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Faculty of Medicine, Health and Life Sciences

Investigating the effects of cannabidiol and its analogues on the regeneration and biomass of *Lumbriculus variegatus*

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MSc in Pharmacology and Toxicology by Research

SWIRL
Swansea Worm Integrative Research Laboratory

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

Cannabis sativa, has seen recent and rapid commercialisation in the form of cannabidiol (CBD) products. Studies in Spain and California detected CBD in 43-80% of sewage sludge samples and THC in 7-100% of sewage samples, with CBD concentrations ranging from 0.1 to 1.5 µM (Black et al., 2019; Mastroianni et al., 2013). Herein, we use *Lumbriculus variegatus,* a novel invertebrate for pharmacological testing, to examine its responses to CBD and its related analogues and the effects of cannabinoids on *L. variegatus* regenerative capacity.

Using in vivo toxicity assays (IVTAs) we were able to determine toxicity thresholds. We found that 14.12 µM of CBD, 11.29 µM of 7-OH-CBD and 15.84 µM of O-1918 displayed toxicity in 50% of test populations (*N* = 6). This was used to determine equimolar concentrations (0-5 µM) used for pharmacological testing. Behavioural assays mirrored methods outlined by Seeley et al., (2021), however, 24-hour exposure to compounds was investigated. CBD was found to decrease helical swimming at 0.5-5 µM *(p*<.05*, N*=8*)* and body reversal at 2.5-5 µM (*p*<.05, *N*=8). Exposure to 5.0 µM (*p*=0.0018) inhibited unstimulated movement with effects persisting at 0.1 µM (*p*=0.0386*, N*=8) during 10 min recovery and at 5.0 µM (*p*=0.0049*, N*=8) at 10 mins and 24 h recovery timepoints. Similarly, 7-OH-CBD inhibited both stereotypical movement behaviours at 5.0 µM (*p*<.05, *N=8*) with no long-term effects. O-1918 exposure decreased stereotypical movement behaviours at 2.5-5 µM (*p*<.05*, N*=8), with persistent effects at higher concentrations and delayed toxicity observed in the free locomotion assay at 0.5 µM (*p*<.05, *N*=8).

The regeneration assay involved dissecting *L. variegatus* and analysing blastema growth over 4 days (Martinez Acosta et al., 2021), results determined the inhibition of regeneration in worms exposed to 0–5 µM CBD (*p*=0.0123, *N≥15*), which was not observed with 7-OH-CBD (*p*>.05, *N≥15*) or O-1918 (*p*>.05, *N≥15*). The biomass assay was conducted over 28 days with weekly replacement of drug solutions (Doohan et al., 2021), a reduction to 0 individuals and 0 mg/worm biomass was observed only at 5.0 µM (*p*<0.0001, *N*=18). Our findings reveal that each compound exhibits varying potency, recovery dynamics, delayed toxicity and dose-response relationships. These results not only advance our understanding of cannabinoid pharmacology in non-mammalian systems but also highlight potential ecological consequences.

Contents

Abbreviations

2- AG - 2- Arachidonoyl Glycerol

7-COOH-CBD 7-Carboxy-Cannabidiol

7-OH-CBD - 7-Hydroxy-Cannabidiol

Abn-CBD - Abnormal Cannabidiol

AChE – Acetyl Choline Esterase

AD - Alzheimer's Disease

AEA - Anandamide

CB_1 - Cannabinoid Receptor 1

CB_2 - Cannabinoid Receptor 2

CBD - Cannabidiol

CBDA - Cannabidiolic Acid

CHS - Cannabinoid Hyperemesis Syndrome

CNS - Central Nervous System

CUD - Cannabis Use Disorder

DAGL - Diacylglycerol Lipase

DMSO - Dimethyl sulfoxide

ECS - Endocannabinoid system

FAAH - Fatty Acid Amide Hydrolase

GPCR - G-Protein Coupled Receptor

HPA - Hours Post-Amputation

IVTA – *in vivo* Toxicity Assay

LGF - Lateral Giant Fibres

LOAEL - Lowest Observed Adverse Effect Level

MAGL - Monoacylglycerol Lipase

MAPK – Mitogen-Activated Protein Kinase

MGF - Medial Giant Fibre

MS - Multiple Sclerosis

NADA - N-Arachidonoyl Dopamine

NO - Nitric Oxide

O-AEA - Virodhamine

ROS – Reactive Oxygen Species

THC - Δ^9-Tetrahydrocannabinol

THCA - Tetrahydrocannabinolic Acid

TRP - Transient Receptor Potential Channels

VNC - Ventral Nerve Cord

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1. Introduction

Globally, the most commonly used illicit drug is cannabis (United Nations, 2024). *Cannabis sativa* has been viewed as a threat to society and associated with drug misuse. It has been outlawed in the UK following the Dangerous Drugs Act of 1928 and the Misuse of Drugs Act of 1971, which further solidified the possession of cannabis as a criminal act and was met with harsh penalties. Since November 2018, medical cannabis has been legalised for specific conditions such as epilepsy and multiple sclerosis (Duddy, 2025) and can only be prescribed by specialist doctors (Reid, 2024). However, some experts argue that cannabis should be legalised for public health equity (National Academies Press, 2024). Therefore, further research is required on cannabis and cannabinoid drugs to understand the pathways and systems through which they exert their effects. This can aid us in better assessing their therapeutic value for wider use.

The earliest known use of cannabis for its psychoactive and psychotropic properties was discovered during an archaeological excavation in China, dating back to 750 BC (Russo et al., 2008). China has some of the earliest recorded medical uses of cannabis in the Shén Nóng, the ancient Chinese book of herbal medicine, for its pain-relieving properties (Yang, 1998). Since then, the cannabis plant has migrated across the globe due to its consistent contact with humans and has been selectively bred for different purposes as an agricultural crop (Charitos et al., 2021).

Cannabis is historically known for its medicinal and recreational use, with effects dependent on the dose, frequency and route of administration, but also on subjective and cultural contexts (Morningstar, 1985). Low-potency preparations are said to be therapeutic, whereas more potent preparations are seen as poisons (Morningstar, 1985). The pharmacological properties of cannabis have produced contradictory results not only in ancient times but also in modern research and are reviewed extensively. This can be attributed to the many cannabinoids, terpenes and flavonoids produced by the female plant that contribute to the plant's ‘entourage effect’ (Christensen et al., 2023; Ferber et al., 2020).

1.2 Endocannabinoid System

First discovered in 1988 by researchers Allyn Howlett and William Devane, the endocannabinoid system (ECS) was initially identified in rats during studies on the psychoactive compound Tetrahydrocannabinol (THC) (Devane et al., 1988), which had been isolated in 1964 (Gaoni & Mechoulam, 1964). Researchers found that the ECS is a neuromodulatory system that influences the brain's neural networks and plays a pivotal role in maintaining homeostasis and central nervous system (CNS) development. It is composed of specialised G-Protein Coupled Receptor (GPCRs) such as Cannabinoid Receptor 1/2 ($CB_{1/2}$), naturally occurring endocannabinoid ligands including anandamide and 2-arachidonoylglycerol, also fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MAGL) enzymes, among others, responsible for the creation and breakdown of these endogenous ligands (Lu & Mackie, 2016).

The ECS is the largest receptor system in the human body, attributed to its CB_1 and CB_2 receptors being the most expressed GPCRs in the human body. Figure 1 depicts receptor localisation and distribution within the human body. The ECS serves as a regulator of key physiological processes, from controlling blood sugar and immune responses to managing muscle and fat tissue functions, hormone release, pain perception and even reward systems (Zou & Kumar, 2018). The ECS ensures that these systems operate together to maintain homeostatic control (Nazarenus, 2019).

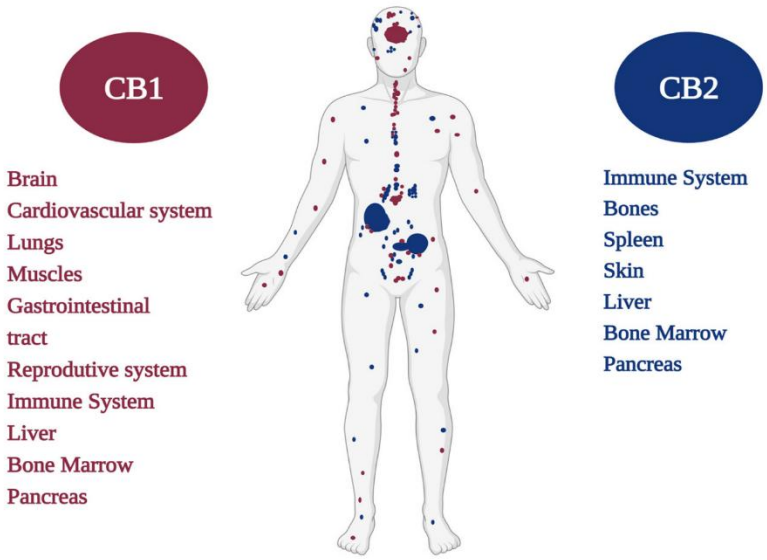

Figure 1: Cannabinoid Receptor Localisation, CB_1 and CB_2 cannabinoid receptors and their distribution in the human body. Taken from Rezende et al., (2023).

1.2.1 G-Protein Coupled Receptors

The effects of cannabinoids and endocannabinoids are mediated by CB_1, CB_2 and other cannabinoid receptors, which belong to the GPCR superfamily. GPCRs are integral membrane proteins that recognise a wide variety of signals ranging from photons to ions, proteins, neurotransmitters and hormones (Rehman et al., 2024). Upon activation of the GPCRs, intracellular signalling is triggered by guanosine diphosphate (GDP) - guanosine triphosphate (GTP) exchange. The signal is regulated through three key processes: desensitisation, internalisation and downregulation (Figure 2), with each stage helping regulate GPCR activity to maintain cellular balance (Black et al., 2016).

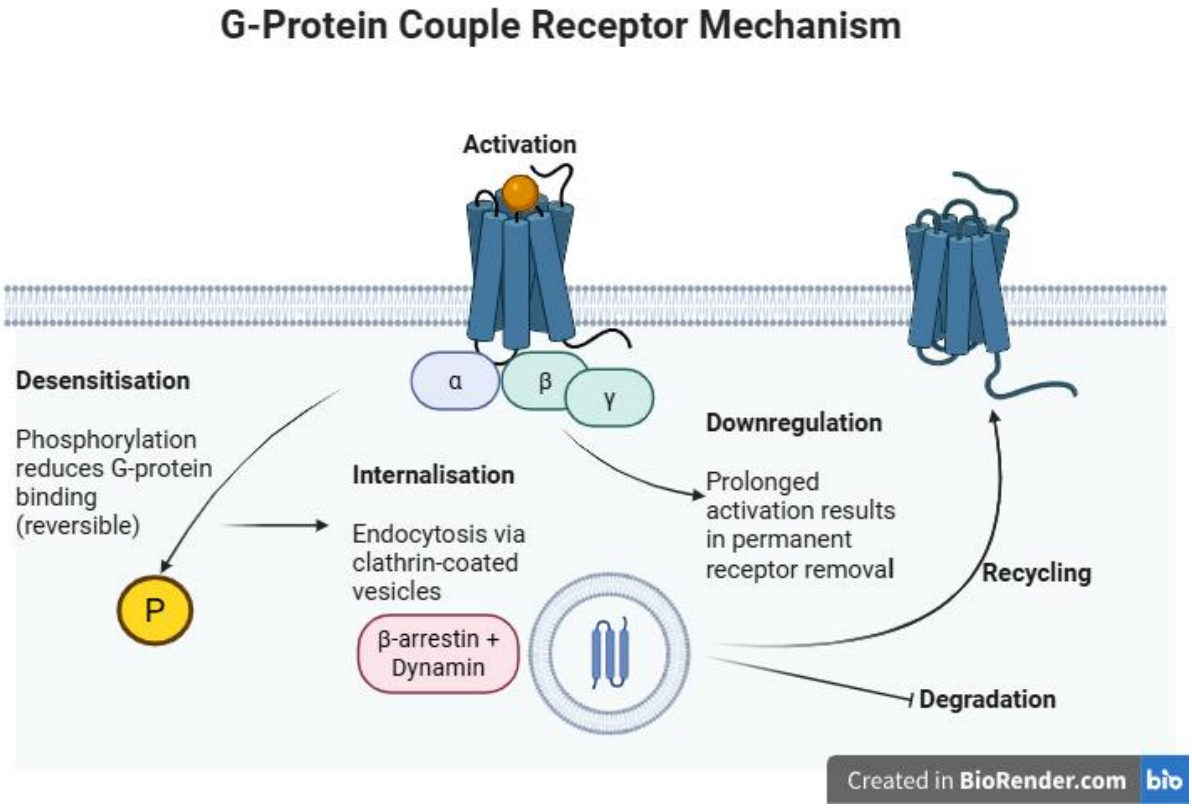

Figure 2: GPCR Mechanism adapted from Rajagopal & Shenoy, (2018), Created in BioRender. The orange ball represents a ligand activating the GPCR, causing GDP-GTP exchange on G-proteins (α,β,γ). Prolonged activation leads to receptor phosphorylation, β-arrestin binding and dynamin-mediated endocytosis via clathrin-coated vesicles. Internalised receptors are either recycled to the membrane or degraded through downregulation, terminating signalling.

Once the GPCR is activated by a ligand (Figure 2), desensitisation begins immediately. This is caused by the phosphorylation of GPCRs by protein kinases, which reduces their ability to bind G-proteins. This process is reversible and does not alter receptor numbers (Rajagopal & Shenoy, 2018). Internalisation happens over several minutes, where GPCRs are internalised via endocytosis through clathrin-coated vesicles. The breakdown of the GPCRs within the endosome is facilitated by β-arrestin and dynamin (Tian et al., 2014). Internalisation removes the receptors from the cell surface temporarily for recycling or lysosomal degradation.

Downregulation and breakdown is caused by prolonged or repeated GPCR activation, which leads to their permanent removal from the plasma membrane through persistent internalisation via endocytosis and sorting to lysosomes for proteolytic degradation, which reduces receptor availability. Prolonged or repeated receptor activation results in permanent receptor loss from the plasma membrane and reduces cellular receptor density and responsiveness (Carman & Benovic, 1998; Rehman et al., 2024). This process of degradation and downregulation due to prolonged activation collectively contributes to drug tolerance. Persistent agonist exposure accelerates desensitisation and downregulation, requiring progressively higher agonist concentrations for the same effect. The molecular mechanism is multifaceted and involves the stabilisation of the β-arrestin and protein complexes, changes in receptor trafficking and compensatory signalling alterations contribute to tolerance (Rajagopal & Shenoy, 2018).

Cannabinoid Receptor 1 (CB_1)

The CB_1 receptor, encoded by the *CB1R* gene, was first discovered and isolated in rats (Devane et al., 1988) and it is activated by two endogenous cannabinoids (eCBs), anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG). The localisation of receptors can provide valuable insight into their function. The CB_1 receptor mediates the more psychoactive effects of cannabinoids (Mackie, 2008). It is highly expressed in brain tissue and neuronal cells (Howlett et al., 1990). CB_1 receptors are present in very high levels in several brain regions and in lower amounts in a more widespread fashion. These receptors play key roles in cognitive functions such as memory and learning, addiction, motor dysfunction (e.g., Huntington's disease) (Fernández-Ruiz, 2009) and mental health conditions like schizophrenia, depression and anxiety (Leweke & Koethe, 2008). These receptors are also involved in neuronal synaptic remodelling as learning takes place but also neurogenesis, neuronal migration and appropriate axonal

targeting and synaptogenesis as the brain develops (Anavi-Goffer & Mulder, 2009; Galve-Roperh et al., 2008; Harkany et al., 2008).

CB_1 receptors are coupled to Gi/o proteins (Howlett et al., 2010). When a ligand binds, the receptor undergoes a conformational change that triggers GDP-GTP exchange on the G-protein. This leads to inhibition of adenylyl cyclase and suppression of protein kinase A (PKA) activity via inhibition of cyclic adenosine monophosphate (cAMP). CB_1 activation also modulates ion channels by inhibiting voltage-gated calcium channels and activating potassium channels, causing hyperpolarisation and reduced neurotransmitter release. Additionally, CB_1 activation stimulates the mitogen-activated protein kinases (MAPK) pathway (Figure 3), which affects gene expression, synaptic plasticity and cell survival (Howlett et al., 2010; Pertwee, 1997). Other cellular pathways activated include ceramide and mTOR pathways, among others (Fletcher-Jones et al., 2020).

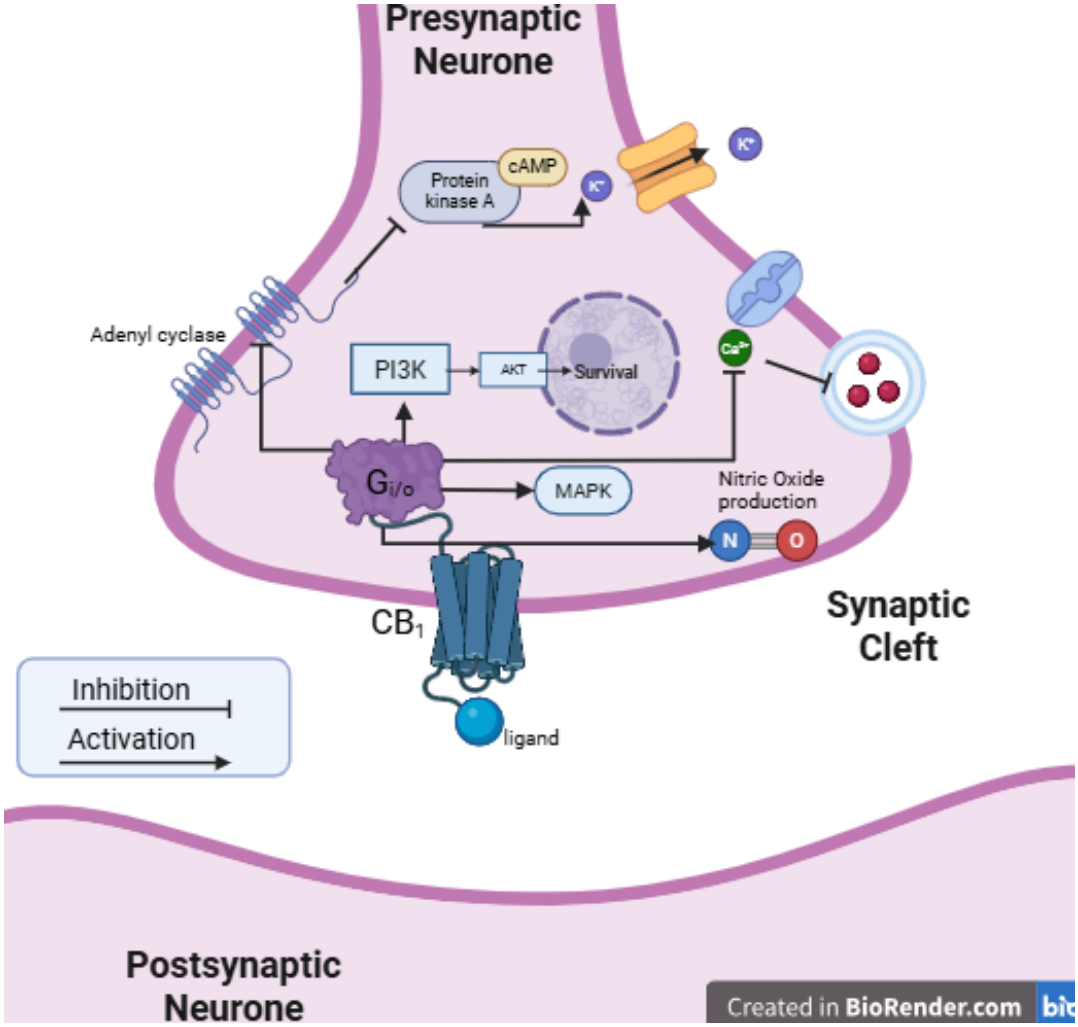

Figure 3: Activation of CB_1 receptor by ligand induces inhibition of adenylyl cyclase, modulation of MAPK pathways and nitric oxide production and depicts other cascading cellular events. Created in BioRender. Adapted from Howlett et al., (2010)

Beyond the brain, CB_1 receptors (Figure 1) are found in the liver, adipose tissues and other organs such as the heart and reproductive system (Maccarrone, 2009; Mallat & Lotersztajn, 2008). Their role in metabolic processes became evident during trials of the CB_1 inverse agonist, rimonabant, for the treatment of obesity; this drug was later withdrawn in 2008 due to psychiatric side effects (Després, 2009). Many cannabinoid drugs have been investigated for their therapeutic potential; however, only two CB_1 agonists, dronabinol (THC) and nabilone, reached the market. Pharmacologically, synthetic CB_1 agonists include potent compounds like HU210 and CP55940, they play an important role in pharmacology research and improving our understanding of the receptor (Bajtel et al., 2022; O'Donnell et al., 2025; Robinson et al., 2007; Soto-Mercado et al., 2021).

Early research into CB_1 focused on pain relief and a non-opioid analgesic such as dronabinol and nabilone (Johnson et al., 1981) but drug development was hindered by side effects like memory impairment and sedation (Jain et al., 1981), they have since been reconsidered for cases of chronic pain and treatment of nausea in cancer chemotherapy (Clark et al., 2005). Recent research has made significant strides in understanding the role of CB_1 receptors in cognitive processes, particularly in reversal learning, where memory traces i.e., stimuli associated to stored memories/ information are modified to form new patterns in response to novel stimuli (Lutz, 2007). This association with memory and learning highlight the role of CB_1 receptors and their influence in addiction (Maldonado et al., 2006).

The orthosteric activation of the CB_1 receptor can be modulated by other ligands, it possesses at least one or more allosteric sites that can be targeted to alter its effects by either inhibiting or enhancing the activation of the receptor by direct agonists (Pertwee et al., 2010). Advancements in cryo-electron microscopy have releveled the conformation of allosteric binding sites, making it possible to design both positive and negative modulators (Shen et al., 2024). These complex mechanisms of receptor activation can be revolutionary in managing unwanted side effects in clinical settings.

Cannabinoid Receptor 2 (CB_2)

CB_2 receptors, first isolated, characterised and cloned in Cambridge (Munro et al., 1993). It is encoded by the *CNR2* gene and is also activated by endocannabinoids 2-AG and AEA, among others. At this cannabinoid receptor, these ligands mediate immune regulation, inflammation and neuroprotection. They are primarily expressed in immune cells and in peripheral tissues

like the spleen, gastrointestinal tract and cardiovascular system, with limited expression in the CNS. However, during inflammation or injury, CB_2 receptor expression significantly increases within the CNS, particularly in microglia and neurons in specific regions like the brain stem and hippocampus (Bie et al., 2018; Cabral & Griffin-Thomas, 2009).

Their functions include regulating immune response by modulating cytokine production and immune cell migration, which translates to controlling inflammation, pain perception and tissue repair (Turcotte et al., 2016). Potential neuroprotective effects of reducing neuroinflammation and restoring microglial function are under investigation (Bie et al., 2018; Turcotte et al., 2016).

