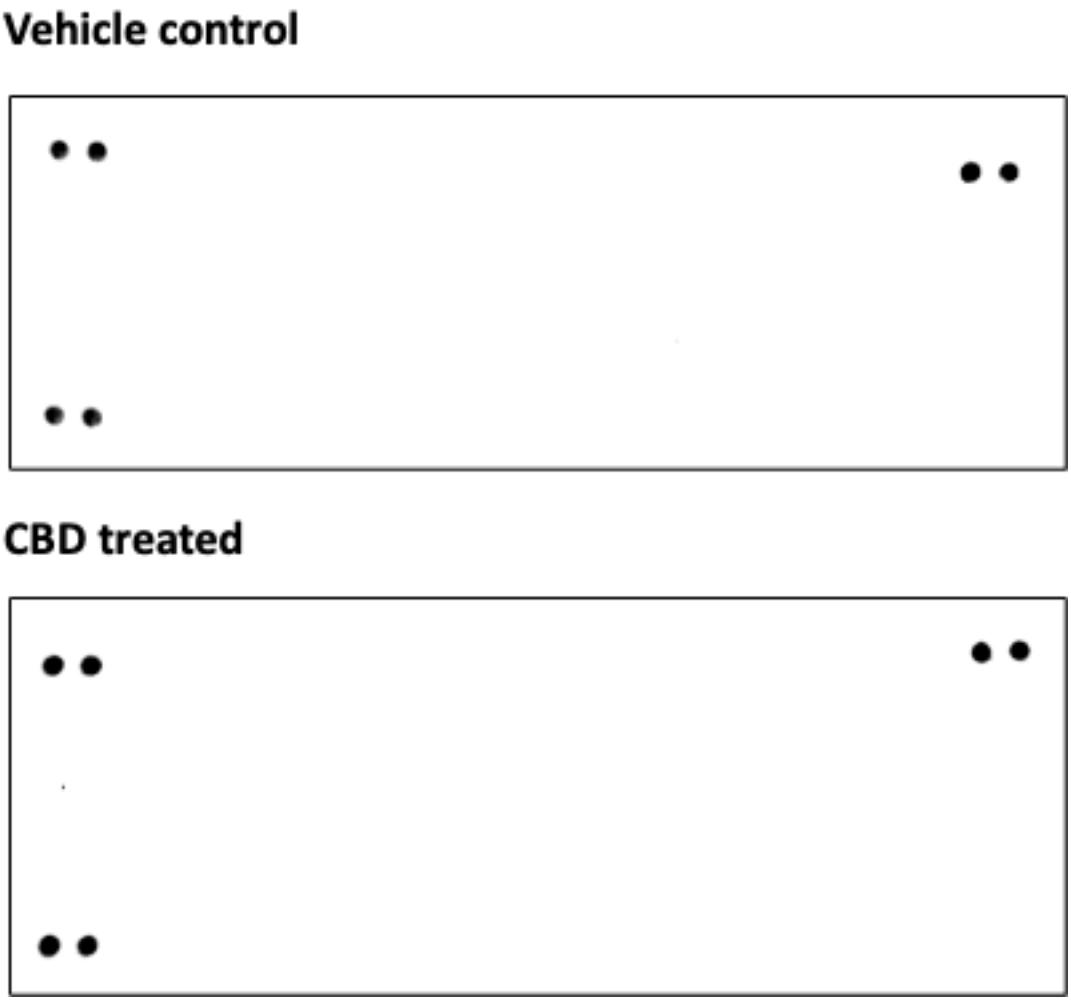
, Storage - 2-8°C

The bio-techne© Proteome Profiler Human Chemokine Array Kit (Product code: ARY003C) includes a Detection Antibody Cocktail, Array Buffers, Lysis Buffer, Chemi Reagents, Streptavidin-HR, and Wash Buffer. All solutions are pre-prepared, and the kits are stored at 2-8°C.

9.2.2. Human Phospho-Kinase Array Kit Methods

Fifteen *L. variegatus* per treatment were transferred to Corning™ Costar™ six-well plates 18-24 hours before completing the experiment. *L. variegatus* were exposed to a vehicle (DMSO 0.5% in artificial pond water) or 20 µM concentrations of CBD for 24 hours. Following exposure, *L. variegatus* were lysed as per the manufacturer's instructions for tissue lysis, and sample protein concentrations were quantified according to Bradford (Bradford, 1976). A total of 600 µg of protein from each condition was added to nitrocellulose membranes and incubated overnight at 4°C. The array was conducted as per the manufacturer's instructions, and images were captured using a BioRad® ChemiDock XRS+.

9.3. Additional Results



Appendix Figure 1. The exploration of signalling mechanisms of CBD in *Lumbriculus variegatus.* *L. variegatus* were exposed to vehicle control (0.5% DMSO in artificial pondwater) or CBD (20 µM) for 24 hours before homogenisation and analysis of protein expression of intracellular signalling proteins using the Human Phospho-Kinase Array, n=1.

Appendix Figure 1. shows the effects of treatment with vehicle control (0.5% DMSO in artificial pondwater) or 20 µM CBD. Reactivity was shown in reference spots only with no reactivity observed for any of the following targets; Akt, beta-Catenin, Chk-2, c-Jun, CREB, EGFR, eNOS, JNK, ERK, Fgr, GSK-3 alpha/beta, HSP27, HSP60, Lck, Lyn, MSK, p38 alpha, p53, p70, PDGF R beta, PLC gamma-1, PRAS40, Pyk2, RSK, Src, STAT, WNK-1 or Yes.

9.4. Additional discussion

An investigation into the effects of CBD on protein function and cell signalling through phosphorylation was assessed using a human microarray immunoassay (n=1). The results, as shown in Appendix Figure 1. indicate that no specific targets were affected by CBD treatment and these results do not display any selectivity on *L. variegatus* cell signalling pathways.

The lack of reactivity for the investigated targets suggests that these targets are not detectable within the selected panel. The absence of reactivity in key pathways associated with cell survival (e.g., Akt, ERK) and stress response (e.g., p38 alpha, HSP27) raises questions about the suitability of this human assay to identify potential targets or mechanisms through which CBD influences the cell signalling pathways of *L. variegatus*, suggesting an organism-specific response in this assay. *L. variegatus* is a new research organism without a fully sequenced genome. As such panels do not currently exist for this organism, and more targeted antibody selection would be required in future studies. It is essential to acknowledge the limitations of the phospho-kinase array, which is designed for human signalling molecules, as they cover a specific set of targets. and may not capture effects of CBD on signalling pathways in other organisms.

Future projects could expand the array to include a broader range of kinases and signalling molecules to explore CBD's molecular impact comprehensively. Additionally, assays targeting specific pathways of interest may provide more detailed insights into the molecular mechanisms underlying CBD's effects on *L. variegatus* behaviour. Further research into alternative targets and species-specific immunoassays could contribute to a more comprehensive understanding of the molecular mechanisms underlying CBD's effects on *L. variegatus*. However, when looking for other studies taking this approach, no other papers could be found showing a lack of activity in other species. Therefore, focusing on the genomic aspects of *L. variegatus* sequencing may provide more fruitful results. By sequencing the genome of *L. variegatus*, we will be able to identify any ECS protein conservation, implying ECS receptor activity in this organism. Studies could focus on developing western blotting or immunohistochemistry methods specific to *L. variegatus*, which quantify protein function and cell signalling through phosphorylation after cannabinoid exposure.