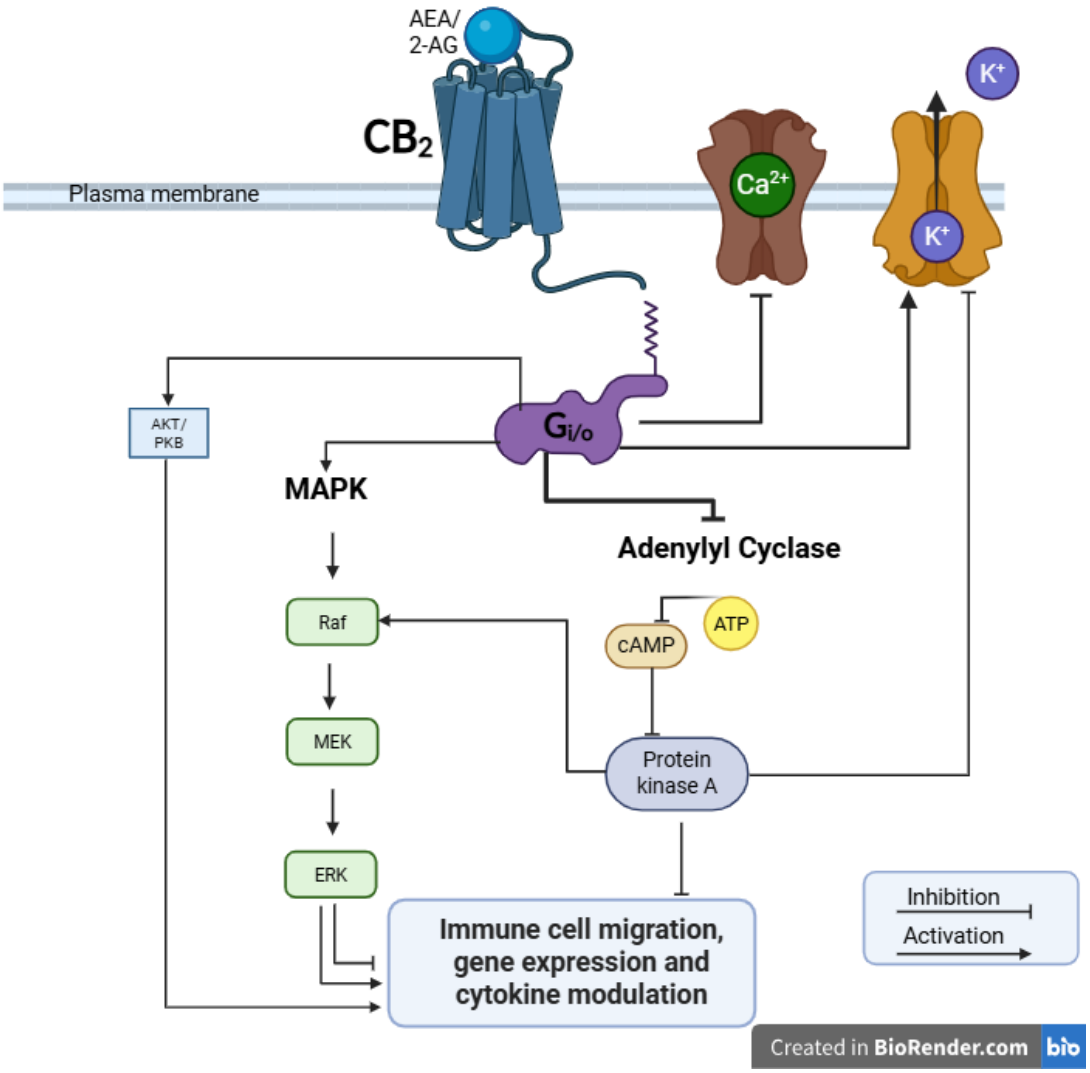

Figure 4: CB_2 Receptor Activation affecting signalling pathways, the inhibition of adenylyl cyclase resulting in decreased cAMP production and PKA activation causing the inhibition of potassium channels which increase RAF to stimulate MAPK, activation of the MAPK cascade and inhibition of calcium channels and activation of AKT, stimulating cell survival and growth, adapted from Dhopeshwarkar & Mackie, (2014), Created with BioRender

CB_2 receptors are also coupled with Gi/o proteins to regulate various signalling pathways (Figure 4). These pathways include inhibition of adenylyl cyclase with reduced cAMP and PKA activity which causes the inhibition of K^+ channels and some gene transcription; activation of MAPK and Akt kinase/protein kinase B cascades, promoting survival, migration, growth and gene regulation; inhibition of Ca^{2+} channels; and PKA reduction that relieves inhibition of Raf further stimulating MAPK and positively regulating gene expression among many other signalling pathways (Cabral & Griffin-Thomas, 2009; Dhopeshwarkar & Mackie, 2014). Although CB_2 receptors activate a wide range of signalling pathways, research on CB_2 ligands has mainly focused on their effects on adenylyl cyclase and ERK1/2. Other pathways, such as those involving arrestin, Akt, ceramide and ion channel modulation, as well as their related physiological processes, remain less explored (Dhopeshwarkar & Mackie, 2014).

Several synthetic agonists and antagonists have been developed to target CB_2 receptors, such as JWH-133, which is a selective CB_2 agonist with anti-inflammatory properties observed *in vivo* (Xu et al., 2007) and MDA7, which has been shown to reduce neuroinflammation and used for the treatment of neuropathic pain (Naguib et al., 2008; Wojcieszak et al., 2016). While THC acts on both CB_1 and CB_2 receptors, its effects on CB_2 are thought to contribute to its anti-inflammatory benefits (Rakotoarivelo et al., 2024). A selective antagonist (SR144528) was created to study the CB_2 receptor functions, which provides contradictory evidence, as it has been shown to block the pro-inflammatory actions of endogenous cannabinoids under certain conditions (Rakotoarivelo et al., 2024; Rinaldi-Carmona et al., 1998). Further confirming the complexity of CB_2 receptor's role in modulating inflammation. The CB_2 receptor is a promising therapeutic target for managing inflammatory and neurodegenerative diseases due to its role in regulating immune responses without causing psychotropic side effects.

Other Receptors

In addition to the well-known CB_1 and CB_2 receptors, several other receptors are associated with the ECS or interact with cannabinoids. These receptors are often referred to as "orphan" GPCRs because their endogenous ligands are not definitively established and therefore their classifications are still under debate (Irving et al., 2017).

Lesser-known cannabinoid receptors include G-protein coupled receptor 55 (GPR55), which can bind to both endogenous cannabinoids and other ligands. GPR55 is widely distributed in brain regions like the hippocampus and peripheral tissues (Godlewski et al., 2009; Ryberg et

al., 2007) with endogenous ligands that include lysophosphatidylinsitol (LPI), which is the most potent ligand, but GPR55 can also be activated with AEA and 2-AG with lower potency. It can also be activated by cannabinoids such as THC and synthetic cannabinoids HU-210 and CP55940 (Fondevila et al., 2021; Oka et al., 2007). It has been proposed to modulate pain perception by regulating intercellular calcium levels, influencing osteoclast activity, regulating metabolism through glucose homeostasis, insulin secretion and is implicated in cancer progression and neurological disorders (Korchynska et al.,2019; Oyagawa & Grimsey, 2021; Tudurí et al., 2017).

G-protein coupled receptor 18 (GPR18) is another orphan receptor which is found in immune cells, lymphoid tissues, brain, lungs, testis and ovaries that has an affinity for cannabinoids. Its endogenous ligands include N-arachidonoylglycine (NAGly), an endocannabinoid metabolite (McHugh, 2012). GPR18 also recognises some CB_1/CB_2 ligands such as THC and some synthetic cannabinoids like O-1918 and O-1602 (Simcocks et al., 2020). The proposed functions of GPR18 include regulating immune responses; it also plays a role in reducing intraocular pressure and therefore can be of potential use in treating glaucoma (Miller et al., 2016). It has also been linked to other ailments such as metabolic disorders and chronic inflammation, it also has potential applications in oncology (Morales et al., 2020)

Other orphan receptors and cannabinoid-relevant receptors include G-protein coupled receptor-119 (GPR119), Transient receptor potential vanilloid-1 (TRPV1) and Peroxisome proliferator-activated receptors (PPARS). These lesser-known cannabinoid-related receptors expand the therapeutic potential of the ECS beyond just CB_1 and CB_2 receptors and make promising targets for a range of different conditions.

1.2.2 Endocannabinoid Ligands

The ECS is a complex lipid-based signalling network that regulates a vast array of physiological processes. Central to this system are endocannabinoids, a class of lipid-derived ligands that interact with cannabinoid receptors and other molecular targets (Lu & Mackie, 2016).

Anandamide (AEA)

The first endocannabinoid identified was N-arachidonoylethanolamide, more commonly called anandamide (AEA) after the Sanskrit word 'Ananda', which means bliss, as it is often referred to as the bliss molecule (Devane et al., 1992). Structurally, it is derived from arachidonic acid and linked to ethanolamine via an amide bond to create N-arachidonoyl phosphatidylethanolamine (Martin et al., 1999). AEA primarily acts as a partial agonist at the CB_1 receptor, with modest affinity at the CB_2 receptor (An et al., 2020). Unlike most neurotransmitters, AEA is synthesised on demand from membrane-phospholipid precursors and its signalling is terminated by enzymatic hydrolysis via fatty acid amide hydrolase (FAAH) to arachidonic acid and ethanolamine (Figure 5) (Ahn et al., 2009; Martin et al., 1999).

AEA can activate both CB_1 and TRPV1 receptors, which play a key role in pain regulation and perception. Although AEA has a low affinity for CB_2 receptors, it can modulate cytokine production in immune cells (Zou & Kumar, 2018).

Studies in mice have shown that AEA exposure results in mild behavioural effects that are short-lived due to rapid degradation by FAAH. However, in FAAH knockout mice, AEA exhibits cannabinoid-like effects including hypomotility, analgesia, catalepsy and hypothermia (Cravatt et al., 2001). FAAH /-/- mice reduced pain sensation that was reversed by CB_1 antagonism SR141716A. Results indicated that FAAH is a key regulator of AEA signalling *in vivo* that translates to the modulation of pain perception (Cravatt et al., 2001). Inhibitors of FAAH are being explored for therapeutic use to elevate AEA levels, potentially treating conditions like pain and anxiety (Ahn et al., 2009).

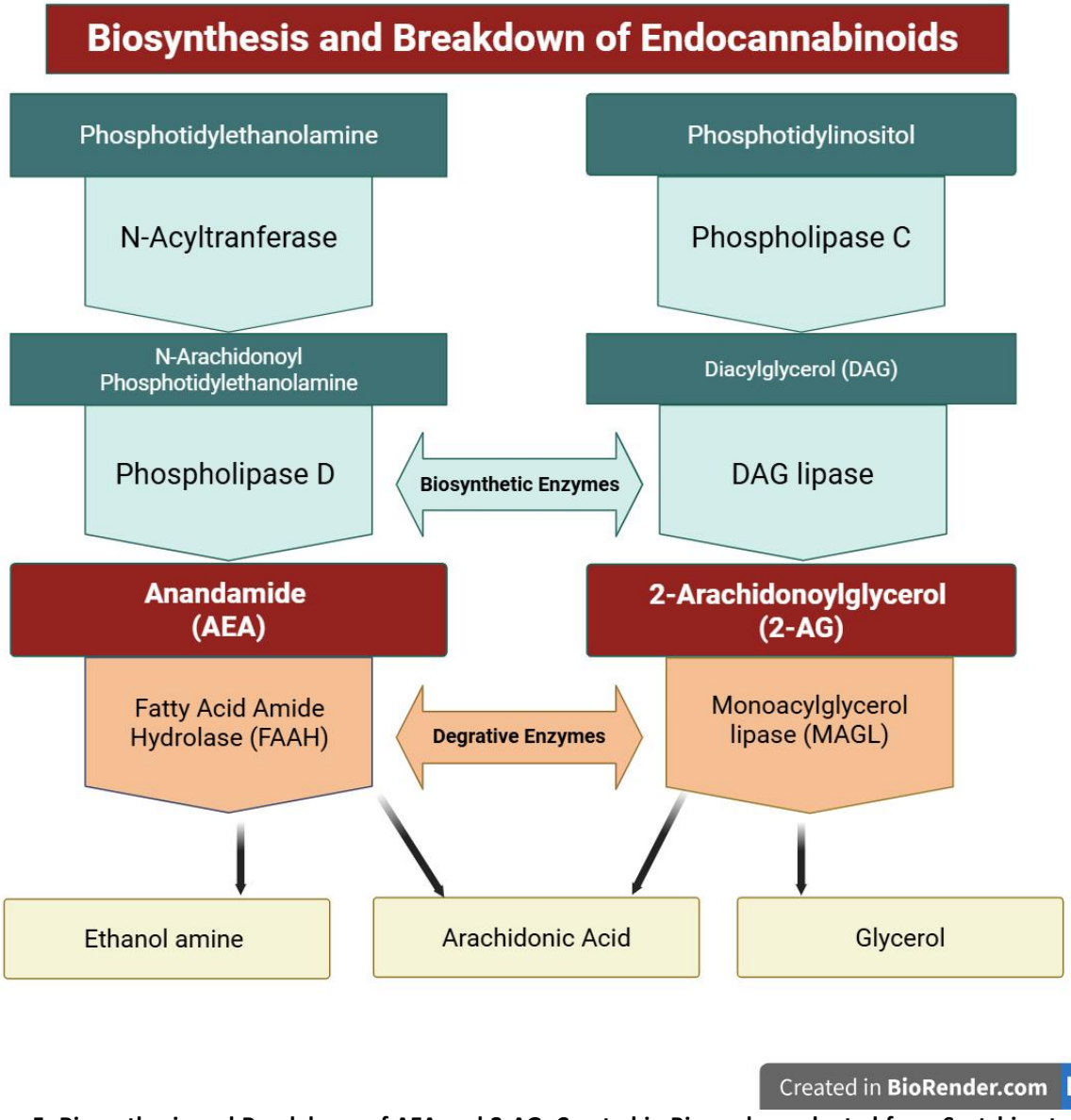

Figure 5: Biosynthesis and Breakdown of AEA and 2-AG, Created in Biorender, adapted from Scotchie et al., (2015).

2-arachidonoylglycerol (2-AG)

2-AG is the most abundant endocannabinoid in the CNS. It is synthesised from membrane phospholipids via diacylglycerol lipase (DAGL) and degraded primarily by monoacylglycerol lipase (MAGL) (Figure 5) (Murataeva et al., 2014). Unlike AEA, 2-AG acts as a full agonist at both CB_1 and CB_2 receptors (Baggelaar et al., 2018).

An important function of 2-AG is retrograde signalling, as it is critical for depolarisation-induced suppression of inhibition/excitation, a process vital for refining synaptic strength (Narushima et al., 2006). Through CB_2 receptor activation, 2-AG attenuates microglial activation and the release of pro-inflammatory cytokines in models of neuropathic pain (Bie et al., 2018). 2-AG also serves as a precursor for prostaglandin synthesis, linking ECS activity to lipid metabolism and inflammatory cascades (Baggelaar et al., 2018).

Research on 2-AG often involves studying its synthesis and degradation pathways (Chen, 2023). Inhibitors of MAGL, such as JZL184, have been used to increase 2-AG levels in mouse *in vivo* models to investigate pain-relieving properties. Chronic administration led to increased tolerance and loss of analgesic activity, which was also observed in MAGL knock-out models (Schlosburg et al., 2010).

Other Endocannabinoid Ligands

Endocannabinoid ligands are still being discovered and investigated; they are highly lipophilic molecules that cross membranes easily and signal locally within the nervous system (Harkany et al., 2008). One such endocannabinoid is N-Arachidonoyl Dopamine (NADA), which combines structural elements of dopamine and arachidonic acid. It exhibits dual agonism at CB_1 receptors and TRPV channels. It is found in high quantities in the hippocampus, cerebellum and striatum, where it modulates synaptic transmission and neuroinflammation (Lawton et al., 2017). Another lesser-known endocannabinoid is Virodhamine (O-AEA), an ester-linked analogue of AEA that displays functional selectivity as a CB_1 agonist and CB_2 agonist. It is shown to be involved in regulating blood pressure and cardiovascular function (Carnevale et al., 2018).

1.3 *Cannabis sativa*

Cannabis is a genus of dioecious flowering plants, with 3 recognised species *C. sativa*, *C. indica* and *C. ruderalis*. All types of cannabis are treated as a subspecies of *C. sativa*. The plant is also known as hemp, although this term is used to refer to the male plant that is cultivated for non-drug use (McPartland, 2018). This is attributed to the low THC content associated with the male hemp plant.

The female *Cannabis* plant, upon maturation, exhibits a higher proportion of capitate-stalked glandular trichomes located in the aerial parts of the plant. These trichomes produce and store resin that contains cannabinoids (Tanney et al., 2021). *Cannabis sativa* produces over 110 cannabinoids, which are split up into 11 subclasses based on their chemical structure. The cannabinoid compounds THC and CBD are two of the most extensively studied phytocannabinoids (i.e., cannabinoid compounds found in plants), along with terpenes, flavonoids, steroids, phenanthrenes, fatty acids, spiroindans, nitrogenous compounds, xanthones and biphenyls (ElSohly & Gul, 2020).

1.3.1 Phytocannabinoid biosynthesis and stability

In natural plant extracts, large amounts of THC and CBD appear in the form of tetrahydrocannabinolic acid (THCA-A) and cannabidiolic acid (CBDA), which are metabolised into THC and CBD by heating (Eichler et al., 2012). It was discovered that when compared, the heated extract resulted in a lower THC plasma concentration but higher levels of THC metabolite, this results in higher active THC and pronounced psychotropic effects and adverse effects. The unheated extracts produced a higher plasma CBD and THC levels but lower metabolite formation, contributing to better tolerance and a lower rate of adverse effects (Eichler et al., 2012). Results suggest that the unheated extracts might offer a more favourable metabolic profile and potentially better tolerability and therapeutic potential (Eichler et al., 2012). This is possibly due to the higher levels of CBD that are lost when heated.

1.3.2 Pharmacological Effects of Phytocannabinoids

The psychotropic “high” effect produced by the cannabis plant is well-documented and attributed to the isolate THC (Paton & Pertwee, 1973; Ranganathan & D’Souza, 2006). However, clinical studies, both *in vivo* and *in vitro*, have also identified other pharmacological effects of cannabinoids. These include anti-nociceptive, anti-epileptic, cardiovascular and immunosuppressive properties (Ameri, 1999), as well as anti-emetic effects and appetite stimulation (Mechoulam & Ben-Shabat, 1999). Additionally, cannabinoids have shown anti-neoplastic (Massi et al., 2004), anti-microbial (Eisohly et al., 1982), anti-inflammatory (Formukong et al., 1988), and neuroprotective anti-oxidant properties (Hampson et al., 1998). Anti-depressant and anxiolytic effects on psychiatric conditions have also been observed (Ferber et al., 2020).

Despite its therapeutic potential, cannabis use has been associated with several adverse effects. Psychoactive side effects include impaired cognition, anxiety, paranoia or psychosis at high doses (Patel & Marwaha, 2025). Long-term use may increase the risk of developing cannabis use disorder (CUD), addiction and cannabinoid induced hyperemesis syndrome (CHS), characterised by cyclic vomiting episodes (Cue et al., 2025). Research shows that cannabinoids like THC can suppress immune function, leading to an increased vulnerability to infections (Maggirwar & Khalsa, 2021). Cardiovascular effects include elevated heart rate and a drop in blood pressure attributed to THC, which could be problematic for older adults or those at risk for heart disease (Dabiri & Kassab, 2021). CBD however, has been shown to attenuate cardiac dysfunction, oxidative stress and inflammation in diabetic cardiomyopathy. Studies also suggest that THC may inhibit hormone secretion, sperm development and embryo implantation, though these effects seem short-lived (Lo et al., 2022). Whole flower cannabis use, which is usually used in conjunction with tobacco, has also been linked to temporarily reducing fertility in both men and women and could potentially interfere with early pregnancy stages (Mack & Joy, 2000).

In contrast, CBD and other cannabinoid compounds have been shown to antagonise some of the undesirable effects of THC, including intoxication, sedation and tachycardia, while contributing to properties in their own right. Phytocannabinoids and essential oil terpenoids share a geranyl pyrophosphate precursor in the glandular trichomes of the plant. This structural similarity contributes to the entourage effect, which describes the synergistic

activities of whole-plant cannabis extracts over those of isolated compounds. they are thought to modulate each other's potency and broaden the therapeutic profile due to the variation in ratios and combinations of compounds (Ferber et al., 2020; Russo & Guy, 2006).

1.4 Cannabidiol (CBD)

CBD is a major non-psychoactive component of the *Cannabis sativa* plant, a characteristic that has contributed to its growing popularity in the commercial market for its potential therapeutic benefits (Blebea et al., 2024). It has attracted medical interest due to its many pharmacological actions. CBD is widely promoted for health benefits, it is know to be neuroprotective (Hampson et al., 1998; Patricio et al., 2020), anti-epileptic (Karler et al., 1982; Wallace et al., 2003), anxiolytic (Blessing et al., 2015; Papagianni & Stevenson, 2019; Sharpe et al., 2020), anti-psychotic (Leweke & Koethe, 2008), anti-inflammatory, analgesic (Atalay et al., 2019; Formukong et al., 1988) and have anti-cancer properties (Velasco et al., 2016; J. Wang et al., 2019). It is also being commercially popularised for chronic pain relief as a natural alternative (Villanueva et al., 2022).

The mechanism of action of CBD remains incompletely characterised with over 70 molecular targets identified and ongoing debate about which are clinically relevant (Peng et al., 2022). Although it is generally well-tolerated, CBD has also been associated with dose-dependent adverse effects. Due to limited safety data, there are concerns about serious side effects, including liver toxicity. With some evidence suggesting that CBD available in the commercial market is expensive, ineffective and potentially harmful in pain management (Amann et al., 2022; Moore et al., 2024). This emphasises the importance of not only investigating the pharmacology of CBD and other cannabinoids but also educating the population on commercially popularised compounds.

1.4.1 Pharmacodynamics/ Mechanism of Action

The pharmacology of CBD is complex as it interacts with multiple physiological systems. It has a multifaceted mechanism of action, interacting with various receptors and exerting its effects on over 70 pathways (de Almeida & Devi, 2020; Martinez Naya et al., 2023). Some of the major pathways influenced by CBD are included in Table 1.

Table 1: CBD's main sites of action across different physiological systems.

System	ECS		Serotonergic System
Molecular Targets	CB_1 and CB_2 receptors	AEA modulation	serotonin 1A receptor (5HT1A)
Main Effect	CBD has a low affinity for cannabinoid receptors as it acts as a negative allosteric modulator, it is thought to reduce the adverse effects associated with THC and other orthosteric antagonism. This explains its lack of psychoactive effects at CB_1 and anti-inflammatory effects at CB_2	CBD inhibits the breakdown of anandamide, by inhibiting FAAH. This leads to increased levels of anandamide in the brain, which may contribute to its anxiolytic and antidepressant effects	CBD acts as an agonist at 5-HT1A which plays a crucial role in regulating mood and anxiety. This interaction is thought to underlie CBD's potential anxiolytic and antidepressant effects
Source	(Laprairie et al., 2015; Martinez Naya et al., 2023)	(Blessing et al., 2015; Papagianni & Stevenson, 2019)	(Blessing et al., 2015)

System	Transient Receptor Potential (TRP) Channels	GABAergic Signalling	Purinergic Signalling
Molecular Targets	TRPV1	GABA Receptors	Adenosine (A2A) Receptors
Main Effect	CBD acts as a weak agonist at TRPV1 and modulates the regulation of pain perception, inflammation and body temperature. This activation contributes to its potential analgesic and anti-inflammatory properties	CBD has been shown to enhance GABAergic transmission. GABA is the primary inhibitory neurotransmitter in the CNS, and its increased activity can reduce neuronal excitability and anxiety	CBD indirectly influences adenosine receptors, which are involved in regulating cardiovascular function, inflammation and neuroprotection
Source	(Martinez Naya et al., 2023).	(Cifelli et al., 2020).	(Martinez Naya et al., 2023)

1.4.2 Pharmacokinetics/ Metabolism

CBD can be administered in various forms, including oral, sublingual, inhalation and topical. The bioavailability (fraction of an administered dose that reaches systemic circulation) of CBD varies significantly depending on the route of administration. For instance, administration of CBD via the oral route is common and convenient for patients (Hossain et al., 2023). However, as CBD is lipophilic, it has low absorption; therefore, what is absorbed is subject to extensive first-pass metabolism, resulting in low bioavailability of approximately 9-13% (Millar et al., 2020), whereas inhalation provides more rapid and effective absorption. Data from artificial membrane assays have shown that CBD is a skin-permeable cannabinoid and is suitable for topical use (Kirk et al., 2022)

Once absorbed, CBD is widely distributed throughout the body, with a high affinity for fatty tissues. It crosses the blood-brain barrier, allowing it to affect the central nervous system (Huestis, 2005). CBD, when administered orally, is primarily metabolised in the liver by the cytochrome P450 enzyme system, particularly CYP3A4 and CYP2C19. It is converted into various metabolites, some of which may have biological activity, such as 7-hydroxycannabidiol (7-OH-CBD). It is then further converted by CYP enzymes into the inactive metabolite 7-COOH-CBD as shown in Figure 6 (Beers et al., 2021; Smith & Gruber, 2023). CBD and its metabolites are excreted primarily through faeces and, to a lesser extent, in urine. The half-life of CBD varies depending on the mode of administration and individual factors, ranging from 18 - 32 hours in humans after chronic oral administration (Chayasirisobhon, 2020; Meissner & Cascella, 2024). CBD is generally well tolerated but may cause mild side effects like fatigue or gastrointestinal discomfort and at high doses can inhibit cytochrome P450 enzymes, potentially altering the metabolism of other drugs (Doohan et al., 2021).

1.5 7-hydroxy-cannabidiol (7-OH-CBD)

7-hydroxy-cannabidiol (7-OH-CBD) is generated by the metabolism of orally administered CBD via the actions of enzymes such as CYP2C19 in the liver (Tchilibon & Mechoulam, 2000). Figure 6 depicts phase 1 metabolism, where enzymes modify CBD to make the compound more water soluble. Phase 2 is where in inactive 7-COOH-CBD is converted into a water-soluble glucuronide by UGT enzymes so it can be excreted in urine and faeces (Caicedo et al., 2025). After oral administration, 7-OH-CBD appears in plasma at concentrations approximately half those of CBD itself, with notable inter-individual variability influenced by sex and body weight, with higher exposure levels observed in females (Zhang et al., 2024).

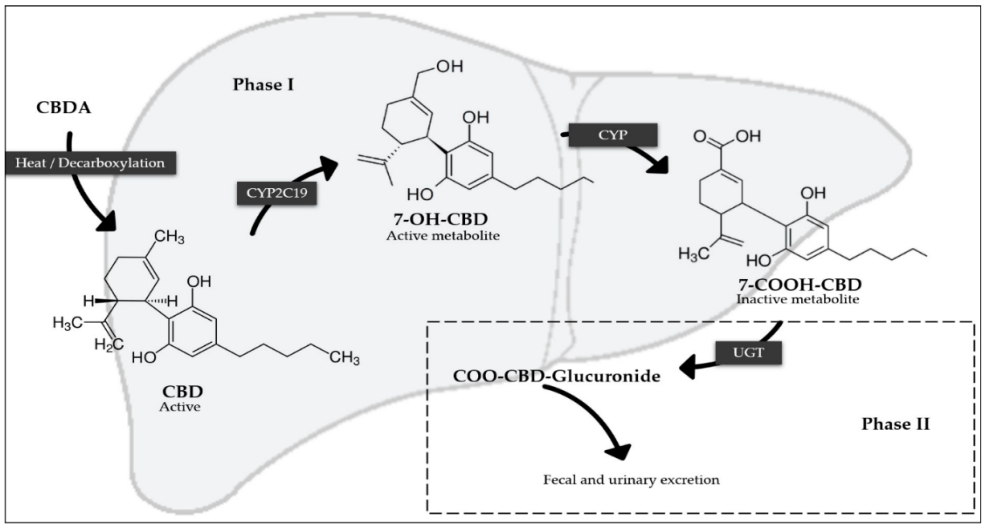

Figure 6 : Metabolism of CBD, taken from Caicedo et al., (2025)

7-OH-CBD exhibits pharmacological effects similar to those of its parent compound CBD. However, the pharmacological activity of the compound is widely debated as some studies consider it to be the active metabolite (Caicedo et al., 2025), while others have reported it to have reduced (Nye et al., 1985), increased (Stott et al., 2015) or equipotent effects to the parent compound CBD (Beers et al., 2023). In mouse models, 7-OH-CBD has been shown to produce significant anti-convulsant effects similar to those of CBD, while its further metabolite 7-COOH-CBD does not share this property (Whalley et al., 2017). However, both 7-OH-CBD and 7-COOH-CBD have demonstrated the inhibition of nitric oxide (NO), reactive oxygen species (ROS) and tumour necrosis factor-alpha (TNF-α) production, suggesting anti-inflammatory potential (Patricio et al., 2020; Ujváry & Hanuš, 2016). Similar to CBD, 7-OH-CBD has been found to inhibit fatty acid amide hydrolase (FAAH), which can influence endocannabinoid signalling (Ujváry & Hanuš, 2016).

1.6 Abnormal-Cannabidiol (Abn-CBD)

Abn-CBD is a synthetic regioisomer of CBD, as depicted in Figure 7. Despite its structural similarity to CBD, Abn-CBD does not induce psychoactive or sedative effects (Adams et al., 1977). Limited data is available on the pharmacokinetic profile of Abn-CBD; however, it is considered to be lipophilic, akin to other cannabinoids and likely undergoes hepatic cytochrome P450-mediated metabolism (Silvaroli et al., 2019).

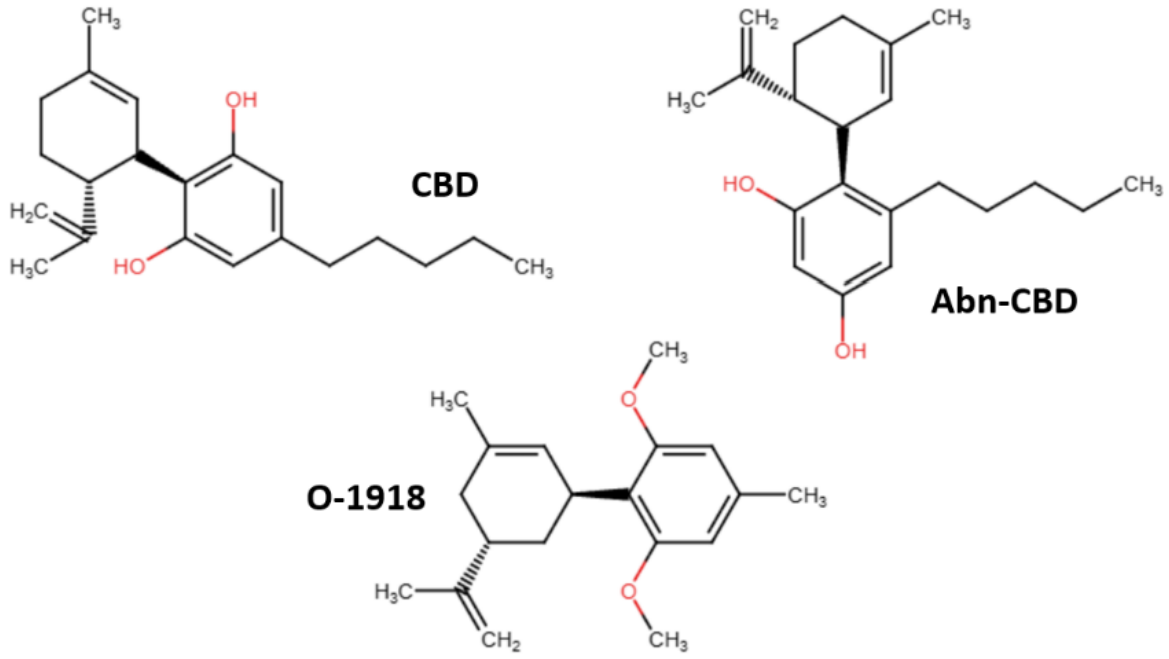

Figure 7: Chemical Structure of CBD, Abn-CBD and O-1918, red labels highlight the oxygen-containing functional groups that act as active sites and undergo changes during metabolism.

Mechanistically, Abn-CBD lacks affinity for $CB_{1/2}$ receptors. Instead, it exerts its effects as a selective agonist of G-protein coupled receptor 55 (GPR55), which is implicated in various physiological processes, including insulin regulation and inflammation (McCloskey et al., 2023). Some actions of Abn-CBD are also mediated through G-protein coupled receptor 18 (GPR18), found primarily in vascular tissues and microglia and is thought to activate MAPK pathways. (McHugh et al., 2010). Abn-CBD produces vasodilator effects through endothelium-dependent mechanisms, explaining its ability to lower blood pressure without neurobehavioral effects (Járai et al., 1999).

Abn-CBD has also been found to exert beneficial immunomodulatory effects in key metabolic tissues in diabetic mouse models (Romero-Zerbo et al., 2020). In addition, Abn-CBD may help regulate microglial activity in neurodegenerative diseases by preventing the accumulation of

misdirected, pro-inflammatory microglia, which contribute to and exacerbate neurodegeneration (McHugh et al., 2010).

Collectively, these properties highlight the therapeutic potential of Abn-CBD for metabolic syndromes, diabetes, inflammatory and vascular disorders. However, further research is required to fully understand its properties and explore therapeutic applications.

1.7 O-1918

O-1918 is a synthetic CBD analogue compound (Figure 7). It is used as a tool for studying endothelial cannabinoid receptors and works as an antagonist at GPR55 and an antagonist or biased agonist at GPR18, which are also receptors engaged by Abn-CBD (Offertáler et al., 2003). O-1918 antagonises vasorelaxant and cell migration effects induced by Abn-CBD and endocannabinoids such as AEA at receptors pharmacologically distinct from $CB_{1/2}$ (Járai et al., 1999). GPR55 and GPR18 are implicated in non-classical cannabinoid signalling in endothelial cells, immune cells, kidney and brain (Silvaroli et al., 2019; Simcocks et al., 2019). O-1918 has been studied for its potential wound healing and bone regeneration effects by increasing cell migration via the MAPK pathway (Simcocks et al., 2019).

The pharmacokinetic data available for O-1918 are limited and its metabolic pathway is not yet characterised in humans or animals. However, based on chemical structure and experimental use *in vivo*, it is speculated to possess lipophilic properties and undergo hepatic cytochrome P450-mediated metabolism akin to other CBD analogues (Simcocks et al., 2020). Further research is required to assess the safety profile and the translational potential of O-1918 beyond preclinical models.

1.8 Therapeutic Potential and Safety

Cannabinoids have shown promise in various therapeutic areas; however, more research is required to fully understand the safety, potential adverse effects and optimise their safety across different medical conditions. Table 2 summarises current findings on the therapeutic and safety profiles of various cannabinoids across multiple conditions.

Table 2: Current findings on the therapeutic and safety profiles of various cannabinoids used in the treatment of multiple medical conditions, along with references.

Condition	Neurodegeneration (glutamate-induced excitotoxicity)	Alzheimer's Disease (AD)	Psychiatric Disorders (anxiety, depression)	PTSD-related symptoms
Cannabinoid Investigated	Endocannabinoids (anandamide)	Targeting oxidative stress and neuro-inflammation	CBD	THC
Effect	Protects neurones from glutamate-induced excitotoxicity by accumulating in immature neurones post-injury as a potential neuroprotective response. An *in vivo* study using neonatal rat models of neurodegeneration due to glutaminergic dysfunction showed that mild to moderate brain injury enhanced endocannabinoid activity via anandamide and CB_1 receptor upregulation.	Potential to prevent inflammation, neuronal dysfunction and cell loss; slows progression.	Anxiolytic effects via modulation of amygdala activity and its connection to the prefrontal cortex.	Temporary relief from symptoms, but can exacerbate anxiety at higher doses.
Study	(Hansen et al., 2001)	(Abubakar et al., 2022; Tiwari et al., 2019)(Pizzinoet al., 2017)(Van Eldik et al., 2016)	(Blessing et al., 2015b; Papagianni & Stevenson, 2019)	(Sharpe et al., 2020)

Condition	Oncology	Autoimmune/ Inflammatory conditions (rheumatoid arthritis, type 1 diabetes, multiple sclerosis and Crohn's disease)	Cardiovascular Health
Cannabinoid Investigated	Cannabinoids (general), THC	Cannabinoids (general)	CBD and THC
Effect	Anti-tumour effects by inducing apoptosis and inhibiting angiogenesis; chemotherapy-induced nausea and vomiting; and enhancing treatment efficacy. THC for increasing appetite.	Reduces inflammation by inhibiting cytokine release from immune cells.	THC increases heart rate and may lower blood pressure acutely; CBD has cardioprotective effects through vasodilation and reduction of oxidative stress.
Study	(Velasco et al., 2016)(J. Wang et al., 2019).	(Katchan et al., 2016)	(Dabiri & Kassab, 2021; Rajesh et al., 2010)

1.9 Animal *in vivo* Models

Animal research plays a crucial role in understanding complex biological mechanisms and disease processes, allowing for controlled experimentation that is not possible in humans. Animal models enable researchers to explore potential treatments and underlying molecular pathways.

Rodent models such as *Rattus rattus* and *M. musculus* are widely used in *in vivo* pharmacology research as they mimic human physiological and biochemical responses. However, the use of different *in vivo* models allows researchers to explore pathways not expressed or affected in other model organisms. *Danio rerio* (zebrafish) model used for fin regeneration and wound healing showed that exposure to CBD supported wound healing and accelerated fin regeneration by modulating inflammation and reducing apoptosis without affecting the number of mitotic regenerating cells. However, this effect was dose-dependent and higher concentrations did not produce these effects (Xu et al., 2021).

A study using a *Rattus norvegicus* model for insulin resistance found that CBD enhances insulin sensitivity under lipid overload by modulating sphingolipid deposition by inhibiting ceramide synthesis and impairing catabolic pathways (Konstantynowicz-Nowicka et al., 2025). This highlights a previously unexplored pathway of hepatic insulin resistance. Another study using a *Mus musculus* model investigated regeneration preceding sciatic nerve injury. They found that the ECS promotes axon regeneration via CB_1R and PI3K-PKB/Akt pathway activation, confirming that the ECS acts as an intrinsic modulator of regeneration (Martinez-Torres et al., 2023). Although these studies using animal models do not yet contribute to any treatment solutions, they clarify possible pathological pathways. These pathways may be evolutionary conserved or differ in informative ways across species and can point to new possible targets for treatments.

1.9.1 Invertebrate *in vivo* Models

In the UK, the Animals (Scientific Procedures) Act 1986 (ASPA) regulates the use of animals in scientific research to ensure welfare and ethical standards. ASPA primarily applies to vertebrates, which encourages the use of invertebrates as alternative models. This encourages ethical values while also providing valuable insights into fundamental biological processes,

while reducing reliance on higher-order organisms. Some commonly used invertebrate models include *Drosophila melanogaster* and *Caenorhabditis elegans.*

D. melanogaster was used as an *in vivo* model to investigate the neuroprotective effects of CBD and THC. Neither compound significantly affected sleep, circadian behaviours or age-related motor decline; however, a two-week CBD (3 µM) treatment enhanced lifespan. When used as a mild traumatic brain injury model, *D. melanogaster* exposed to CBD (3 µM) showed a decrease in 48-hour post-injury mortality rate and improved overall longevity. Study showed that CBD and THC at the doses examined had a modest impact on basal neural function, while CBD demonstrated significant neural protective properties (Candib et al., 2024). Cannabinoids were also shown to suppress food intake; interestingly, CB_1 receptor agonist AM251 mitigated this effect. AEA was shown not only to reduce food intake but also to enhance starvation resistance by modulating lipid metabolism. Findings suggest a cannabinoid receptor-independent mechanism influencing feeding behaviour in *D. melanogaster* (He et al., 2021).

CBD exposure in *C. elegans* resulted in mild hypoactivity. Whole-life exposure to (10-100 µM) CBD extended the lifespan of *C. elegans* by up to 18% and increased late-stage motility by 206% without acute toxicity (Land et al., 2021). Although *C. elegans* lacks CB_1 and CB_2 receptors, it possesses functional orthologs of human cannabinoid receptors, such as Natriuretic peptide receptors (NPR). NPR-1, which exerts its effects on endocannabinoid-like signalling pathways (van Es-Remers et al., 2022). Functional orthologs of the mammalian GPR18 and GPR55 are considered to be NPR-32 within invertebrates. Within *C. elegans*, NPR-32 is thought to regulate regenerative axon navigation and activate monoaminergic signalling cascades in nociception, feeding, development and ageing (Clarke et al., 2021). Axon regeneration was found to be promoted by the JNK and MAP kinase pathways. Studies have found that AEA inhibits axon regeneration by suppressing these pathways acting via NPR-19 and NPR-32 (Pastuhov et al., 2016). These pathways require further investigation in alternative *in vivo* models for further confirmation of their translational potential. This can be demonstrated within *L. variegatus*, an annelid capable of regeneration and an ecological indicator species also used within pharmacology research (Seeley et al., 2021).

1.10 *Lumbriculus variegatus*

L. variegatus, commonly known as the California Blackworm, is a freshwater aquatic annelid found in ponds and marshes across North America and Europe and has been used extensively as an ecological indicator species. They detect food with their anterior end, feeding on decaying vegetation and microorganisms, and they use the posterior end to perform gas exchange. *L. variegatus* grow between 1-10 cm and reproduces both asexually and sexually. Additionally, they exhibit regenerative abilities, restoring lost body parts through epimorphosis and neural morphallaxis (Cook, 1969; Martinez Acosta et al., 2021; Seeley et al., 2021). Due to their invertebrate status, they are not subject to regulation under the Animals (Scientific Procedures) Act of 1986. As such, scientific work involving *L. variegatus* does not require the same legal oversight or licensing as procedures involving vertebrate animals.

Seeley et al. (2021) introduced *L. variegatus* as an invertebrate model for *in vivo* pharmacology education, addressing the decline in animal use in teaching due to regulatory restrictions imposed by the Animal Scientific Procedures Act of 1986. Two novel behavioural assays were developed, one measuring stereotypical movements after tactile stimulation and another assessing unstimulated locomotion. Using these assays, the effects of various drugs can be evaluated. *L. variegatus* can be used as a cost-effective, ethical alternative for pharmacology education without regulatory constraints (Seeley et al., 2021).

1.10.1 Behaviour

L. variegatus exhibits anterior-posterior differentiation in behaviour, particularly in response to potential predatory threats, as shown in Figure 8. When the posterior part of the worm is stimulated, it withdraws its tail, while stimulation of the anterior part results in head withdrawal or a reversal of the movement. These reflexes are essential for survival and allow for toxicological testing using behavioural assays (Drewes, 1984). Their primary mode of reproduction is regenerating segments into a new individual when separated from the rest of the animal. In most populations, this is their primary mode of reproduction. *L. variegatus* have also been observed to form tangled balls of several individuals to conserve heat and moisture and prevent desiccation (Tuazon et al., 2022)

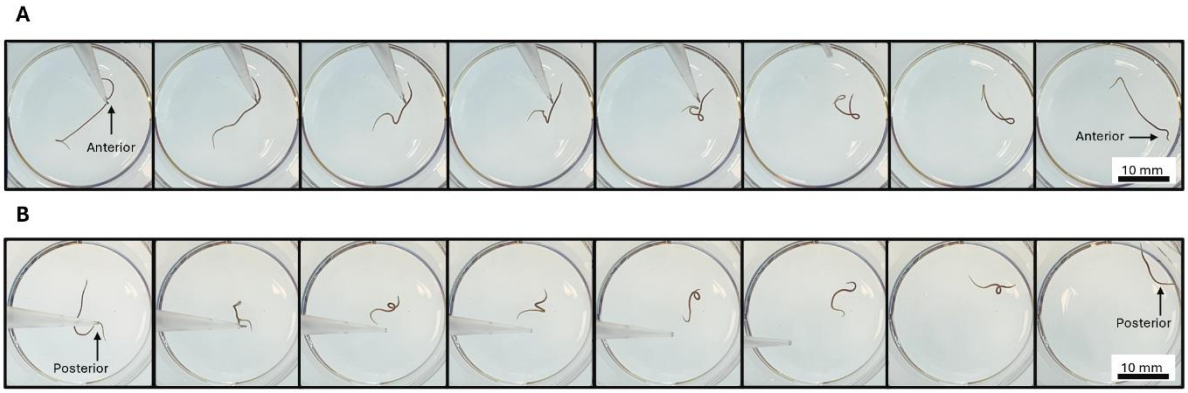

Figure 8: Stereotypical movements of *L. variegatus* following tactile stimulation. Images show the stereotypical movement **(A)** body reversal, where the head and tail positions are reversed as *L. variegatus* bends around, and **(B)** helical swimming, characterised by helical body bends, of *L. variegatus* following tactile stimulation with a pipette tip. Images show the stereotypical movements over the 2 – 3 seconds following stimulation. Taken from Seeley et al., (2025)

1.10.2 Anatomy

L. variegatus undergoes peristaltic movements, where its body stretches and shortens along the posterior-anterior axis. The central nervous system (CNS) of *L. variegatus* consists of a cerebral ganglion (brain) connected to a ventral nerve cord (VNC) through circumesophageal connectives (Martinez Acosta et al., 2021). The VNC runs through the length of the worm, giving rise to segmental nerves that control sensory and motor functions in each segment, except the first two (Hessling & Westheide, 1999). The VNC contains various neurons (sensory, motor, interneurons) and the neuropil, which integrates synaptic events to control behaviour (Purschke, 2015). *L. variegatus* possesses three giant nerve fibres (one medial and two lateral) in the VNC, which are essential for the rapid escape reflex. These giant fibres are segmented, connected by gap junctions and ensheathed in myelin. The medial giant fibre (MGF) controls head withdrawal, while the lateral giant fibres (LGF) control tail withdrawal (Martinez Acosta et al., 2021).

1.10.3 Regeneration and reproduction

Regeneration is the ability to restore and regrow body parts lost to injury. Although most animals demonstrate the capacity for wound healing, the ability to initiate a developmental process leading to partial or complete replacement of a lost structure varies widely among animal taxa, with more complex organisms showing lower regenerative abilities and varying through developmental stages and anatomical locations within a species (Bely & Nyberg, 2010; Zhao et al., 2016).

The nervous system plays a prominent role in the animal regenerative process (Kumar & Brockes, 2012). In *L. variegatus* and many other invertebrates, the presence of the VNC is necessary for regeneration; injuries to the VNC can induce limb regeneration known as epimorphosis (Boilly et al., 2017). Regenerating worm fragments show rapid recovery of nervous system function, with observable neuronal activity within 24 hours post-amputation (HPA). The fragments undergo morphallaxis, where existing tissues transform to match the fragment's new positional identity along the body axis. This process is particularly evident in the nervous system, where the MGF and LGF pathways adapt quickly to the fragment's new role (Lybrand & Zoran, 2012).

L. variegatus are important models in regenerative research due to its ability to regrow an entire body from a small fragment; they have demonstrated the ability to do this even under nutrient deprivation (Martinez Acosta et al., 2021). Therefore, *L. variegatus* worms were among the first annelids studied for regeneration by Bonnet in 1745, demonstrating that each of 16 worm fragments could regenerate a complete worm. Further research into regeneration by Bülow (1883) and Harriet Randolph (1892) explored the origin of cells in regenerated organs, introducing the concepts of neoblasts, which are somatic adult stem cells that are abundant in planarians and crucial for regeneration. However, the role played by these migrating neoblasts in *L. variegatus* and other clitellates remains unclear.

L. variegatus reproduces asexually by fragmentation through a process called architomy (Figure 9 B). In this process, the worm spontaneously breaks into two or more body pieces at predetermined fission zones, and each fragment then regenerates the missing anterior or posterior segments to form a complete, genetically identical individual.

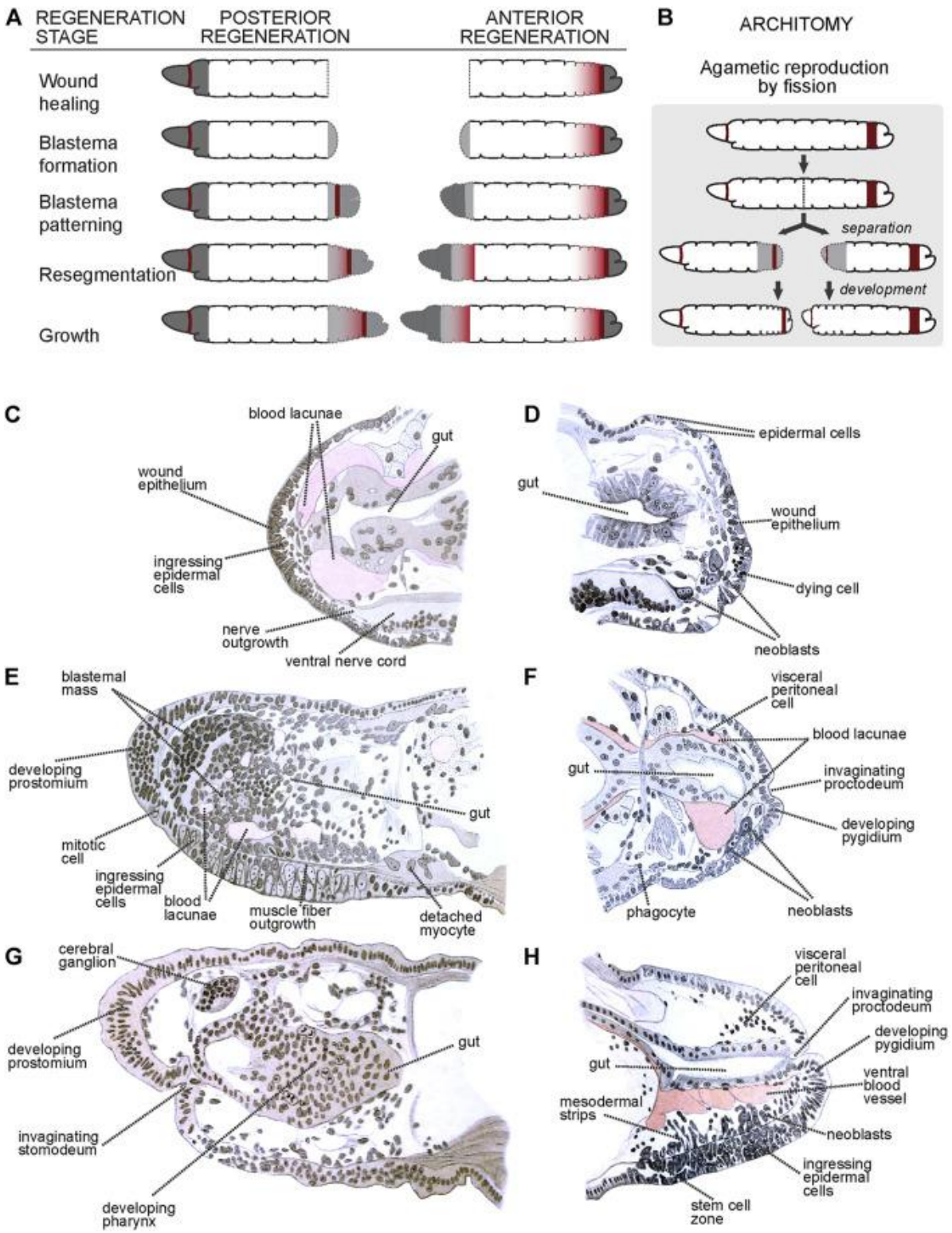

Figure 9: Regeneration and asexual reproduction in *L. variegatus*, (A) Generic stages of annelid regeneration. Dashed line: cut/regenerated tissue; dark grey: non-segmental tissues; dark red: mitotically active areas; grey shading: differentiating segmental tissues. (B) Asexual reproduction by fission. Colouring as in A. (C–H) Histological sections through early (C), middle (E), and late (G) anteriorly regenerating individuals, and early (D), middle (F) and late (H) posteriorly regenerating individuals. (C–H) After Iwanow (1903); all labels are direct or interpreted translations of the original German labels, taken from Martinez Acosta et al., (2021).

There are notable differences between head and tail regeneration and how various factors affect regenerative outcomes. Regeneration of both anterior (head) and posterior (tail) is described in 5 stages (Figure 9 A). Starting with wound healing, immediately after amputation, circular muscles contract to close the exposed coelomic cavity (Bely, 2014). Epithelial cells extend to seal the wound. Mitotic activity is reduced and damaged cells are cleared by phagocytes (Iwanow, 1903), while blood lacunae fill the interstitial spaces. (Figure 9 C,D). Blastema formation is the next step, where nerves invade the wound site (Figure 9 C), cell proliferation is upregulated. Proliferating cells form a mass of undifferentiated cells known as the blastema (Figure 9 E)(Iwanow, 1903). Neoblasts migrate to the wound site (Figure 9 D, F, H). After which, the blastema undergoes differentiation into the anterior (head) (Figure 9 E, G) and the posterior (tail) (Figure 9 F and H). In anterior regeneration, structures such as the cerebral ganglion, stomodeum and pharynx develop (Figure 9 G). In posterior regeneration, a new posterior growth zone and VNC ganglia develop (Iwanow, 1903; Martinez Acosta et al., 2021). The blastema then organises into clusters of dorsal, lateral and ventral cells. Dorsal and lateral clusters develop into chaetal sacs and nerve cord ganglia. New segments begin to form, known as resegmentation (Martinez Acosta et al., 2021). The regenerated structures complete their differentiation and grow in size, restoring the original proportions and full functionality of the organism (Martinez Acosta et al., 2021).

The role of ROS is a key contributor in orchestrating the regenerative process (Bideau et al., 2021). ROS are highly reactive products of oxygen metabolism. In animals, major sources of ROS are the mitochondrial electron transport chain and NADPH oxidases (NOX) (Palma, 2023). These partially reduced metabolites are detoxified by anti-oxidant enzymes to protect against oxidative damage. ROS are not purely toxic by-products of cellular metabolism but can act as key components of cellular signalling at relevant concentrations. ROS interact with signalling molecules critical for a variety of necessary cellular processes such as proliferation, survival and apoptosis (Bardaweel et al., 2018; Palma, 2023).

ROS production in response to injury is highly conserved in animals (Suzuki & Mittler, 2012). Accumulation of ROS at wound sites acts as a secondary messenger. Although regeneration closely follows wound healing, variations in the accumulation of ROS make these two mechanisms distinct (Owlarn et al., 2017). In *L. variegatus,* amputation resulted in ROS accumulating at the wound site within 15 minutes post-amputation; however, this diminished

in most worms by 6 HPA. Inhibition of this ROS burst impaired regeneration and decreased survival (Beinart & Gillen, 2024).

The mechanism for how ROS promote regeneration is still being uncovered, with many studies suggesting it exerts its effects by activating MAPK/ERK pathways that are required in regeneration (Pagano et al., 2023; Wang & Arnold, 2024; Zhang et al., 2022). Comparative studies of cellular and molecular processes in regeneration could reveal conserved genomic pathways, potentially advancing regenerative medicine (Martinez Acosta et al., 2021).

L. variegatus worms can reproduce asexually by fragmenting into two or more parts, which regenerate into complete individuals. They have the ability to do this in water or in the absence of water within desiccation-resistant cysts (Cook, 1969; Stephenson, 1924). Unlike injury-driven regeneration, fragmentation in *L. variegatus* is due to an autotomy reflex, where circular muscles contract at a specific location, leading to the worm splitting (Lesiuk & Drewes, 1999). The “breaking plane” is marked by an epidermal serotonin-immunoreactive ring. One study found that treatment with nicotine, a cholinergic agonist, blocked the autotomy reflex (Lesiuk & Drewes, 1999). Therefore, it is possible that the autotomy reflex might be controlled by acetylcholine-mediated activation of serotonergic neurons (Lesiuk & Drewes, 1999; Martinez Acosta et al., 2021; Zattara, 2012).

1.10.4 CBD’s effect on *L. variegatus*

Swansea Worm Integrative Research Laboratory (SWIRL) focuses on *L. variegatus as part* of a larger objective to study drugs of abuse. The effects of CBD and other cannabinoids and endocannabinoids were tested to explore the existence of an endocannabinoid system within the previously unexplored organism. Preliminary data collected within our laboratory have shown Gas Chromatography Mass Spectrometry (GC-MS) evidence of CBD present within *L. variegatus* succeeding exposure to ≥ 25 µM CBD, matching the established toxic threshold (Figure 22). Significant reduction in behavioural effects was also observed. Despite the limited conservation of classical cannabinoid receptors, *L. variegatus* shows sensitivity to CBD exposure, suggesting off-target effects or undiscovered pathways (Carriere et al., Unpublished). The research contributions presented within this thesis aim to improve our understanding of the toxicology and pharmacology of CBD and its analogues. To provide insight into the mechanism of action and ecological impact of cannabinoids.

1.11 Aims and objectives

This project aims to investigate and characterise the pharmacological effects of CBD and its structural analogues on the toxicological response, behavioural patterns and regenerative capacities of the novel *in vivo* model *L. variegatus*. Cannabinoid products have gained recent commercial popularity in the form of CBD products that claim to be anti-nociceptive, anti-inflammatory and anti-emetic, among other benefits (Zou & Kumar, 2018).

However, recent research suggests that CBD products available to the public do not contain clinically relevant concentrations of CBD to back up these claims and could potentially cause sub-toxic effects if not regulated (Moore et al., 2024). It highlights the importance of educating the population and raising awareness on the risks and side effects associated with the overconsumption of these compounds. Due to the lipophilic nature of cannabinoids, chronic administration of low doses can cause bioaccumulation and toxicity within certain populations; therefore, chronic exposure to sub-lethal doses is also investigated within this project. The study further seeks to develop preliminary mechanistic hypotheses regarding the invertebrate ECS and the molecular pathways influenced by cannabinoid exposure.

The specific objectives of the study are to:

- Determine concentrations of CBD, 7-OH-CBD, Abn-CBD and O-1918 that produce a toxic response in 50% of the tested population.
- Employ the use of behavioural assays to further test for toxicity by assessing the effects of tested drugs on stereotypical movement and free locomotion.
- Examine the effects of cannabinoid compounds on the regenerative abilities of *L. variegatus*.
- Investigate the effects of cannabinoid drug exposure on the biomass of *L. variegatus* using 28-day exposure.

During the toxicological testing of O-1918, we observed splitting within *L. variegatus* and hypothesised that O-1918 would encourage axon navigation akin to pathways previously identified in *C. elegans*.

1. Materials and Methods

2.1 General

All inorganic products used in this project were analytical grade. Drug solutions were prepared on the day of the experiments before initiating drug exposure.

2.2 Health and Safety

All waste materials were managed in compliance with the manufacturer's guidelines and all cannabinoid compounds were chemically disposed of due to environmental concerns obtained from studies conducted within SWIRL (Williams et al., 2025). Through analysis of all experimental procedures, formal risk assessments were carried out and documented in Control of Substances Hazardous to Health (COSHH) forms.

2.3 Ethical Guidelines

Treatment of the *in vivo* model, *L. variegatus*, throughout this project was conducted in accordance with the 3 R's of ethical guidelines for animal research. To minimise any known pain and suffering while maintaining high animal welfare standards by adhering to ethical principles such as replacement, reduction and refinement. The research minimises the use of higher-order animals; the number of worms needed for statistically significant research was carefully considered and procedures were refined to minimise any unnecessary stress and harm. The worms were kept in an environment that mimics their natural habitat (See section 2.5 for further details), with appropriate substrate and water conditions to promote natural behaviours and well-being. Due to their invertebrate classification, *L. variegatus* is not covered under the Animal (Scientific) Procedures Act of 1986 and therefore, the study does not require approval by an ethics committee.

2.4 Reagents and Solutions

Below is a list of reagents and solutions, where they were supplied from and how they were stored within the laboratory.

Reagent	Supplier	Storage
Abn-CBD	Tocris (#1297)	-20°C
O-1918	Tocris (#2288)	-20°C
Methyl Acetate	Sigma- Aldrich	Room temperature
(-) Cannabidiol	Tocris (#1570)	-20°C
Dimethyl sulfoxide (DMSO)	Sigma- Aldrich	Room temperature
Calcium nitrate tetrahydrate	Duchefa Biochemie	Room temperature
HEPES	Melford Laboratories	Room temperature
Magnesium sulphate heptahydrate	Duchefa Biochemie	Room temperature
Potassium chloride	Melford Laboratories	Room temperature
Sodium chloride	Melford Laboratories	Room temperature
Ethanol	Fisher Chemical	Room temperature
7- hydroxy- cannabidiol	Merck (#C-180)	-20°C
Methanol (MeOH)	Fisher Chemical	Room temperature

Table 3: Reagents and solutions inventory.

2.5 Maintaining *Lumbriculus variegatus* Cultures

Cultures were established using specimens sourced from ALFA Fish Foods and cultivated in laboratory aquariums filled with a custom artificial pond water mixture. The artificial pond water recipe consisted of the following components: 1mM NaCl, 13µM KCl, 4µM $Ca(NO_3)•4H_2O$, 17µM $Mg(SO_4)• 7H_2O$; 71µM HEPES buffer (O'Gara et al., 2004). The aquarium was maintained at room temperature and subject to a 16-hour light and 8-hour dark cycle. To maintain water quality, the APW in the aquariums are consistently filtered and aerated using air stones. The cultures were sustained on TetraMin flakes, and a concentration of 10 mg/L of spirulina was replaced weekly. Prior to conducting any experiments, cultures were kept for a minimum of 3 months. Individual worms chosen for experimentation were randomly selected and displayed no noticeable morphological abnormalities. *L. variegatus* specimens were transferred from the aquarium to plates containing APW approximately 18-24 hours before the commencement of any experiments.

L. variegatus were aspirated following assay endpoints and euthanised by exposure to 70% ethanol and then incubated for 24 hours before disposal.

2.6 Storage and Preparation of Drugs and Solutions

All drugs were dissolved in artificial pond water (APW) with a vehicle of either methanol, methyl acetate or DMSO in order to ensure the drugs solubility in APW. All drugs were stored at -20°C and aliquoted to prevent freeze-thaw cycles.

2.6.1 CBD

CBD was supplied as a powder. A 5 mM master stock was prepared by diluting in 100% DMSO. Prior to drug exposure, CBD was dissolved in corresponding volumes of undiluted DMSO and APW to achieve a concentration of 0.5% DMSO and 0-25 µM CBD

2.6.2 7-OH-CBD

7-OH-CBD was supplied as a 1 mg/ml pre-dissolved solution in MeOH, giving a 3 mM master stock solution. Prior to drug exposure, 7-OH-CBD was dissolved in corresponding volumes of 100% MeOH and APW to achieve a final concentration of 0.5% of MeOH and 0-15 µM 7-OH-CBD.

2.6.3 Abn-CBD

Abn-CBD was supplied pre-dissolved in methyl acetate as a 15.9 mM solution, which was diluted to a 15 mM stock solution with 100% methyl acetate. Prior to drug exposure, Abn-CBD was dissolved in corresponding volumes of 100% methyl acetate and APW to achieve 0.1% Methyl acetate and 0-15 µM Abn-CBD.

2.6.4 O-1918

O-1918 was supplied as a 10 mM master stock solution which was dissolved and diluted in corresponding volumes of 100% DMSO and APW to give a final concentration of 0.5% DMSO and 50 µM of O-1918.

2.7 Establishing the Toxicity of Compounds

In vivo Toxicological Assays (IVTA) were employed to establish the dose of a compound that produces a toxic response in half the population; this was used to determine the Lowest Observed Adverse Effect Level (LOAEL).

The experimental design consisted of 18 *L. variegatus* worms collected in a 24-well plate with one worm in each well and left to acclimatise for 18-24 hours before the experiment. On the day of the experiment, CBD (0-25 µM), 7-OH-CBD (0-15 µM) or O-1918 (0-50 µM) were made by diluting with APW to give 0.5% DMSO in CBD and O-1918 solutions and 0.5% MeOH in 7-OH-CBD solutions and their vehicle controls, respectively.

APW and any debris were replaced with 1 ml of drug solution and *L. variegatus* was left for 24 hours in the drug solution. The number of worms that displayed signs of toxicity, such as pallor and decomposition (O'Gara et al., 2004) was recorded, and 6 experimental repeats were conducted with 3 technical repeats. The technical repeats were conducted for repeat measurements on the same sample to assess assay variability, whereas an experimental repeat is repeating the whole experiment.

2.8 Behavioural assays

The novel *in vivo* model, *L. variegatus*, exhibits certain behavioural characteristics that can be quantified and allows for toxicological testing and for objective comparison of drug effects, as previously demonstrated (Seeley et al.,2021). *L. variegatus* worms were collected from the aquarium and transferred to 6-well plates containing APW 18-24 hours preceding the experiments to allow for acclimatisation.

Behavioural assays were performed at four timepoints:

1. Baseline (before drug exposure)
2. Drug exposure (10 minutes for acute/ 24 hours for chronic)
3. 10 minutes after recovery (i.e. removal from drug solutions)
4. 24 hours after recovery from drug solutions.

All experiments were conducted in equimolar concentrations of CBD, 7-OH-CBD, Abn-CBD and O-1918

2.8.1 Stereotypical Movement

The stereotypical movement assays assess the ability of *L. variegatus* to perform stimulated movement. The impact of drug exposure is evaluated by eliciting stereotypical behaviour such as body reversal and helical swimming as depicted in Figures 10 and 11. This is done by stimulating the worm with a 20-200 µL pipette tip five times, alternating between the anterior and posterior ends, allowing for 5-10 second intervals between stimulations. Movement was recorded using a scoring sheet (Table 4).

The observed stereotypical movements are documented using a three-point scoring system where movement is graded as; 1 = no movement, 2 = some movement, 3 = complete stereotypical movement.

Baseline behaviour was recorded in APW on the day of the experiment. To initiate drug exposure, APW was removed and replaced with drug solutions of varying concentrations, ensuring worms were not left out of aqueous solutions for more than 30 seconds to minimise malaise. Stereotypical movement was measured after 10 minutes in drug solutions to assess acute drug exposure and 24 hours in drug solutions to evaluate chronic exposure. Following drug exposure, the recovery stage involved rinsing the worms with APW once to remove any residual drug traces and then adding fresh 4 ml APW. Stereotypical movement was recorded 10 minutes and 24 hours after recovery from the drug solutions. Methods were adapted from Seeley et al. (2021).

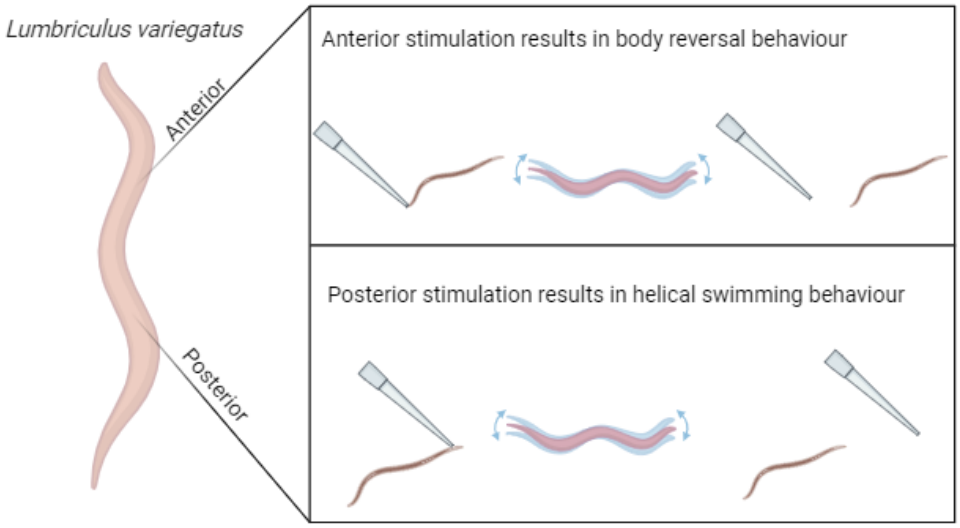

Figure 10 : Stereotypical movement of *L. variegatus*. Stimulation of the anterior using a pipette tip resulted in body reversal behaviour and stimulation of the posterior resulted in helical swimming behaviour. The purpose of exhibited behaviour is to move away from stimulus. Created using BioRender.com

2.8.2 Free locomotion

The free locomotion assay serves to assess the impact of drug exposure on the spontaneous unstimulated movement of *L. variegatus* within a 6-well plate.

On the day of the experiment, APW is replaced with 2 ml of APW to limit any vertical movement, as images taken to quantify movement were 2D. The free locomotion is also documented at 4 different time points, mirroring the Stereotypical movement assay.

For recording movement 6-well plates were placed onto a light stage, sequential images were captured using a 13MP camera programmed to capture one image per second for a duration of 50 seconds. These images are analysed using ImageJ 1.53t software (Figure 11), where a Z-stack is created to superimpose all 50 images in (Figure 11 B) and a scale is set. The total area covered by the worm is isolated by selection (Figure 11 C) and adjusting the threshold to cover the worm (Figure 11 D). The software function 'analyse particles' is used to quantify the surface area covered by the worm.

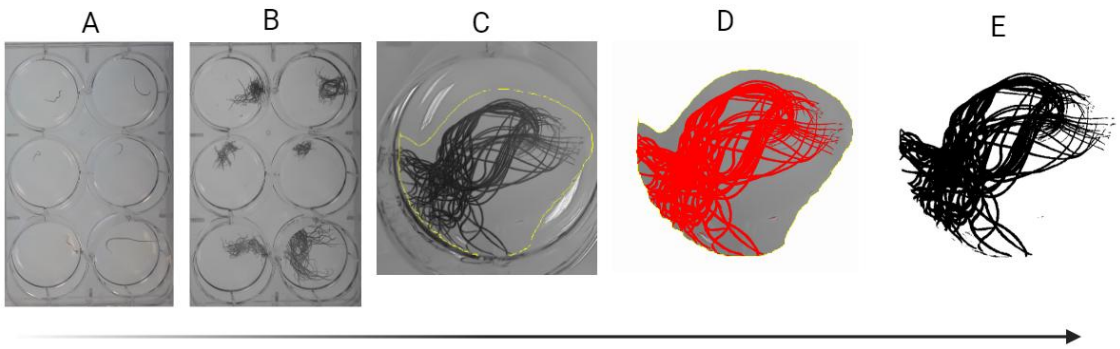

Figure 11: Calculating free locomotion using Image J. (A) 50 sequential images are taken over 50 seconds and uploaded to Image J (B) All images are superimposed and the scale is set. (C) area covered by the worm is selected. (D) Adjust threshold function is used to accurately select area covered by worm. (E) Analyse particle function is used to calculate the surface area covered by the worm.

2.9 Regeneration assays

This assay aims to observe the impact of cannabinoid compounds and their effects on the regenerative capabilities of *L. variegatus*. This experiment was conducted in triplicate, three worms were selected per concentration, in a six-well plate and left to acclimatise for 18-24 hours (Figure 12 A). On the day of the experiment, worms were dissected in half using scissors, their anterior and posterior ends were placed in separate wells (Figure 12 B). These ends were then exposed to 0 µM to 5 µM of CBD, 7-OH-CBD and O-1918 (Figure 12 C). The surface area of the blastema was measured and recorded using a Nikon® SMZ1270i stereomicroscope at 0 hours post-amputation (HPA), 24 HPA, 48 HPA and 72 HPA (Figure 12 D), measurements were taken at timepoints outlined by Tellez-Garcia et al (2021). Growth was converted to a fold change relative to measurements at 0 HPA.

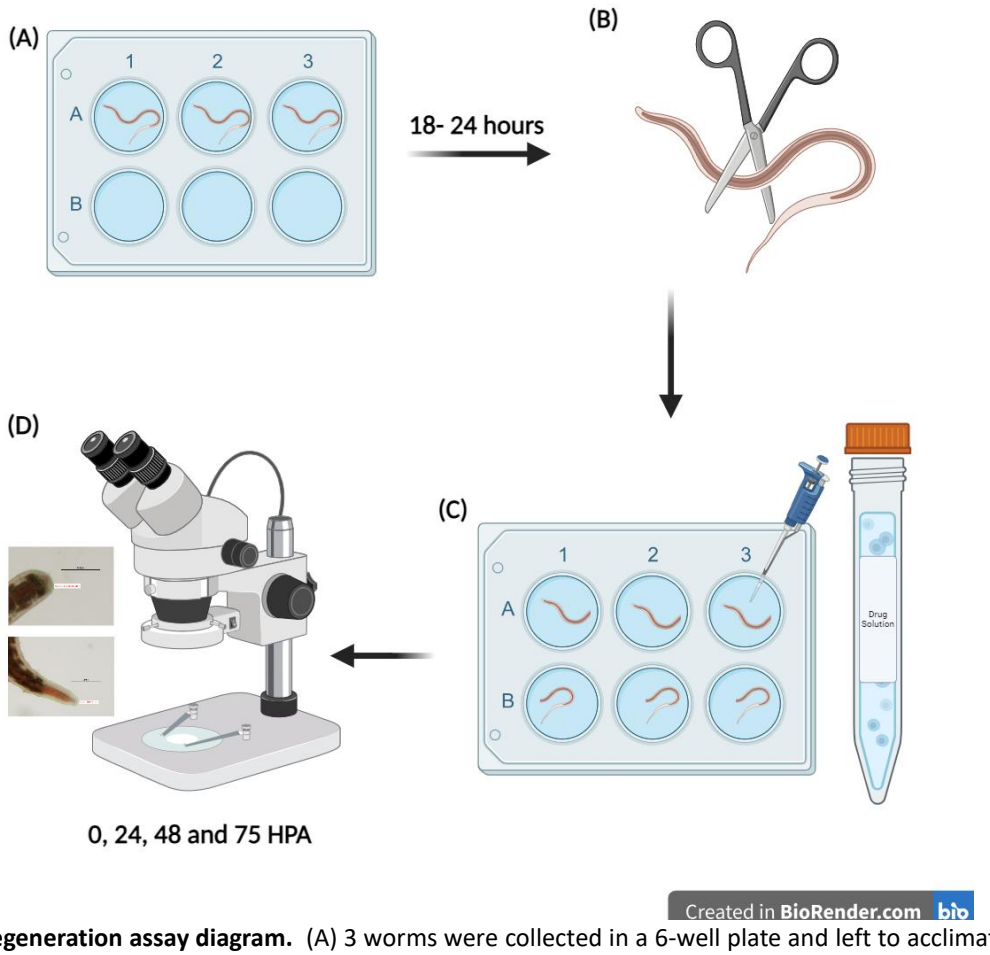

Figure 12: Regeneration assay diagram. (A) 3 worms were collected in a 6-well plate and left to acclimatise for 18-24 hours. (B) worms were dissected in half (C) Worms were separated into anterior and posterior sections and exposed to drug solutions (D) worms were imaged at 0, 24, 48 and 75 HPA, the images next to the microscope show the posterior section of the control group, the image at the top was taken at 0 HPA and the image at and the bottom was taken at 75 HPA. Created with BioRender.com.

2.10 Biomass

The assay investigates the effects of long-term CBD exposure on the biomass of *L. variegatus*. Ten worms were collected per condition and placed in a 13 ml sample tube containing APW and 5 g of aquarium gravel to allow for barrowing behaviours, 18-24 hours before the experiment.

On Day 1, CBD solutions (0-5 µM) were prepared and administered. The worms were left in these CBD solutions for 7 days, after which the solutions were removed.

On Days 7, 14 and 21, fresh CBD solutions were prepared; however, APW was replaced with conditioned APW containing nutrients, oxygen and microorganisms to better mimic a natural, established aquatic environment over long-term exposure. Fresh drug solutions were administered after removing the previous week's solutions. After 28 days of exposure, worms were collected, counted and recorded from the sampler tubes for analysis.

Empty microcentrifuge tubes were weighed and recorded before transferring the worms in. The worms were flash-frozen using liquid nitrogen and stored in -80°C. The frozen samples were placed onto a heating block at 40°C for 48 hours to dehydrate. The Eppendorf tubes containing the dried worm samples were then reweighed. The value of the empty micro centrifuge tubes was subtracted to calculate the total and individual worm biomass. Methods were modified from Silva et al., (2021).

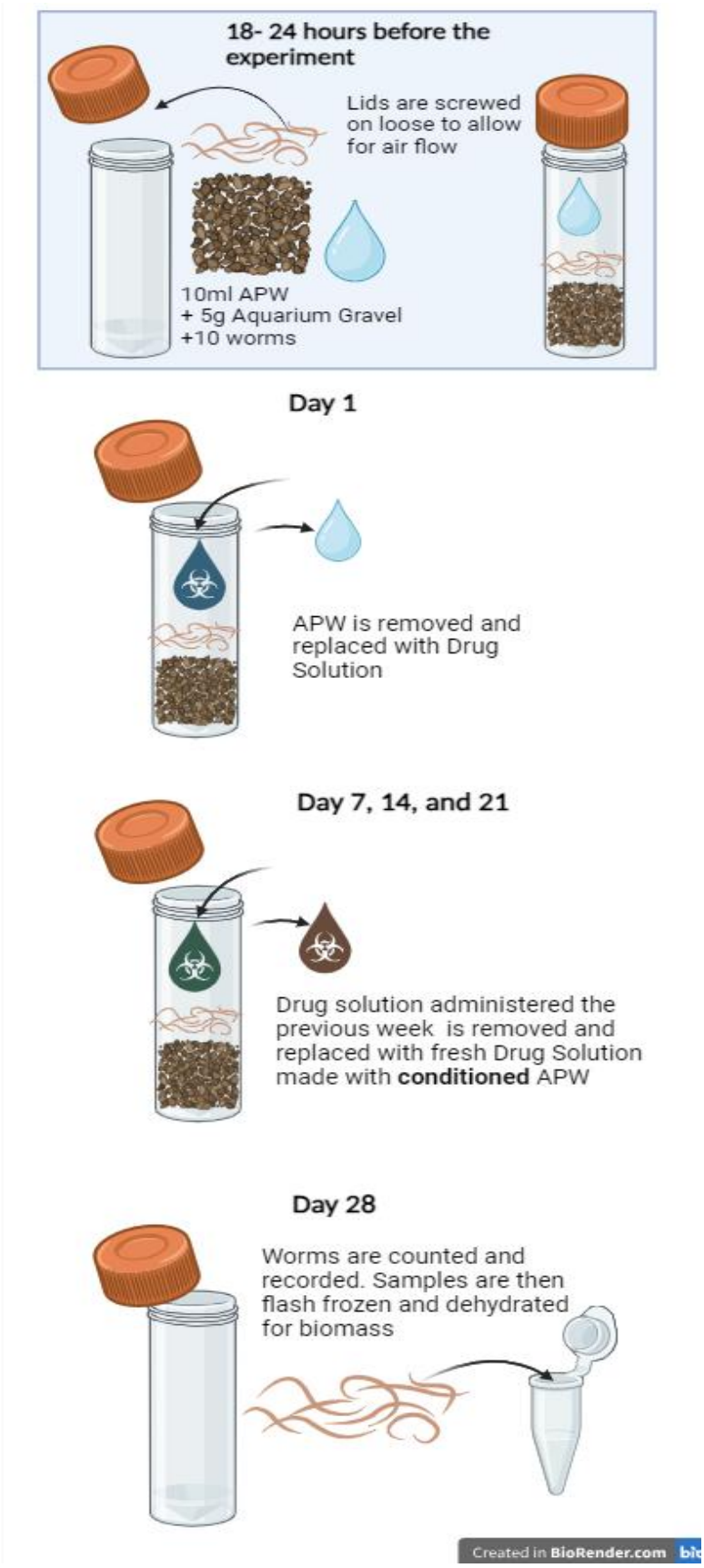

Figure 13: Biomass Assay, Created with BioRender.com.

2.11 Data Analysis

All data were reported as the standard error of the mean (± SEM) of each data set. Statistical significance was assessed using GraphPad Prism 10, with the significance value set at $p < 0.05$. Data is compared to the untreated control conditions/pre-exposure for each *L. variegatus* per condition.

IVTA results were assessed using nonlinear regression to produce a dose-response simulation to produce an EC_{50} and 95% confidence interval. Drug exposure conditions were tested against baseline conditions using a paired nonparametric two-tailed t-test for stereotypical movement assays and a paired parametric two-tailed t-test to assess free locomotion assays. Recovery time points of 10 minutes and 24 hours were compared to baseline conditions using a two-way ANOVA with Dunnett's post-test. Regeneration assay results were tested for significance using two-way ANOVA to compare variables of time and dose. Biomass assay used nonparametric mixed one-way ANOVA with Dunnett's multiple comparisons test to assess significance, defined at $p < 0.05$.

3.1: Results Chapter 1: Toxicity and Behavioural Responses

3.1.1 Determining Toxicity of CBD, 7-OH-CBD, Abn-CBD and O-1918

To determine a dose range that can be used in equimolar concentrations throughout the project, we started by determining the toxicity by exposing *L. variegatus* to CBD (0-25 µM), 7-OH-CBD (0-15 µM), O-1918 (0-50 µM) and Abn-CBD (0-15 µM).

Following a 24-hour exposure period, the concentration that produced 50% toxicity within the tested population was observed as 14.12 µM (95% Cl: 12.28-15.90 µM) for CBD (Figure 14 A), 11.29 µM (95% Cl: 10.53-12.09 µM) for 7-OH-CBD (Figure 14 B), and 15.84 µM (12.88-19.22 µM) for O-1918 (Figure 14 C). The concentration that produced observed toxic effects within 50% of the tested population could not be determined with Abn-CBD (Figure 14 D).

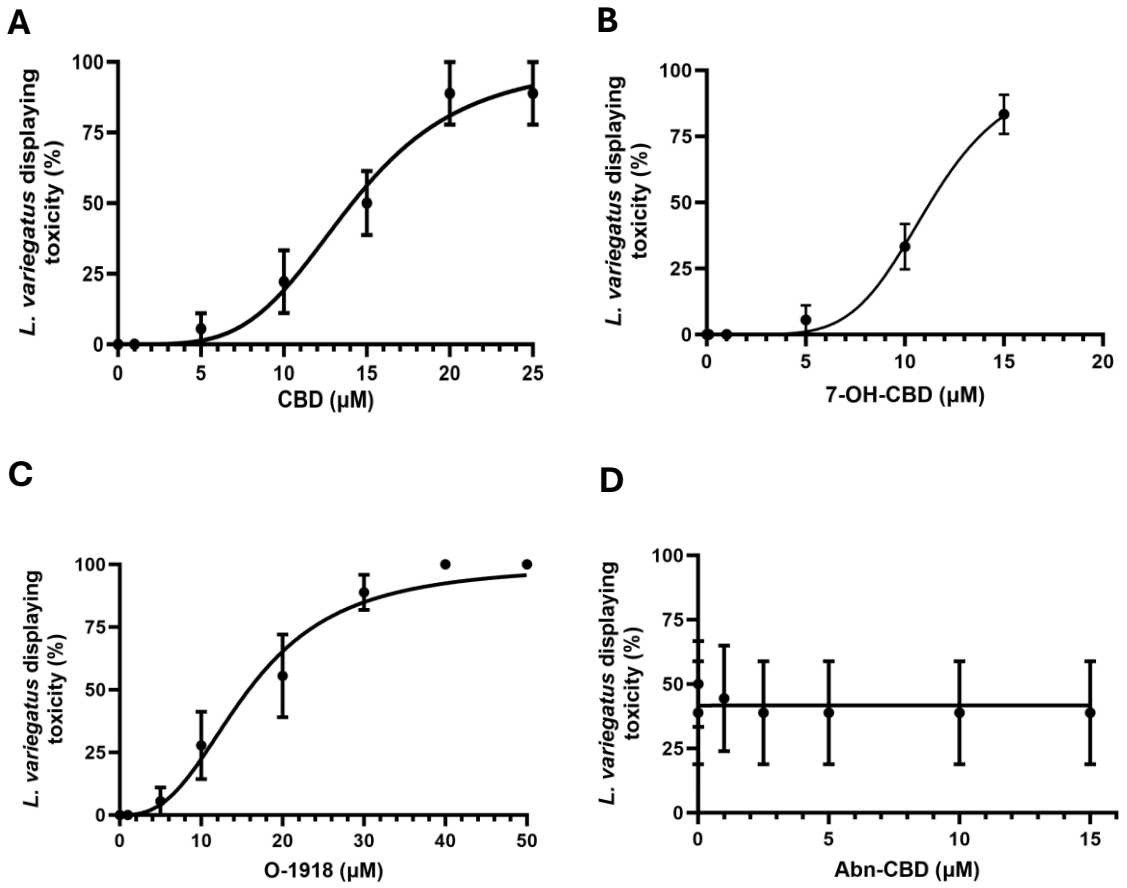

Figure 14: Toxicity of CBD, 7-OH-CBD, Abn-CBD and O-1918 in *L. variegatus*. Exposure to **(A)** CBD (0-25 µM), **(B)** 7-OH-CBD (0-15 µM), **(C)** O-1918 (0-50 µM) and **(D)** Abn-CBD (0-15 µM) for 24 hours and examined for observed signs of whole organism toxicity i.e., worms displaying decomposition, as determined by visible partial or complete tissue degeneration and whole organism tissue pallor, were counted. The percentage of *L. variegatus* displaying signs of toxicity, relative to Veh:0.5%(v/v) DMSO in artificial pond water was calculated (CBD and O-1918; 0.5% DMSO and artificial pond water. 7-OH-CBD; 0.5% methanol and artificial pond water. Abn-CBD; 0.1% Methyl acetate and artificial pond water). Error bars represent ± SEM. *N* = 6. Data was collected in collaboration with Ben Williams, James McRobbie-Aston, Megan Flanagan and Grace Hawkes

3.1.2 Behavioural Effects of *Lumbriculus variegatus* when exposed to CBD

We investigated the impact of 24-hour CBD exposure on the tactile response and locomotor activity of *L. variegatus*. We assessed the organism's ability to perform stereotypical behaviours such as body reversal and helical swimming following exposure to varying concentrations of CBD (0-5 µM). The results demonstrated that CBD exposure impaired stereotypical motor responses. The inability to respond to stimulation of the anterior and elicit body reversal behaviour was observed at ≥ 2.5 µM CBD (p = 0.0078, N = 8, Figure 15 A). Whereas, the inability to respond to stimulation of the posterior and elicit helical swimming behaviour was observed at ≥ 0.5 µM CBD ($p \leq 0.05$, N = 8, Figure 15 B).

Following removal from CBD solutions and subsequent incubation in artificial pond water the inability to respond to stimulation persisted. Following the 10-minute recovery period, *L. variegatus* exposed to 0.5 µM CBD (p = 0.0042, N = 8) and ≥ 2.5 µM CBD ($p < 0.0001$, N = 8, Figure 15 C) continued to exhibit an inability to perform body reversal when stimulated. Similarly, helical swimming behaviours remained inhibited at 1.0 µM CBD (p = 0.022, N = 8) and ≥ 2.5 µM CBD ($p < 0.0001$, N = 8, Figure 15 D). Notably, a concentration of 5.0 µM CBD inhibited both stereotypical movement behaviours after 24-hour recovery in artificial pond water ($p < 0.0001$, N = 8, Figure 15 C-D).

Furthermore, the locomotor abilities of *L. variegatus* was reduced to 45.12 ± 11.23 % (p = 0.0018, N = 8) at 5.0 µM CBD following 24-hour exposure (Figure 15 F). Removal of CBD solutions followed by a 10-minute recovery in artificial pond water showed that effects persisted and were observed at a lower concentration of 2.5 µM CBD, where movement was reduced to 74.20 ± 9.06 % (p = 0.0381, N = 8). At 5.0 µM CBD, movement reduced to 42.41 ± 6.17 % ($p < 0.0001$, N=8) at the 10-minute recovery time point. After 24 hours of recovery in artificial pond water, effects were documented at 0.1 µM CBD with a decrease in movement to 76.82 ± 8.60 % (p = 0.0386, N = 8). At 5.0 µM CBD 24-hour recovery showed an increase in movement to 68.01 ± 11.05 % (p = 0.0049, N = 8). (Figure 15 G).

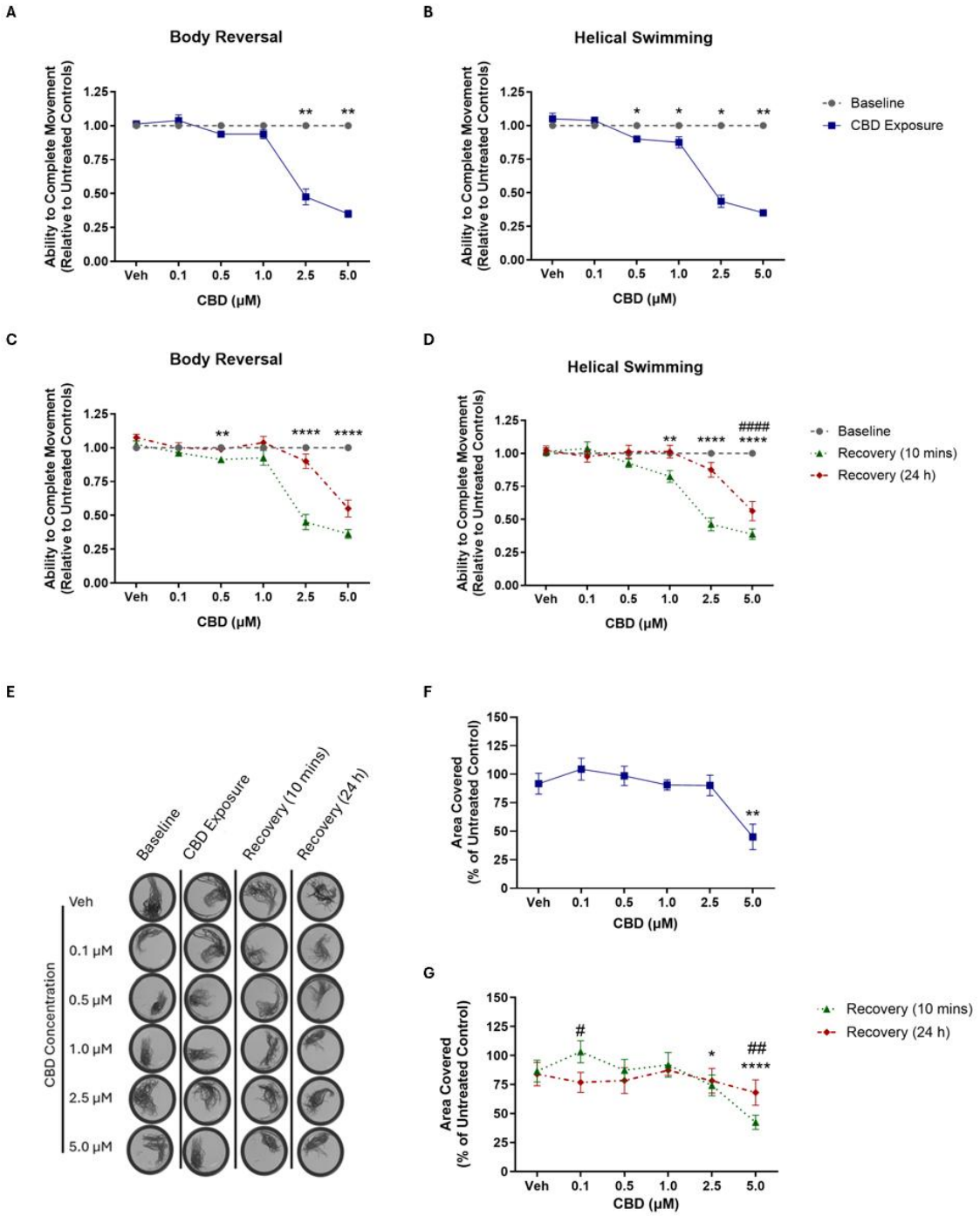

Figure 15: Behavioural Assays of *L. variegatus* exposed to CBD. Behavioural assays were conducted on *L. variegatus* following exposure to CBD (0-5 µM) for 24 hours. Behavioural responses were investigated using stereotypical movement assays that tested **(A)** body reversal and **(B)** helical swimming. Ability to conduct **(C)** body reversal and **(D)** helical swimming were tested after removal from drug solutions at Recovery 10 mins and Recovery 24 h. **(E) R**epresentative images for free locomotion assay **(F)** 24 hours of CBD exposure on *L. variegatus* ability to conduct unstimulated movement **(G)** effects on unstimulated movement after Recovery from CBD solutions were tested at Recovery 10 mins and Recovery 24 h. Area covered is expressed as a percentage of movement relative to baseline. All data is reported as the ratio of movement of the worm at CBD exposure and at Recovery (10 mins) and Recovery (24 h), relative to the movement at baseline. Error bars represent ±SEM.*refers to either CBD Treatment or Recovery (10 mins), # refers to Recovery (24 h) ; */# $p < 0.05$, **/## $p < 0.01$, ****/#### $p < 0.0001$. $N = 8$. Data was collected in collaboration with Ben Williams, James McRobbie-Aston, Megan Flanagan and Grace Hawkes

3.1.3 Behavioural Effects of *Lumbriculus variegatus* when exposed to 7-OH-CBD

We assessed the impact of 24-hour exposure to 7-OH-CBD on the tactile response and locomotor activity of *L. variegatus* following exposure to varying concentrations of 7-OH-CBD (0-5 µM). The results demonstrated that *L. variegatus* ability to conduct stereotypical movements of body reversal and helical swimming was inhibited by 7-OH-CBD exposure. At 5.0 µM 7-OH-CBD, *L. variegatus* displayed a significant inability to respond to stimulation with body reversal ($p = 0.0156$, $N = 8$, Figure 16 A) and helical swimming ($p = 0.0078$, $N = 8$, Figure 16 B).

The impaired responsiveness persisted after removal from 7-OH-CBD solutions and subsequent incubation in artificial pond water for 10-minute and 24-hour recovery time points. Notably, after 10 minutes in recovery, *L. variegatus* exposed to 2.5 µM 7-OH-CBD ($p = 0.0012$, $N = 8$, Figure 16 C) exhibited the inability to perform the stereotypical movement of body reversal, which was not observed previously in Figure 16 A-B during drug exposure. At the highest concentration of 5.0 µM, body reversal 7-OH-CBD ($p < 0.0001$, $N = 8$, Figure 16 C) and helical swimming CBD ($p < 0.0001$, $N = 8$, Figure 16 D) remained significantly inhibited. However, no adverse effects were observed after a 24-hour recovery period in artificial pond water

Interestingly, despite the acute effects on stereotypical movement, 24-hour exposure to 7-OH-CBD (0-5 µM) showed no effect on the locomotor abilities of *L. variegatus* ($p > 0.05$, $N = 8$, Figure 16 F-G).

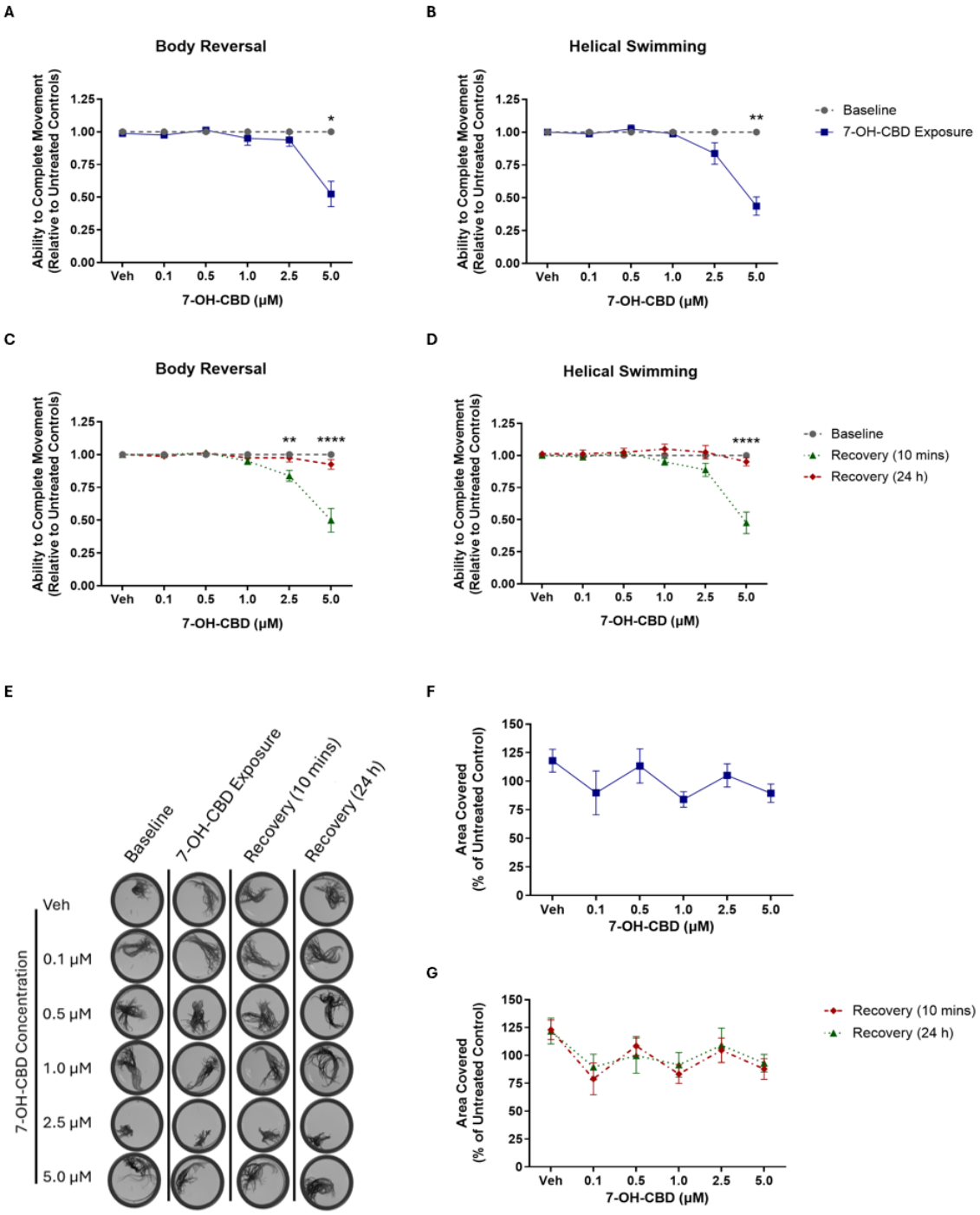

Figure 16: Behavioural Assays of *L. variegatus* exposed to 7-OH-CBD. Behavioural assays conducted on *L. variegatus* following exposure to 7-OH-CBD (0-5 µM) for 24 hours. Behavioural responses were assessed using Stereotypical movement assays which assessed **(A)** body reversal and **(B)** helical swimming. Ability to conduct **(C)** body reversal and **(D)** helical swimming at 10 mins and 24 h after Recovery from drug solutions was recorded. **(E)** Z-stack images taken during the different stages of treatment for free locomotion assay were used in quantification. **(F)** 24 hours of 7-OH-CBD exposure on *L. variegatus* ability to conduct unstimulated movement **(G)** effects on unstimulated movement 10 mins and 24 h after Recovery from drug solutions. Area covered is expressed as a percentage of movement relative to baseline. All data is reported as the ratio of movement of the worm at Drug exposure and at Recovery (10 mins) (24 h), relative to the movement as baseline. Error bars represent the standard error of the mean ±SEM.*refers to either Drug Treatment or Recovery (10 mins), # refers to Recovery (24 h) ; */# $p < 0.05$, **/## $p < 0.01$, ***/### $p < 0.001$, ****/#### $p < 0.0001$. $N = 8$. Data was collected in collaboration with Ben Williams, James McRobbie-Aston, Megan Flanagan and Grace Hawkes

3.1.4 Behavioural Effects of *Lumbriculus variegatus* when exposed to O-1918

We examined the effects of 24-hour exposure to O-1918 on the motor responses and locomotor activity of *L. variegatus*. Exposure to (0 - 5 µM) of O-1918 revealed that *L. variegatus* ability to conduct stereotypical movements of body reversal and helical swimming was inhibited by O-1918 exposure. The inability to respond to stimulation of the anterior and elicit body reversal behaviour was observed at 5 µM O-1918 ($p = 0.0156$, $N = 8$, Figure 17 A). However, the inability to respond to stimulation of the posterior and elicit helical swimming behaviour was observed at ≤ 2.5 µM O-1918 ($p \leq 0.05$, $N = 8$, Figure 17 B).

Following removal from O-1918 solutions and subsequent incubation in artificial pond water for recovery time points of 10 minutes and 24 hours, the inability to respond to stimulation persisted. After 10 minutes in recovery, *L. variegatus* exposed to 2.5 µM O-1918 ($p = 0.0346$, $N = 8$) and 5.0 µM O-1918 ($p < 0.0009$, $N = 8$, Figure 17 C) exhibited an impaired inability to perform body reversal when stimulated. Similarly, 2.5 µM O-1918 ($p = 0.0037$, $N = 8$) and 5.0 µM O-1918 ($p < 0.0001$, $N = 8$) exhibited impaired helical swimming behaviour after 10 minutes in recovery (Figure 17 D). After 24-hours in recovery, concentration of 5.0 µM O-1918 showed impairment of body reversal, Figure 17 C, and helical swimming ($p < 0.0001$, $N = 8$, Figure 17 D).

Interestingly, locomotor abilities of *L. variegatus* were not affected by O-1918 during the 24-hour drug exposure period. However, movement decreased to 76.16 ± 5.11 % ($p = 0.0162$, $N = 8$) at 0.5 µM O-1918 following 24 hours in recovery.

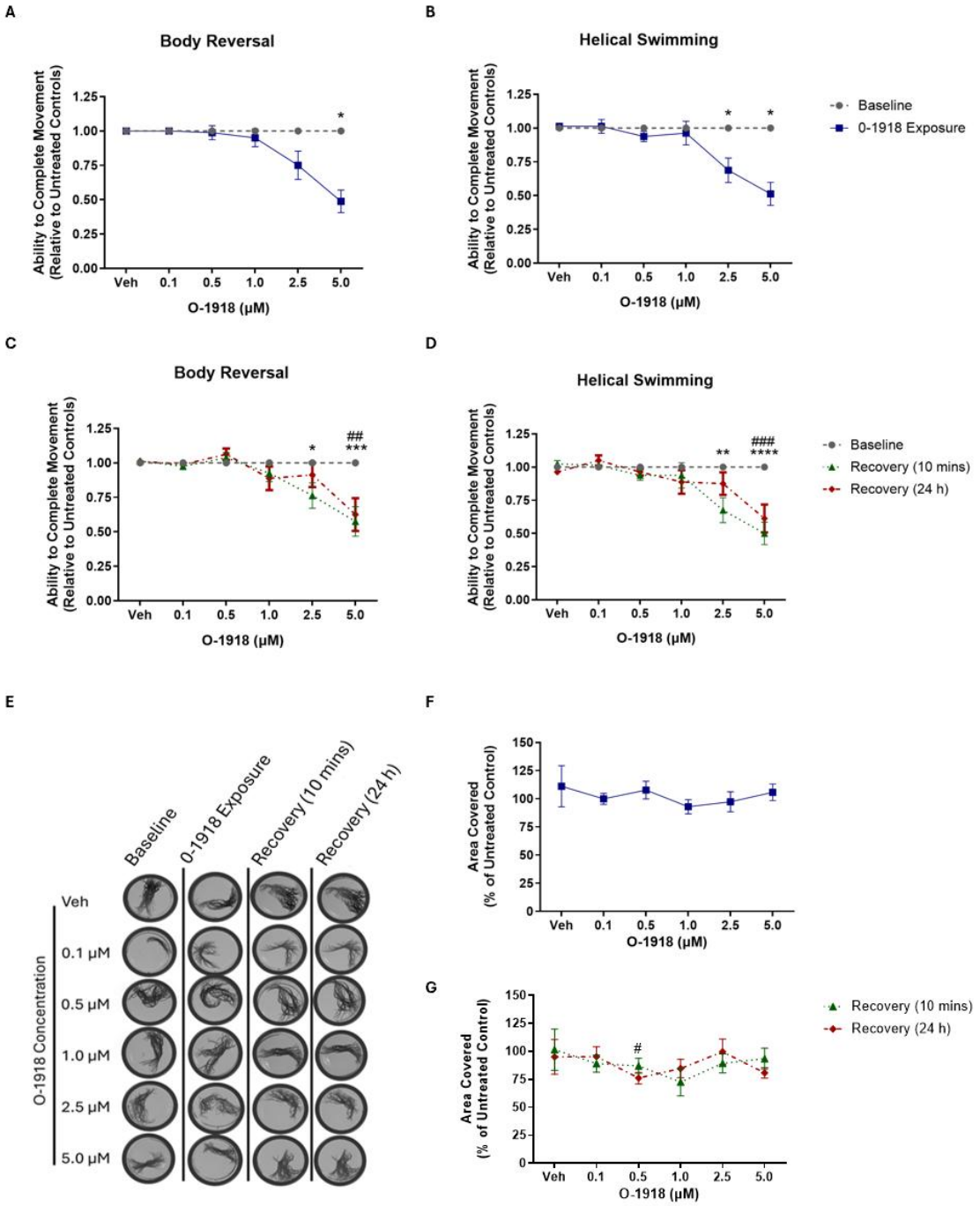

Figure 17: Behavioural Assays of *L. variegatus* exposed to O-1918. Behavioural assays conducted on *L. variegatus* following exposure to O-1918 (0-5 μM) for 24 hours. Behavioural responses were assessed using, Stereotypical movement assays which assessed **(A)** body reversal and **(B)** helical swimming. Ability to conduct **(C)** body reversal and **(D)** helical swimming at 10 mins and 24 h after Recovery from drug solutions was recorded. **(E)** Z stack images taken during the different stages of treatment for free locomotion assay were used in quantification, **(F)** 24 hours of O-1918 exposure on *L. variegatus* ability to conduct unstimulated movement **(G)** Effects on unstimulated movement 10 mins and 24 h after recovery from drug solutions. Area covered is expressed as a percentage of movement relative to baseline. All data is reported as the ratio of movement of the worm at O-1918 exposure and at recovery (10 mins, 24 h), relative to the movement as baseline. Error bars represent the standard error of the mean ± SEM.*refers to either Drug Treatment or Recovery (10 mins), # refers to Recovery (24 h) ; */# $p < 0.05$, **/## $p < 0.01$, ***/### $p < 0.001$, **** $p < 0.0001$. $N = 8$. Data was collected in collaboration with Ben Williams, Megan Flanagan and Grace Hawkes

3.2 Results Chapter 2: The Effects of Cannabinoids on the Regeneration and Biomass of *Lumbriculus variegatus*

L. variegatus specimens were bisected and exposed to varying concentrations of CBD (0-5 µM) for 72 hours post-amputation (HPA). Images of the regenerating blastema were captured at 24 HPA, 48 HPA and 72 HPA, and the blastema formation was monitored and measured at each time point .

3.2.1 The effect of CBD on the regenerative abilities of *L. variegatus*.

We observed significant effects of CBD exposure over time on tissue regeneration in *L. variegatus*. Regeneration capacity was assessed by measuring head and tail growth at various concentrations (0 - 5 µM) of CBD and time points (24, 48 and 72 hours post-amputation (HPA)).

At concentrations ≤ 2.5 µM CBD, significant effects were observed at both 48 HPA ($p < 0.01$, $N \geq 15$) and 72 HPA ($p < 0.0001$, $N \geq 15$) when compared to 24 HPA, demonstrating a time-dependent effect on regeneration. However, no significant growth was noted at 2.5 - 5.0 µM CBD between 24 HPA to 48 HPA ($p > 0.05$, $N \geq 15$). An increase in growth was observed at 72 HPA compared to 24 HPA ($p > 0.05$, $N \geq 15$), Figure 18 A). Head regeneration of the posterior segment demonstrated a significant effect of time ($F_{(1.277, 21.70)} = 39.61$, $p < 0.0001$, $N \geq 15$), but no significant effects of dose were detected ($F_{(3.238, 55.05)} = 0.65$, $p > 0.05$, $N \geq 15$).

Similarly, at concentrations ≤ 2.5 µM CBD, significant tail regeneration was observed at 48 HPA ($p < 0.001$, $N \geq 15$), with further growth by 72 HPA ($p < 0.001$, $N \geq 15$), compared to 24 HPA, further demonstrating time-dependent effects. Exposure to 5.0 µM CBD, showed limited initial growth at 48 HPA ($p = 0.0356$, $N \geq 15$), however, an increase in growth was seen at 72 HPA ($p = 0.0123$, $N \geq 15$, Figure 18 B) when compared to 24 HPA.

In contrast to head regeneration, tail regeneration was influenced by both dose ($F_{(2.678, 45.53)} = 4.224$, $p = 0.0127$, $N \geq 15$) and time ($F_{(1.052, 17.88)} = 33.29$, $p < 0.0001$, $N \geq 15$).

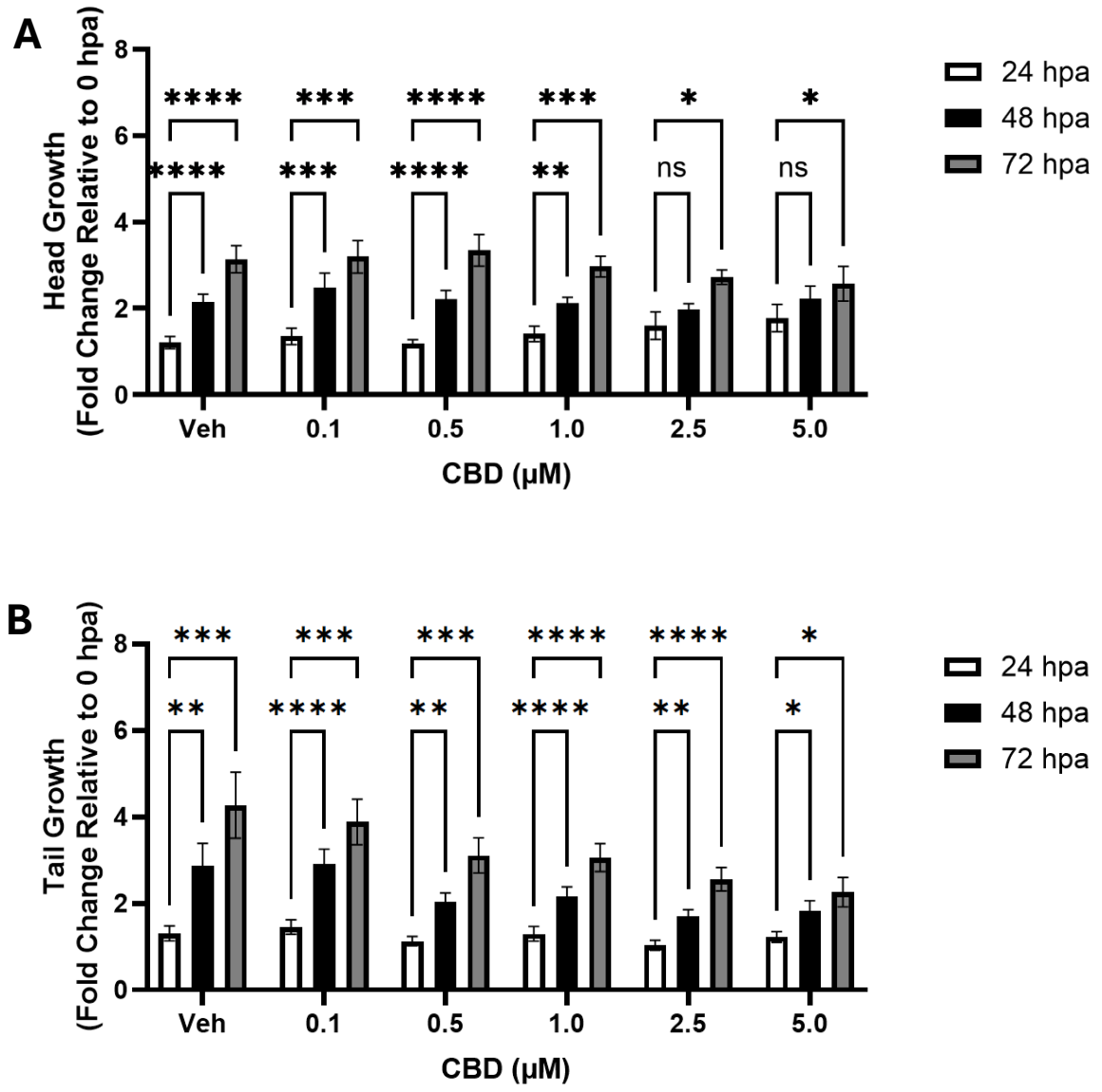

Figure 18: Regeneration Assays of *Lumbriculus variegatus* exposed to CBD

Regeneration assays conducted *on L. variegatus* following CBD (0-5 µM) exposure. Regeneration capacity was assessed by measuring blastema **(A)** head growth and **(B)** tail growth at 0, 24, 48 and 72 hours post amputation (HPA). Error bars represent the standard error of the mean ± SEM.* is used to show significance ; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. $N \geq 15$. Data was collected in collaboration with Ben Williams and Megan Flanagan

3.2.2 The effect of 7-OH-CBD on the regenerative abilities of *L. variegatus*.

Investigating the effects of 7-OH-CBD exposure over time on tissue regeneration in *L. variegatus* revealed a significant effect of time on regeneration. Regeneration capacity was assessed by measuring head and tail growth at various concentrations (0-5 µM) of 7-OH-CBD and time points (24, 48 and 72 hours post-amputation (HPA)).

At all concentrations of 7-OH-CBD, fold increases in head growth were observed between 24 HPA and later time points of 48 ($p > 0.05$, $N \geq 15$) and 72 HPA, with the highest growth noted from 24 HPA to 72 HPA ($p > 0.05$, $N \geq 15$). At the highest dose of 5.0 µM, a similar trend was observed although variability increased. Significant effects of time were observed ($F_{(1.281, 21.77)} = 43.58$, $p < 0.0001$, $N \geq 15$), demonstrating a time-dependent increase in head regeneration across all concentrations. However, no significant effects of dose ($F_{(2.807, 47.72)} = 0.35$, $p > 0.05$, $N \geq 15$) was detected. (Figure 19 A).

Tail regeneration increased significantly over time across all concentrations (Figure 19 B). Significant growth was seen from 24 HPA to 48 HPA ($p > 0.01$, $N \geq 15$) and at 72 HPA compared to 24 HPA ($p > 0.05$, $N \geq 15$), confirming the effect of time ($F_{(1.175, 18.27)} = 30.16$, $p < 0.0001$, $N \geq 15$) on the regeneration of *L. variegatus* when exposed to 7-OH-CBD. However, no overall significant effect of dose ($F_{(3.132, 53.25)} = 1.368$, $p > 0.05$, $N \geq 15$) was observed.

The results indicate a pronounced time-dependent effect of 7-OH-CBD on both head and tail regeneration in *L. variegatus*.

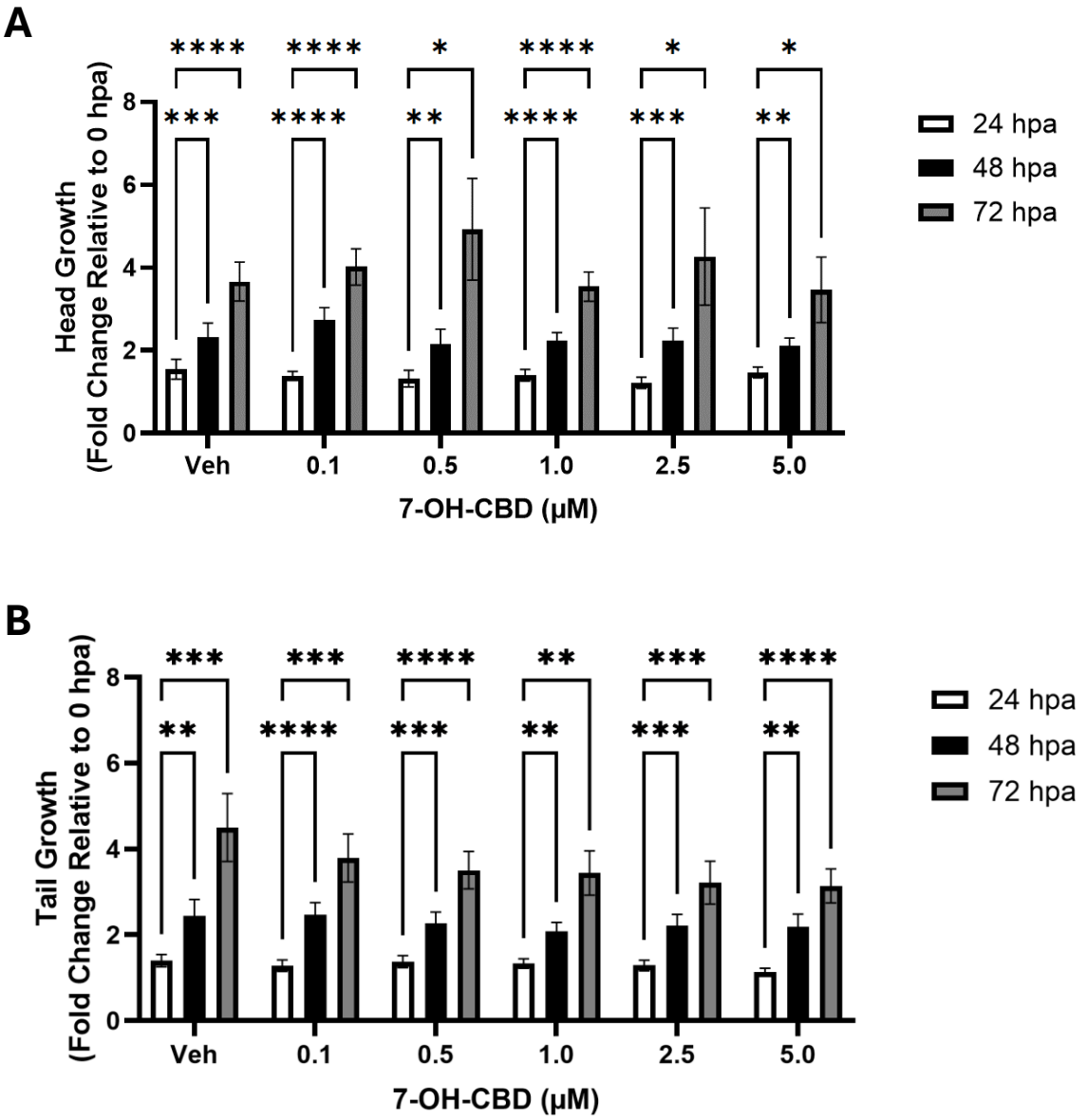

Figure 19: Regeneration Assays of *L. variegatus* exposed to 7-OH-CBD

This figure displays the results of regeneration assays conducted *on L. variegatus* following 7-OH-CBD (0-5 µM) exposure. Regeneration capacity was assessed by measuring blastema **(A)** head growth and **(B)** tail growth at 0, 24, 48 and 72 hours post amputation (HPA). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $N \geq 15$. Data was collected in collaboration with Megan Flanagan

3.2.3 The effect of O-1918 on the regenerative abilities of *L. variegatus*.

The effects of O-1918 exposure on the regeneration in *L. variegatus* were assessed by measuring fold changes in head and tail growth at various concentrations (0-5 µM) of O-1918 and at different time points (24, 48 and 72 hours post-amputation (HPA)).

Head regeneration, Figure 20 A, showed significant growth at all time points and at all concentrations ($p < 0.0001$, $N \geq 15$). A significant effect of time were observed ($F_{(1.352, 22.98)} = 107.5$, $p < 0.0001$, $N \geq 15$) demonstrating a time-dependent increase in head regeneration across all concentrations. However, no significant effects of dose ($F_{(3.572, 60.72)} = 1.11$, $p > 0.05$, $N \geq 15$) was detected.

Tail regeneration, Figure 20 B, showed significant growth at all time points and concentrations apart from the vehicle group. Vehicle control tail growth at 48 HPA when compared to 24 HPA showed no significant growth ($p > 0.05$, $N \geq 15$). However, growth from 24 HPA and 72 HPA ($p > 0.0001$, $N \geq 15$) showed significant growth. At 5.0 µM a significant effect of dose was observed at 48 HPA when compared to vehicle control ($p = 0.0239$, $N \geq 15$). A significant effect of time was observed ($F_{(1.105, 18.79)} = 44.76$, $p < 0.0001$, $N \geq 15$) but there was no significant effect of dose ($F_{(3.584, 60.93)} = 0.63$, $p > 0.05$, $N \geq 15$) .

O-1918 displayed consistent regenerative trends across time regardless of dose. Dose sensitivity was only observed for tail regeneration at later time points and at higher doses.

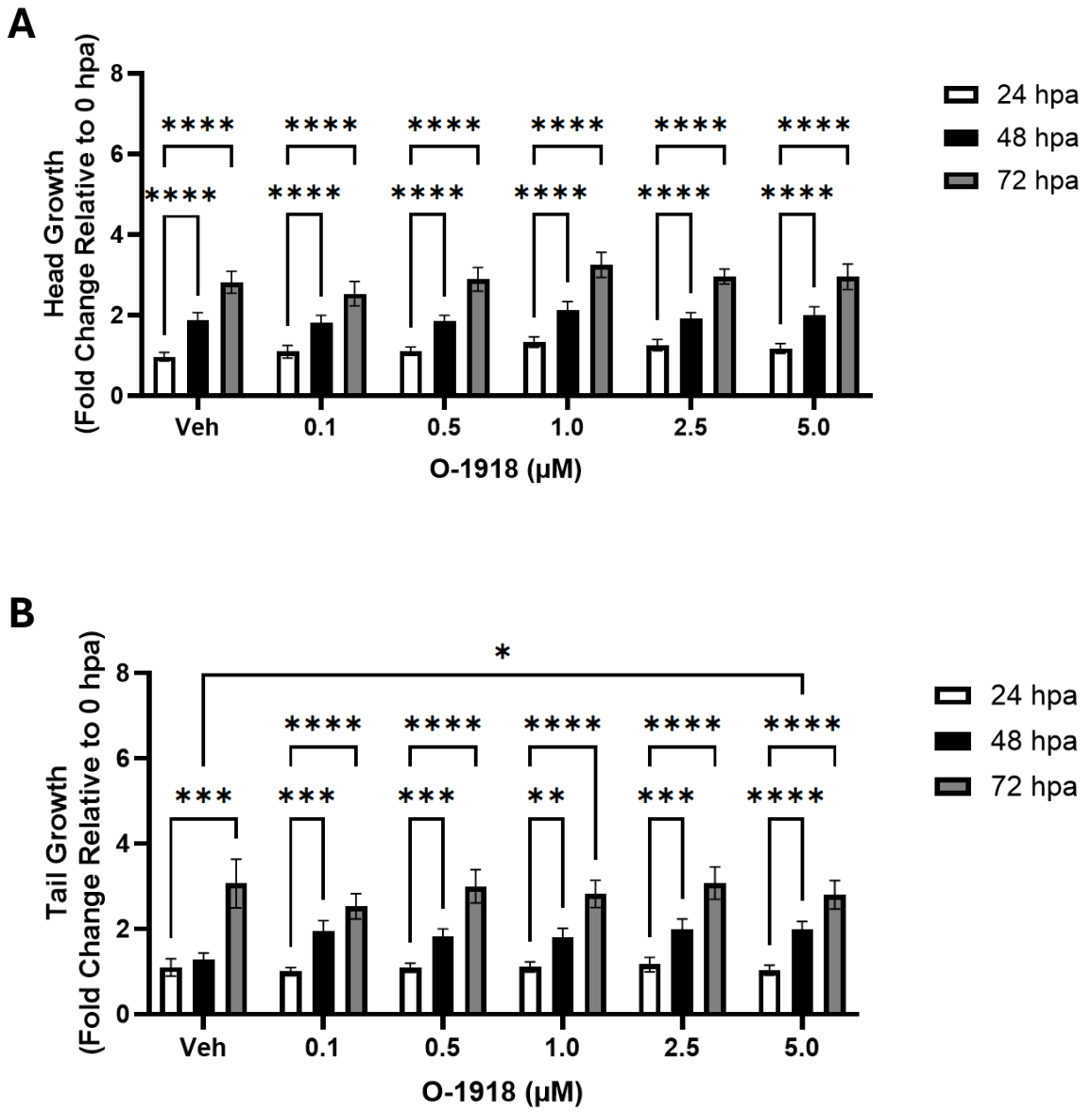

Figure 20: Regeneration of *Lumbriculus variegatus* exposed to O-1918. Regeneration capacity was assessed by measuring blastema **(A)** head growth and **(B)** tail growth at 0, 24, 48 and 72 hours post amputation (HPA). When exposed to O-1918 (0-5 µM); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $N \geq 15$. Data was collected in collaboration with Megan Flanagan

3.2.4 The effect of CBD on the biomass of *L. variegatus*.

In the experiment, *L. variegatus* underwent 28-day exposure to varying concentrations of CBD (0-5 µM). The biomass remained consistent at 0.1 mg/worm across CBD concentrations of 0 – 2.5 µM (Figure 21) . However, at the highest concentration of 5.0 µM ($p < 0.0001$, $N = 18$), the biomass drops sharply to 0mg/ worm, indicating a lethal or highly toxic effect at this concentration. Figure 21 B similarly shows the number of worms is consistently 10 across CBD concentrations 0 – 2.5 µM. At 5.0 µM the number of worms plummets to 0 ($p < 0.0001$, $N = 18$), indicating complete mortality at this concentration.

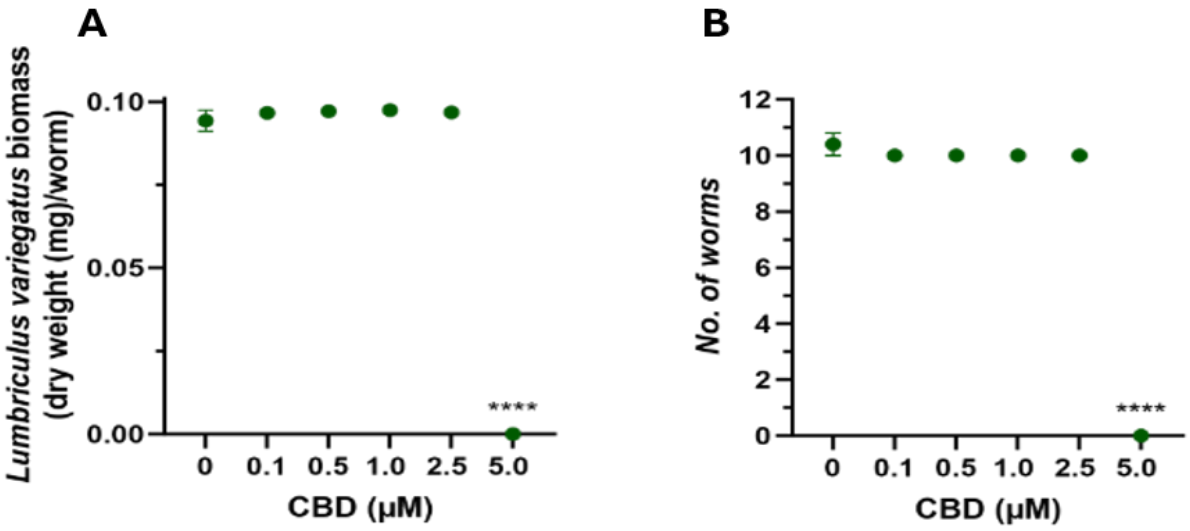

Figure 21: Biomass of *Lumbriculus variegatus* exposed to CBD. Experiments were done in conjunction over 28 days measuring Biomass **(A)** and Reproduction **(B)** on the 28th day of exposure to CBD (0-5 µM); **** $p < 0.0001$, $N \geq 15$. Data was collected in collaboration with Ben Williams

4.0 Discussion

Our data shows the pharmacological effects of cannabinoid drugs CBD, its metabolite 7-OH-CBD, the synthetic cannabinoid Abn-CBD and the antagonist O-1918 on the novel *in vivo* model *L. variegatus*, by assessing the toxicity, behavioural effects and the regenerative capacity of *L. variegatus* and its potential impact on biomass.

While *L. variegatus* cannot be directly equated with more complex vertebrate models such as rodents, it represents a valuable starting point for *in vivo* pharmacological investigations. This species offers a novel system for elucidating drug mechanisms and simultaneously characterising the functions of a proposed endocannabinoid system within the species.

4.1 Comparative toxicity of cannabinoids in *L. variegatus*

The acute toxicity of CBD, its metabolite 7-OH-CBD and synthetic compounds Abn-CBD and O-1918 was assessed using a 24-hour toxicity assay that has been optimised from previously established methods (O'Gara et al., 2004; Seeley et al., 2021). These experiments revealed distinct toxicity profiles highlighting differences in potency, physiological responses and methodological challenges.

4.1.1 Comparison of CBD and 7-OH-CBD toxicity within *L. variegatus*

The toxicity of CBD on *L. variegatus* is depicted in Figure 14 A, showing the percentage of *L. variegatus* displaying toxicity in response to increasing concentrations of CBD. The graph displays a classic sigmoidal, S-shaped dose-response pattern, indicating a threshold-dependent effect of CBD on *L. variegatus*. We observed that CBD displayed toxicity at higher concentrations. Similarly, exposure to 7-OH-CBD also produced a dose-response curve depicted in Figure 14 B. The sharp increase in toxicity shows a clear dose-dependent toxic effect that is consistent with both compounds. The time point of 24-hour exposure to compounds to test toxicity was previously established (O'Gara et al., 2004). They found that almost all worms that succumbed to copper exposure died during the first 24 hours of exposure. Other studies testing the toxicity of active compounds on *L. variegatus,* such as nicotine, also had an exposure time point of 24 hours (Davies et al., 2025). This consistency within exposure time points allows for a dependable comparison. Nicotine is considerably more toxic in most models, with an LD_{50} of 0.5-1.0 mg/kg considered to be lethal in humans (Kamble et al., 2020). Comparatively, CBD LD_{50} within rhesus monkeys has been established at 212 mg/kg (Rosenkrantz et al., 1981). These findings are not consistent with toxicity doses established within *L. variegatus*, as nicotine toxicity was established within a millimolar range whereas CBD produced similar toxicity results within the micromolar range (Davies et al., 2025).

The comparison of these two similar compounds shows that CBD exhibited toxicity within 50% of the tested population at concentrations of 14.12 µM CBD, as opposed to 7-OH-CBD, which produced similar toxic effects at a lower value of 11.29 µM 7-OH-CBD. This minor difference suggests that toxicity to 7-OH-CBD may have increased potency. This increased potency could be related to differences in metabolic processing between invertebrates and mammals, as *L. variegatus* lacks hepatic first-pass metabolism; the presence of cytochrome P450 (CYP) enzymes or other drug-metabolising enzymes within the invertebrate model is in question and requires further investigation. The absence of these drug-metabolising enzymes could potentially allow the analogue 7-OH-CBD to exert more direct effects within *L. variegatus*. Results from toxicity assay conducted on the inactive metabolite 7-COOH-CBD (0-14 µM) showed a value of 15.36 µM (95% CI: 14.0319.94 µM) where 50% of the tested population exhibited signs of toxicity, confirming it to be less toxic than CBD and 7-OH-CBD. Furthermore,

exposure to concentrations ≤ 20 µM resulted in near-complete mortality at 20 µM (Williams et al., 2025). These findings indicate a heightened sensitivity of *L. variegatus* to CBD, suggesting a greater toxicological susceptibility compared to other invertebrate models (Land et al., 2021).

A previous experiment conducted within SWIRL established an LD_{50}, Figure 22. The *in vivo* toxicity assay was conducted to narrow down the concentration range by drugging the worms individually as opposed to in groups. *L. variegatus* is known to form tightly entangled structures or worm "blobs" as a survival mechanism (Tuazon et al., 2022). This was observed when comparing the two experiments, as worms exhibited a higher tolerance when grouped, as outer worms may absorb or delay penetration of contaminants, protecting internal members temporarily.

Interspecies comparisons can further elucidate the variability in CBD sensitivity.

For instance, *C. elegans* that underwent whole-life exposure to 10-100 µM CBD increased lifespan. They also found that *C. elegans* exposed to 0.4-4000 µM CBD showed no lethality and exhibited a reduction in motility at a relatively high concentration of 4000 µM CBD, which exceeds physiologically relevant exposure levels. Moreover, *C. elegans* demonstrated enhanced tolerance to CBD, with increased activity reported in the late stage of life when exposed to CBD concentrations of 10-100 µM (Land et al., 2021).

These findings highlight the extreme differences in tolerance, potentially due to species-specific pharmacodynamic mechanisms or distinct molecular targets for CBD action. Another feasible explanation for the variation in concentration between the two studies is due to the medium used, as Land et al., 2021 reported the use of nematode growth media (NGM) plates and that CBD was formulated in liquid NGM, whereas in our experiments, CBD was formulated and cultured in artificial pond water. This can significantly alter CBD bioavailability and stability as NGM components can bind or metabolise compounds. Whereas the artificial pond water is a simpler aqueous medium that may increase immediate compound availability but reduce long-term stability, potentially explaining the heightened sensitivity in *L. variegatus* compared to *C. elegans*. The timepoints for both studies also vary. Land et al., (2021) tested behavioural effects of acute exposure at 0, 3 and 8 hours and lifelong toxicity to locomotor behaviour was tested at days 5, 8, 12 and 15, as opposed to the 24-hour exposure followed by 10 minutes and 24-hour recovery time points demonstrated within this study. This

difference in methodology reflects the requirements of the respective *in vivo* models. *C. elegans* and *L. variegatus* differ in physiology and exposure route, with *C. elegans* primarily absorbing compounds via ingestion and cuticular contraction on solid media, while *L. variegatus* allows direct uptake across a permeable body wall. These differences are likely to influence compound bioavailability, effective dosing and tolerance.

Studies conducted on *Manduca sexta* (tobacco hornworm), showed survival to CBD concentrations of 10-2,000 µM CBD (Park et al., 2019). A concentration of 1.69 µM was determined to be the lethal concentration that killed 50% of the tested population of *D. rerio* (Carty et al., 2018), however, contradictory evidence was also published where 3.2-12.7 µM CBD exposure caused significant decreases in *D. rerio* body length (Ahmed et al., 2018), however, both studies conducted on *D. rerio* reported developmental abnormalities when exposed to CBD. The binary measurement of toxicity within these experiments cannot capture sublethal effects such as cellular or physiological dysfunction without causing death, as the techniques are reliant on observational endpoints.

These results from these studies demonstrate the increased toxicity of CBD in *L. variegatus* when compared with other *in vivo* models. For instance, bisphenol A (BPA) exerts observable effects in *L. variegatus* in the picomolar range, whereas *D. magna* and *C. elegans* typically require nanomolar to micromolar concentrations to show comparable responses (Cp et al., 2025; Y. Wang et al., 2023; Y. Wang & Wang, 2021). This can also be observed with nicotine exposure; *L. variegatus* exposed to 0.5 mM nicotine for 24 hours showed 8.3.33 ± 16.67% mortality (Davies et al., 2025), in contrast to 100% survival in *C. elegans* at equivalent doses (Kanteti et al., 2015). Additionally, CBD elicited locomotor suppression and oxidative stress responses in *L. variegatus* at concentrations nearly 200 times lower than those required to elicit similar effects in *C. elegans* (Land et al., 2021; Williams et al., 2025).

4.1.2 Investigating the toxicity of O-1918 within *L. variegatus*.

Exposure to the synthetic antagonist O-1918 (0-50 µM) shows concentration-dependent toxicity (Figure 14 C). Concentrations below 5 µM showed no adverse effects, at the 10 µM to 25 µM range, toxicity is shown to increase with 15.84 µM CBD (12.88-19.22 µM, *N*=6), eliminating half of the tested population. It is shown to be less toxic than CBD and 7-OH-CBD, as a higher concentration is needed to reach comparable toxicity levels. Toxicity approaches 100% at 30-40 µM, with a plateau seen between 40-50 µM.

Notably, during toxicity assays, some worms exhibited autotomy or spontaneous splitting. This response can be due to a mechanical stress response, which has been previously observed (Lesiuk & Drewes, 1999) or due to a pharmacological response, though the precise mechanism of action remains unclear.

A plausible mechanism of the autotomy observed is associated with the presence of an epidermal serotonin immunoreactive nerve ring; the autotomy reflex is considered to be influenced by acetylcholine-mediated activation of serotonergic neurones, as previously described (Martinez Acosta et al., 2021). Due to the absence of cannabinoid receptors, it is proposed that O-1918 exerts its effects via previously established pathways. Such as, O-1918, influencing either acetylcholine, nicotinic acetylcholine receptor (nAChRs) located on serotonergic neurones may lead to autotomy via stimulation of the epidermal serotonin immunoreactive ring (Martinez Acosta et al., 2021). However, the theory that O-1918 can encourage axon regeneration by disinhibiting JNK signalling through the cannabinoid ortholog NPR32, which is found in *C. elegans* and assumed to be present in *L. variegatus* (Clarke et al., 2021; Pastuhov et al., 2016). This can also be a possible mechanism for why splitting is induced. O-1918 may activate pathways to encourage head regeneration and in turn induce splitting, but this requires further investigation.

4.1.3 Understanding the toxicity of Abn-CBD within *L. variegatus*.

Exposure to Abn-CBD (0-15 µM, *N* = 6) produced a flat response curve depicted in Figure 14 D. The large error bars show high variability at each concentration and no statistically significant differences between doses. Due to the absence of a sigmoidal increase depicted in the graph, an EC_{50} could not be established.

Assessment of Abn-CBD toxicity was complicated by the use of methyl acetate as a solvent. Abn-CBD was supplied pre-dissolved in methyl acetate, which was toxic to the worms. Methyl acetate, due to its low boiling point and hydrophobic nature (Graczová et al., 2018), causes erratic toxicity results, affecting even the control groups. The solvents' volatility and poor solubility in artificial pond water likely led to vapour exposure and non-specific toxicity, making it difficult to accurately assess the effects of Abn-CBD itself. Future studies should employ alternative solvents to avoid confounding results.

Over all these findings highlight that 7-OH-CBD is the most acutely toxic of the tested cannabinoids in *L. variegatus*, while O-1918, despite its lower lethality, can induce a notable response to drug exposure. These results also emphasise the importance of rigorous methodological controls, particularly regarding solvent selection.

4.2 Effect of cannabinoid drugs on the behaviour of *L. variegatus*

Herein, we discuss the effects produced by the exposure of *L. variegatus* to cannabinoids CBD, 7-OH-CBD and O-1918 at equimolar concentrations of 0-5 µM to assess the effects on the ability to conduct both stimulated and unstimulated movements using previously established methods (O'Gara et al., 2004; Seeley et al., 2021). Comparatively, the methods used here differ due to chronic exposure of 24 hours where as previously established methods investigated 10-minute exposure of compounds (Seeley et al., 2024).

4.2.1 Comparison of behavioural responses of *L. variegatus* to CBD Vs 7-OH-CBD exposure

L. variegatus when exposed to CBD, revealed dose-dependent behavioural impairments (Figure 15 (A)(C)), with persistent effects even after recovery periods (Figure 15 (B)(D)). Immediate impairment in response to stimuli was observed at lower concentrations for helical swimming, compared to body reversal, where higher concentrations were required (Figure 15 (C)). As helical swimming exhibited less sensitivity to stimuli when exposed to CBD compared to body reversal, this further confirms that the tail (posterior segment) of *L. variegatus* is less sensitive than the anterior, possibly due to differing neural/muscular pathways (Martinez Acosta et al., 2021).

The anterior circuit that governs the head relies on glutaminergic signalling and is electrically coupled to the MGF (Lybrand et al., 2020), this signalling pathway could be disrupted by CBD by either impairing glutamate release or receptor function. In contrast, the posterior circuit, which develops later is less centralised and therefore explains the decreased response of the posterior as the anterior governs the escape response and due to its electrical coupling to the MGF it evokes a faster response.

Quantitative analysis of the movement area depicted in Figure 15 (F) shows that 24-hour exposure to CBD significantly reduced unstimulated movement at 5.0 µM, further confirming the behavioural impairments caused by CBD exposure. During the 10-minute recovery period, delayed impairment was observed at 2.5 µM (Figure 15 (G)). Mobility remained impaired at 5.0 µM for the 24-hour recovery time point, and further delayed effects emerged at lower concentrations, suggesting delayed toxicity. Partial recovery was observed at 5.0 µM at the 24-hour recovery time point, however, this was still below control levels. The observed

decrease in movement could be explained by *in vitro* research that has demonstrated CBD can inhibit acetylcholinesterase (AChE) activity (Puopolo et al., 2022). Cholinesterase activity has also been documented in *L. variegatus* homogenate, with nicotine indicating the presence of cholinergic motor neurones (Davies et al., 2025). Reduced movement in *L. variegatus* may be due to the inhibition of AChE, resulting in the accumulation of acetylcholine (ACh) at neuromuscular junctions and causing prolonged stimulation of cholinergic receptors in the body wall muscles. Through AChE inhibition, CBD may produce its effects on movement through hyper-contraction paralysis, thereby reducing movement in *L. variegatus* (Čolović et al., 2013). Additionally, the dorsal blood vessel pulse rate in *L. variegatus* is generated by rhythmic, peristaltic contractions of the body wall muscles (Crisp et al., 2010; Lesiuk & Drewes, 1999). If CBD-induced AChE inhibition leads to hyper-contraction paralysis, it could impair peristaltic movements and contribute to the decrease in dorsal blood vessel pulse rate, which has been observed (Williams et al., 2025).

Exposure to CBD resulted in *L. variegatus* exhibiting behavioural sensitivity with concentrations ≥ 2.5 µM, causing long-lasting motor deficits, even after 24-hour recovery. Locomotor impairment at 0.1 µM at the 24-hour recovery time point suggests delayed toxicity, possibly caused by unknown mechanism .

When examining CBD metabolism, *L. variegatus* showed distinct responses to the active metabolite 7-OH-CBD (Figure 16). Higher concentrations (5.0 µM) of 7-OH-CBD were required to disrupt stereotypical movement behaviours; the steeper dose-response curve suggests that once a critical concentration is reached, impairment occurs rapidly (Figure 16 (A)(C)). Full behavioural recovery was observed at the 24-hour recovery time point (Figure 16 (B)(D)), suggesting reduced potency compared with CBD or a reversible mechanism of action for 7-OH-CBD. Exposure to 7-OH-CBD had no significant effects on free locomotion and did not exhibit any signs of delayed toxicity at recovery timepoints (Figure 16 (F)(G)). The contrast in sensitivity can be caused by differences in bioaccumulation, membrane permeability or interaction with neuromuscular targets. The complete recovery from 7-OH-CBD suggests enhanced clearance compared to CBD. This could be due to hydroxylation, resulting in faster elimination of 7-OH-CBD as it is more water soluble and its reduced lipophilicity results in lower tissue accumulation (Rosowsky et al., 1990; Tihăuan et al., 2025). This data suggests that 7-OH-CBD is less neurotoxic than CBD.

Prior studies have established that acute exposure (10 minutes) to pharmacologically active compounds can elicit significant behavioural alterations in *L. variegatus* (Carriere et al., 2023; Davies et al., 2025; Seeley et al., 2021, 2024). The acute effects of a 10-minute exposure to CBD on *L. variegatus* have been previously established (Figure 22). CBD was observed to cause a sustained dose-dependent reduction in responsiveness to tactile stimuli. 24-hour exposure was found to impair stereotypical movements at concentrations far lower than those of 10-minute exposure.

D. rerio exposed to a concentration of 127.1 µM CBD showed reduced swimming distance and velocity in a novel tank test (Jensen et al., 2018). Conversely, it was also reported that *D. rerio* embryos exposed to 20-300 µM CBD displayed a temporary increase in motor activity at 24 hours post-fertilisation (Brigante et al., 2018). These findings suggest that CBD's effects on locomotion are age, dose and time dependent, which aligns with the findings presented here, as both time and dose are common variables explored.

Developmental exposure to CBD in *D. rerio* resulted in notable behavioural abnormalities by 96 hours post-fertilisation, with morphological and behavioural disruptions (Carty et al., 2018). It was found that CBD exposure led to increased expression of the *c-fos* gene, which is often associated with neuronal activity and stress response, indicating potential neurobehavioral alterations and impairments during early development. Significant hypoactivity was observed in later life stages and in the F1 generation, suggesting transgenerational neurobehavioral consequences of CBD exposure (Carty et al., 2018). The contrast underscores the complexity of CBD's biological effects and highlights the need for species-specific studies to clarify mechanisms in invertebrates and to assess its translational potential in mammalian models.

In mammalian models, adult mice treated with intraperitoneal administration of 20 mg/kg CBD for 6 weeks exhibited reduced spontaneous locomotion in open field tests without impairing motor coordination on the rotarod test (Calapai et al., 2022). This suggests selective suppression of voluntary activity as opposed to a motor deficit. Interestingly, a similar study exploring acute CBD exposure (30 mg/kg for 6 days) found no significant variation in locomotion (Viudez-Martínez et al., 2019). This suggests that chronic exposure can possibly result in neuroadaptive changes and modulation of neurotransmitter systems via receptor downregulation, altered neurotransmitter levels or changes in neural circuit function. Despite

reduced activity, spatial learning, memory and anxiety-related behaviours remain unaffected, further indicating specific modulation of motor output rather than broader neurotoxicity (Calapai et al., 2022). In mammals, such as adult mice, CBD exposure results in more modulatory effects compared to the toxic effects observed in *L. variegatus*. These interspecies differences may be attributed to variations in neuroanatomy, cannabinoid receptor expression or metabolic capacity.

Compared to *L. variegatus*, other model organisms exhibit notably greater tolerance to CBD-induced locomotor impairment. Both *C. elegans* and *D. rerio* show behavioural alterations at concentrations several magnitudes higher, with some reports even indicating enhanced activity. The pronounced sensitivity of *L. variegatus* to low-dose CBD suggests a more vulnerable neuromuscular system, potentially linked to cholinergic dysfunction through acetylcholinesterase inhibition. Unlike vertebrates, where CBD often exerts proactive or regulatory actions, *L. variegatus* responds with persistent motor deficits, highlighting its value as an invertebrate model for detecting subtle neurotoxic effects.

4.2.2 Evaluating the behavioural responses of *L. variegatus* to O-1918 exposure

Behavioural responses to O-1918 exposure (Figure 17) showed a reduced ability to perform stereotypical movement behaviours, Figure 17 (A) depicted body reversal behaviour disruption and exhibited higher tolerance in comparison to, Figure 17 (B) helical swimming which is consistent with findings from CBD exposure. O-1918's post-exposure results (Figure 17 (C)(D)) show delayed hypoactivity at the 10-minute recovery time point, with effects persisting yet showing partial recovery at high concentrations during the 24-hour recovery time point. Similar effects were also observed with CBD and 7-OH-CBD, suggesting either receptor binding or tissue damage occurring at high concentrations that decrease sensitivity to stimulus or result in impairment of motor functions. The delayed toxicity can also suggest bioaccumulation or metabolite-driven toxicity.

As *L. variegatus* is thought not to possess any mammalian cannabinoid receptors and produces contrasting effects, the cannabinoids administered possibly affect already recognised pathways such as serotonin and acetylcholine (Martinez Acosta et al., 2021). *C. elegans* possess invertebrate orthologs of the mammalian cannabinoid receptors, such as NPR-32, which mediate endocannabinoid-like signalling (Clarke et al., 2021). These orthologs have not yet been sequenced within *L. variegatus*; however, the possibility of their presence cannot be entirely dismissed. Therefore, it can also be assumed that the cannabinoids administered could modulate this receptor-mediated pathway.

4.3 The effect of cannabinoids on the regeneration and biomass of *L. variegatus*

L. variegatus possesses regenerative abilities that allow it to recover from injury or reproduce asexually through segmental regeneration. Regeneration within *L. variegatus* relies on coordinated cell proliferation and migration, as well as critical signalling pathways such as ROS accumulation at the wound site (Beinart & Gillen, 2024; Martinez Acosta et al., 2021). Herein, we discuss the effects of cannabinoid exposure on the regenerative abilities of *Lumbriculus variegatus*.

4.3.1 The effect of CBD exposure on the regeneration of *L. variegatus*

Exposure to CBD (0-5 µM) significantly impaired the regenerative capacity of *L. variegatus*, with distinct effects on head and tail regeneration (Figure 18). At ≤2.5 µM CBD, both head and tail regeneration showed inhibition of growth, while at 5.0 µM CBD, a delay or partial recovery was observed at 72 HPA. This contrasts with lower doses and suggests a negatively correlating non-linear relationship between CBD concentrations and regenerative outcomes. However, this observation could also be due to the degradation of CBD over time, as the drug solutions were not replaced. CBD is chemically unstable in water, especially when exposed to heat or light. Studies show that non-encapsulated CBD in aqueous solution is prone to degradation, with significant loss of potency over 72 hours (Crew et al., 2025). However, the use of DMSO in drug solutions acts as a solvent to improve the solubility of CBD in artificial pond water.

Notably, the variation observed between the regeneration of the head and tail showed the tail to be more sensitive to CBD, exhibiting dose-dependent inhibition, whereas head regeneration remained unaffected by dose. This disparity may reflect intrinsic biological limits, as anterior (head) regeneration in *L. variegatus* ceases once a complete functional structure is restored, whereas posterior (tail) regeneration continues indefinitely (Beinart & Gillen, 2024), potentially making it more susceptible to cumulative or prolonged CBD exposure. The differences in neural expression between the head and tail regenerative tissues also provide valid explanations as the primary mechanism of regeneration for the head is morphallaxis, whereby existing tissue is remodelled to regenerate the head. Whereas, tail regeneration

follows the mechanism of epimorphosis for new tissue growth and is comparatively much slower and follows posterior segmental duplication. The regeneration of the Giant fibre systems also varies. The LGF are restored late for segmental coordination as opposed to the MGF, which is critical for brain and head and therefore restored rapidly (Martinez et al., 2008).

Mechanistically, studies have shown that annelid tail regeneration relies on cell proliferation and migration (Randolph, 1892), a process that CBD may disrupt by modulating the production of ROS. The production of ROS was measured within *L. variegatus* using H2DCFDA probes. Researchers observed that within minutes of amputation, a controlled burst of ROS (H_2O_2, O_2^-) occurs at the wound site. H_2O_2 acts as a secondary messenger to trigger cellular responses for regeneration (Beinart & Gillen, 2024). CBD acts as a potent anti-oxidant at low concentrations and directly neutralises ROS (H_2O_2, O_2^-). This has been observed previously in *D. rerio* models as CBD reduced H_2O_2 levels by 40-60% (Lachowicz et al., 2023). The primary sources for ROS production post-amputation are NADPH oxidases (NOX enzymes) in epidermal cells and mitochondrial respiration in adjacent cells. CBD has been found to decrease NOX4 expression, in turn reducing oxidative stress in mammalian cells (Rajesh et al., 2010). It also decreases mitochondrial ROS in *C. elegans* (Y. Zhang et al., 2022). Therefore, excessive ROS scavenging by CBD in *L. variegatus* may interfere with the early redox signalling required for regeneration, potentially impairing wound healing by disrupting essential oxidative cues.

Furthermore, CBD exposure reduces carbohydrate stores and increases lipid levels in *L. variegatus* when exposed to 2.5 µM CBD for 72 hrs (Williams et al., 2025). The dose and time frame used here align with the data depicted in Figure 18, with the observed reduction in regeneration at concentrations >2.5 µM CBD . This provides evidence that CBD limits the metabolic resources required for regeneration. Since regeneration in annelids requires both cell proliferation and migration (Beinart & Gillen, 2024; Cook, 1969; Martinez Acosta et al., 2021), CBD's known anti-proliferative effects can be defined as the cause of inhibiting regeneration in *L. variegatus*, as it can be observed in other models. A study investigating the effect of CBD on stem cells found that the molecule demonstrated anti-proliferative effects on some cancer stem cells (Mesas et al., 2025). The concentrations used here (0-5 µM CBD) are lower than the previously established lethal toxicity thresholds (14.12 µM CBD); however, even at such low concentrations CBD exhibits sublethal but functionally significant impacts on

L. variegatus, which further emphasises CBD's dose-dependent modulatory effects that can be observed across species

Interestingly, the effects of CBD on regeneration are not universally inhibitory. In *D. rerio*, for example, exposure to CBD (≤ 2.5 µM) has been shown to enhance fin regeneration at 48 HPA and 72 HPA (Lachowicz et al., 2023), likely by modulating inflammation and apoptosis. This study uses similar doses and methods of administration as CBD was administered in water. However, the contrasting results underscore the complexity of CBD's biological effects and highlight the importance of species-specific studies when evaluating the environmental and therapeutic implications of CBD exposure. The variation observed between species could be attributed to differences between the ECS of vertebrates and invertebrates. The ECS is well-conserved within vertebrates and underpinned by distinct cannabinoid receptors and well-defined roles in physiology and homeostasis (Lu & Mackie, 2016). In invertebrates, ECS components are variable, often less specialised and may be missing entirely, notably in insects, but similar endocannabinoid-like systems play important roles in some groups, suggesting evolutionary precursors to the vertebrate ECS (Clarke et al., 2021; Elphick, 2012; Lutz, 2007).

4.3.2 The effect of 7-OH-CBD exposure on the regeneration of *L. variegatus*

By contrast, 7-OH-CBD exposure demonstrated a strong time-dependent progression in regeneration across all tested doses (0-5 µM) (Figure 19, $N \geq 15$). Head regeneration under 7-OH-CBD showed consistent fold increases from 24 to 72 HPA; tail regeneration similarly progressed over time. Indicating CBD-specific ROS interference, it is also possible that the metabolite 7-OH-CBD selectively modulates ROS signalling by mildly amplifying ROS bursts or possibly has targeted anti-oxidant effects to reduce pathological ROS without disrupting regenerative ROS gradients (Pagano et al., 2023). This highlights the importance of metabolite-specific studies. While CBD disrupts regenerative processes, its oxidative metabolite 7-OH-CBD appears less inhibitory, allowing time-dependent tissue repair. The findings underscore that minor structural modifications, such as hydroxylation, can profoundly alter the bioactivity of compounds (Rosowsky et al., 1990). This further highlights the importance of metabolism and investigating the presence of CYP enzymes or other drug-metabolising enzymes within *L. variegatus*.

4.3.3 The effect of O-1918 exposure on the regeneration of *L. variegatus*

One of our key observations was understanding the effects of the antagonist O-1918 as it was hypothesised that O-1918 exposure might enhance the regenerative capabilities of *L. variegatus*. NPR-32 found in invertebrates is considered to be a functional homologue to GPR18 and GPR55; these homologues are thought to regulate generative axon navigation and activate monoaminergic signalling cascades in nociception, feeding, development and ageing (Clarke et al., 2021). A study by Pastuhov et al (2016) in *C. elegans* found that endocannabinoid signalling regulates axon regeneration by promoting the JNK/MAPK pathway. They found that AEA acts to inhibit axon regeneration response by suppressing JNK signalling pathway via NPR19 and NPR32 (Pastuhov et al., 2016). Therefore, it was hypothesised that O-1918, a known antagonist at mammalian receptors GPR18 and GPR55 (Simcocks et al., 2019), would block invertebrate homologs NPR-32 and work similarly to AEA by blocking JNK signalling, encouraging axon regeneration.

Contrary to the hypothesis, O-1918 did not improve regeneration in *L. variegatus* (Figure 20, $N \geq 15$), instead, inhibition of tail regeneration was observed at 5.0 µM ($p < 0.05$). In *C. elegans*, NPR-19 /NPR-32 activation by AEA suppressed JNK/MAPK signalling, impairing regeneration (Clarke et al., 2021; Pastuhov et al., 2016). Blocking these receptors via O-1918 was expected to disinhibit JNK and promote regeneration. The lack of such effects can be due to the possibility that *L. variegatus* may not possess the receptor/ homolog function or perhaps that regeneration is regulated in *L. variegatus* via alternative pathways (Huizen et al., 2022). An alternative explanation is that O-1918 antagonism of GPR18/GPR55 does not work in invertebrate homologs in the same manner. A study found that O-1918 antagonism of GPR18 inhibited Resolvin-D2-mediated muscle regeneration in Duchenne Muscular Dystrophy (DMD) models by reducing myogenesis and impairing functional recovery (Dort et al., 2021). This suggests that although O-1918's antagonism of GPR18/GPR55 enhances regeneration in some models, it has contrasting effects that can vary not only between species but also between disease models.

4.3.4 The effect of long-term CBD exposure on the reproduction of *L. variegatus*

The methodology was derived from previous studies (Silva et al., 2021) conducted similarly over 28 days to assess the effects of chronic exposure; however, this paper used wild-type *L. variegatus* obtained from Mau River in central Portugal, which had an average dry weight of 0.8 mg per worm. Whereas the cultured worms used in our experiments had an average weight of 0.1 mg per worm, other studies suggest a dry weight of 0.2 mg per worm for lab-cultured worms (Betz-Koch et al., 2025). This suggests that for future biomass experiments, the use of wild-type worms will be a better indicator of changes in biomass. The medium used to treat the worms also varies between these two experiments, as Silva et al., 2021 used purified water with a suspension of fish food, whereas within our experiment, we used conditioned artificial pond water. However, both experiments used sediment to allow for natural borrowing behaviours.

The data from the 28-day exposure experiment reveal a sharply dose-dependent response of *L. variegatus* to CBD. The findings suggest that *L. variegatus* is tolerant to low micromolar levels of CBD but highly susceptible to levels approaching or exceeding 5.0 µM. This data further supports the sublethal physiological and behavioural effects reported, the reduction in locomotor activity, inhibition of regeneration and disrupted energy reserves (Williams et al., 2025). These sublethal effects have the potential to cascade through the population as they reduce their chances of survival.

This current data demonstrates a steep toxicity curve and potential threshold between 5.0-14.1 µM CBD. Similar reproductive and developmental toxicity has been observed in other aquatic species, such as *D. rerio* (Ahmed et al., 2018; Carty et al., 2018), indicating that CBD's disruptive effects may affect other organisms. Should such concentrations occur in the environment, they could lead to severe impacts on aquatic environments. Future research should explore intermediate concentration intervals to narrow down the concentration range of toxicity onset and inform regulatory benchmarks to protect aquatic environments from potential CBD contamination.

5. Limitations

Assessment of Abn-CBD toxicity was complicated by the use of methyl acetate as a solvent. Abn-CBD was supplied pre-dissolved in methyl acetate, which was toxic to the worms. Methyl acetate, due to its low boiling point and hydrophobic nature (Graczová et al., 2018), causing erratic toxicity results, affecting even the control groups. The solvents' volatility and poor solubility in artificial pond water likely led to vapour exposure and non-specific toxicity, making it difficult to accurately assess the effects of Abn-CBD itself.

In addition to solvent-related issues, the experimental design of the stereotypical movement assay presents certain limitations, such as subjectivity in the 3-point scoring scale used, it not only lacks the granularity to capture subtle behavioural changes it also risks bias as interpretation could be inconsistent between observers.

The method of stimulation with a pipette further complicates the interpretation of the results as it can sometimes damage the worm, affecting its response and the results if injury occurs or if the tail is separated from the worm. It can also trigger a stress response that is unrelated to the drug. A solution to this problem is using vibrotactile stimuli or light pulse as *L. variegatus* is photosensitive (Bohrer, 2006).

The free locomotion assay, however, is conducted in a constrained environment to restrict 3D movement; this also constricts natural behaviours like burrowing. The image stacking used to analyse the raw data does not account for overlap i.e; if a worm crosses its own path again.

5. Future Directions and Wider Implications

In order to implement these findings effectively, future work must bridge mechanistic studies in model organisms with relevant clinical and environmental applications. Firstly, clarifying metabolic pathways and identifying detoxification mechanisms in *L. variegatus* by characterising CYP450 homologs will enhance our understanding of its utility as an *in vivo* pharmacological model (Snyder, 2000). Using magnetic resonance spectroscopy (MRS) to directly measure acetylcholine esterase activity in *L. variegatus* exposed to CBD to confirm that CBD inhibits AChE activity and therefore reduces movement and alters physiological function. Additionally, exploring CBD's interaction with ROS signalling, quantified using H2DCFDA probes (Beinart & Gillen, 2024), can reveal unknown mechanisms of action relevant to both regeneration and toxicity.

Exploring the synergistic effects of cannabinoid compounds can assist us in alleviating any unwanted side effects or modulating their effects (Ferber et al., 2020). Exploring CBD metabolites have also highlighted the importance of secondary modifications, such as hydroxylation other modifications such as halogenation and glycosylation can be investigated as they may enhance or modulate cannabinoid properties (Ujváry & Hanuš, 2016). Long-term exposure studies can also be conducted to investigate tolerance in *L. variegatus*.

Environmentally, CBD presents high risks due to persistent motor deficits observed at ecologically relevant concentrations, which raises concerns for aquatic ecosystems. Two separate studies conducted in Spain and California discovered that CBD was found in 43% to 80% of sewage sludge samples tested, while THC was present in 7% to 100% of sewage samples. Reported CBD concentrations ranged from 0.1 µM to 1.5 µM (Black et al., 2019; Mastroianni et al., 2013). Interestingly, cannabinoid compounds show higher tolerance within mammalian systems; therefore, the modulation observed within *L. variegatus* at low concentrations can help us understand the mechanisms targeted and reveal conserved targets for regeneration and toxicity.

Advancing our mechanistic understanding of cannabinoid action in invertebrate models, alongside continued clinical innovation and environmental monitoring, will be crucial for harnessing the therapeutic benefits of cannabinoids while minimising their ecological risks.

This can assist in informing safer drug development and also contribute to managing any ecological concerns that may arise due to rising cannabinoid use.

7. Conclusion

This study provides new insights into the pharmacological and toxicological effects of CBD, its metabolite 7-OH-CBD and the antagonist O-1918 in the aquatic invertebrate *L. variegatus*. Our findings reveal that CBD exposure leads to significant dose-dependent impairments in behaviour, movement and regenerative capacity, with persistent effects observed at sub-lethal concentrations. Notably, 7-OH-CBD displayed lower acute toxicity and did not impair regeneration, which highlights the importance of metabolite-specific effects. The synthetic antagonist O-1918, contrary to the hypothesis, did not enhance regeneration and instead showed inhibitory effects on tail regrowth, suggesting species-specific differences in receptor function and signalling pathways.

Comparative analysis with other invertebrate models demonstrated that *L. variegatus* was particularly sensitive to CBD, with toxic and behavioural effects occurring at much lower concentrations than those reported in *D. rerio*, *C. elegans* or mammals. The results obtained support the hypothesis that CBD disrupts neuromuscular function or ROS signalling (Puopolo et al., 2022), ultimately impairing both movement and tissue regeneration. These effects are further established by CBD-induced metabolic shifts, including reduced carbohydrate stores and increased lipid levels, which limit the organism's capacity for repair and recovery.

Beyond the laboratory, our findings raise important ecological concerns. CBD and related cannabinoids are increasingly detected in aquatic environments at concentrations investigated with this study that have been shown to cause persistent motor deficits and reduce the regenerative and reproductive capacity in detritivore species like *L. variegatus*. Given the ecological role of these organisms in nutrient cycling and sediment health, such impacts could have cascading effects on freshwater ecosystems.

This study underscores the need for environmental monitoring of cannabinoid contaminants and highlights *L. variegatus* as a sensitive model for detecting subtle neurotoxic and regenerative effects of emerging pollutants and pharmacologically active compounds. Further research should focus on clarifying the molecular mechanisms underlying these effects, exploring the broader ecological implications and both environmental policy and the safe development of cannabinoid-based therapeutics.

8. Appendices

Worm	Treatment	Body Reversal Movements					Helical Swimming Movements				
		1	2	3	4	5	1	2	3	4	5
A1	Baseline										
	Control										
	Rescue										
	24 Hour Rescue										
A2	Baseline										
	Rescue										
	24 Hour Rescue										
A3	Baseline										
	Rescue										
	24 Hour Rescue										
B1	Baseline										
	Rescue										
	24 Hour Rescue										
B2	Baseline										
	Rescue										
	24 Hour Rescue										
B3	Baseline										
	Rescue										
	24 Hour Rescue										

Table 4: Scoring sheet used to measure *L. variegatus* stereotypical behaviours.

8.1: Previous CBD experiments done by Carriere et al., 2023 (Unpublished)

Work conducted within the Studies To Observe Novel *in vivo* Endocannabinoid-related Drugs (STONED) project has contributed to the optimisation of methods within this study

8.1.1: CBD Toxicity

The results demonstrated a dose-dependent toxicity of CBD in *L. variegatus*. Figure 22 A tested CBD concentration of 0-250µM and the graph demonstrated a steep decline in survival rate with 100% fatality rate at concentrations of ≥30 µM CBD (*N*=6). The concentration range was narrowed to 0-50µM in Figure 22 B. The findings determined an LD50 of 23.00 µM (95% CI: 21.74-24.26 µM, *N*=9) for CBD in *L. variegatus*.

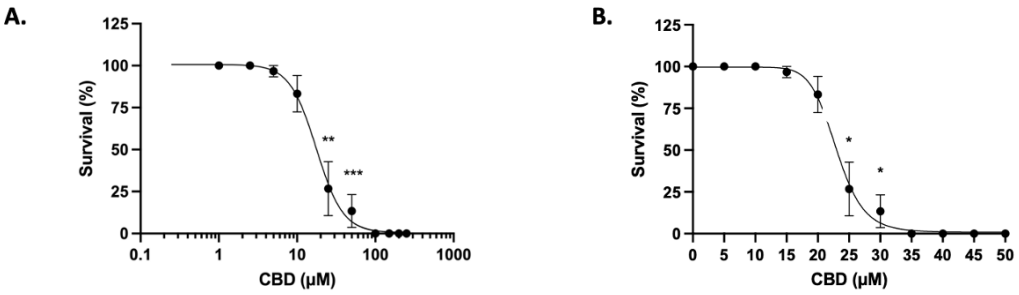

Figure 22: Dose response of CBD in *L. variegatus*.

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 the population of *L. variegatus* after 24-hour CBD exposure. Survival is expressed as a percentage of the untreated control after exposure to CBD. Veh: 0.5% DMSO in artificial pond water. Error bars represent the standard error of the mean. **p*<0.05, ***p*<0.01 or **** *p*<0.0001

8.1.2: Behavioural Effects of Acute CBD Exposure

In Figure 23 A and B, it was observed that CBD significantly inhibits *L. variegatus* body reversal and helical swimming movements, when exposed to concentrations ≥5 µM for 10 minutes (p<.05, Figure 23 A-B). After 10-minutes of recovery in drug-free artificial pondwater, we observed that body reversal movement continues to be significantly reduced at CBD concentrations ≥5 µM (p<.05, Figure 23 C), however, 5 µM did not have prolonged effects on helical swimming when removed (p>.05, Figure 23 D). Helical swimming was significantly restricted at concentrations of ≥10 µM (p<.05, Figure 23 D). However, after a 24 hour recovery period in drug-free artificial pondwater, we observed no long-term effects at 5-10 µM on helical swimming (p>.05, Figure 23 C-D) or body reversal movement (p<.05, Figure 23 C), and helical swimming (p<.05, Figure 23 D) are significantly inhibited at the ≥15 µM concentrations, in comparison to baseline.

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 23 E). Contrastingly, during the 10-minute rescue period in drug-free artificial pondwater, a significant increase in movement was observed by 27±8.68% at 5 µM (p=.0212, Figure 23 F). After a 24-hour rescue period in drug-free artificial pondwater, we observed that unstimulated behaviour is significantly reduced to 71±11.64% in comparison to baseline at 15 µM (p=.0188 Figure 23 F) and 20 µM, where movement is reduced to 61±13.52% compared to the baseline (p=.0120, Figure 23 F).

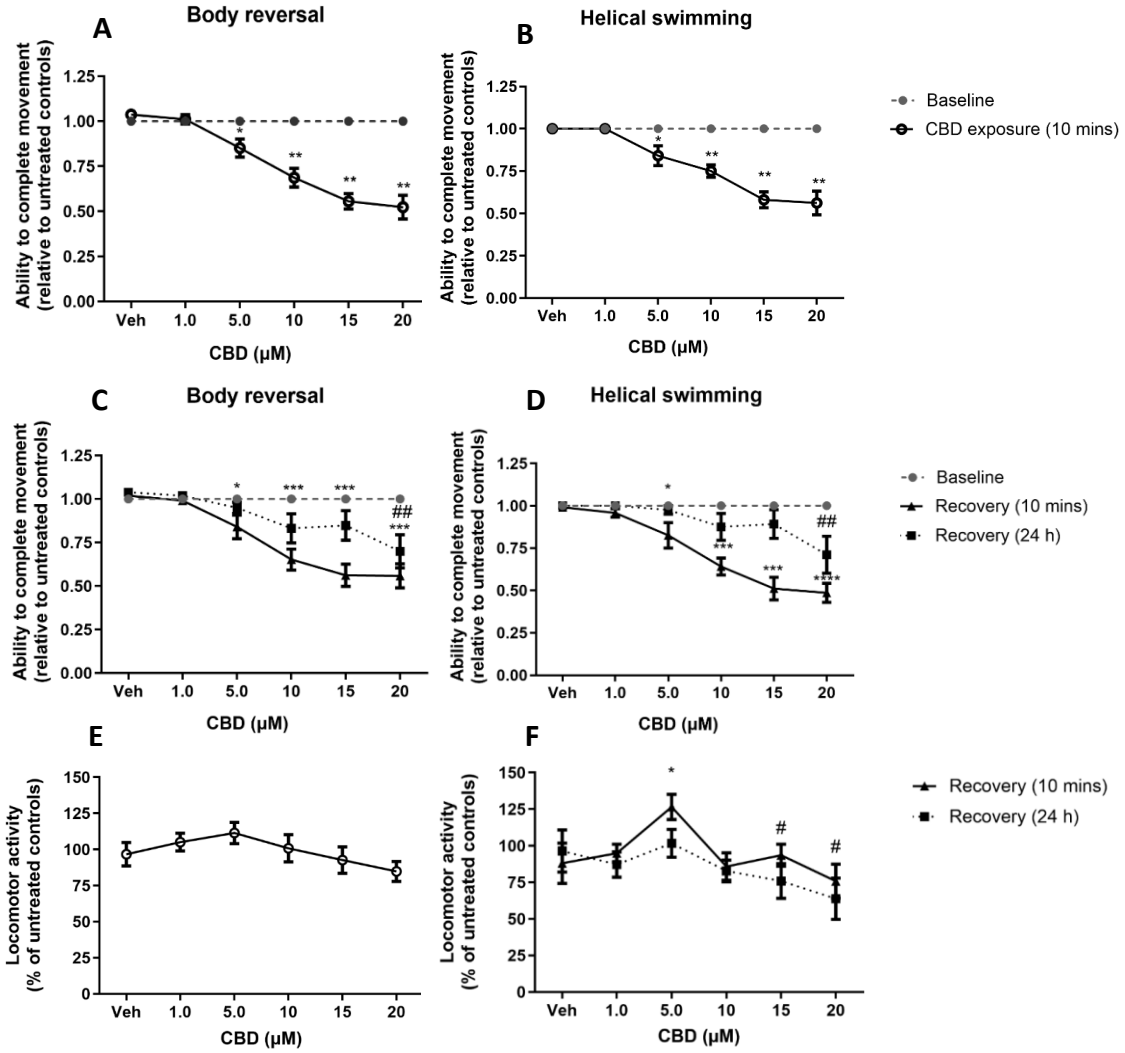

Figure 23: Behavioural assays were conducted on *L. variegatus* following 10-minute exposure to CBD (0-5 µM). Behavioural responses were investigated using stereotypical movement assays that tested **(A)** body reversal and **(B)** helical swimming. Ability to conduct **(C)** body reversal and **(D)** helical swimming were tested after removal from drug solutions at Recovery 10 mins and Recovery 24 h **(E)** 24 hours of CBD exposure on *L. variegatus* ability to conduct unstimulated movement (**F**) effects on unstimulated movement after Recovery from CBD solutions were tested at Recovery 10 mins and Recovery 24 h. Area covered is expressed as a percentage of movement relative to baseline. All data is reported as the ratio of movement of the worm at CBD exposure and at Recovery (10 mins) and Recovery (24 h), relative to the movement at baseline. Error bars represent ±SEM.*refers to either CBD Treatment or Recovery (10 mins), # refers to Recovery (24 h) ; */# p<0.05, **/## p<0.01, ****/#### p<0.0001. N=8.

8.2 Behavioural Effects of *Lumbriculus variegatus* when exposed to Abn-CBD

Exposure to Abn-CBD (0-5 µM) over 24 hours showed that *L. variegatus* ability to conduct stereotypical movements of body reversal and helical swimming was inhibited by Abn-CBD exposure. The inability to respond to stimulation of the posterior and elicit body reversal behaviour was observed at 2.5 µM Abn-CBD (p=0.0234) and 5 µM Abn-CBD (p=0.0312), Figure 24 A). Whereas, the inability to respond to stimulation of the anterior and elicit helical swimming behaviour was observed at 2.5 µM Abn-CBD (p=0.0156, Figure 24 B).

Following removal from Abn-CBD solutions and incubation in APW, the inability to respond to stimulation persisted. 10 minutes after recovery, effects were observed in *L. variegatus* exposed to 2.5 µM Abn-CBD (p=0.0004) and 5.0 µM Abn-CBD (p=0.0074), effects persisted 24 hours after recovery. This was observed at 2.5 µM Abn-CBD (p=0.0022) and 5.0 µM Abn-CBD (p=0.0257), Figure 24 C.

Inability to perform helical swimming was documented at 0.5 µM Abn-CBD (p=0.0492), 2.5 µM Abn-CBD (p=0.0002) and 5.0 µM Abn-CBD (p=0.0045) at 10 minutes after recovery. Effects persisted at 2.5 µM Abn-CBD (p=0.0001) and 5.0 µM Abn-CBD (p=0.0073) after 24 hours of recovery (Figure 24 D).

The locomotor abilities of *L. variegatus* were inhibited at 0.1 µM Abn-CBD (p=0.0412) and 2.5 µM Abn-CBD (p=0.0175) when exposed for 24 hours (Figure 24 F). Removal of Abn-CBD solutions showed full recovery; no significant changes were observed at either 10 minutes or 24 hours after recovery.

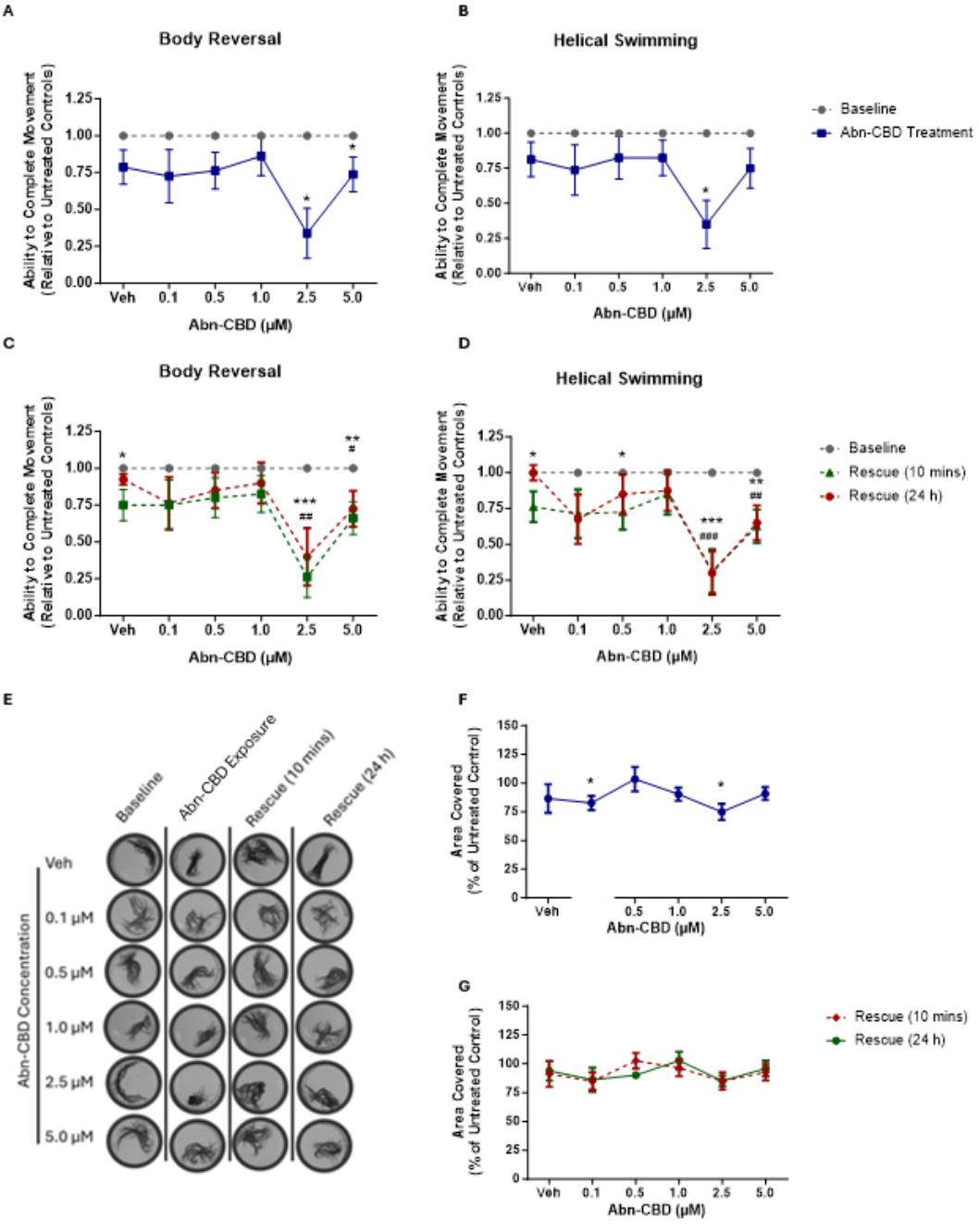

Figure 24: Behavioural Assays of *Lumbriculus variegatus* exposed to Abn-CBD. Behavioural effects on *L. variegatus* following exposure to Abn-CBD (0-5 µM) for 24 hours. Behavioural responses were assessed using, stereotypical movement assays that tested **(A)** body reversal and **(B)** helical swimming. Ability to conduct **(C)** body reversal and **(D)** helical swimming at 10 mins and 24 h Recovery from Abn-CBD solutions was recorded. **(E)** representative images for free locomotion assay, **(F)** 24 hours of Abn-CBD exposure on *L. variegatus* ability to conduct unstimulated movement **(G)** effects on unstimulated movement 10 mins and 24 h after rescue from Abn-CBD solutions. Area covered is expressed as a percentage of movement relative to baseline. All data is reported as the ratio of movement of the worm at Abn-CBD exposure and at rescue (10 mins) (24 h), relative to the movement at baseline. Error bars represent ±SEM.*refers to either Abn-CBD Treatment or Recovery (10 mins), # refers to Recovery (24 h) ; */# $p<0.05$, **/## $p<0.01$, ***/### $p<0.001$. (N=8).

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