A Neurodevelopmental Study of Mice following *In Utero* and Early Postnatal Exposure to Imidacloprid, A Neonicotinoid Pesticide

by

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Abstract

Imidacloprid (IMD), a neonicotinoid insecticide, is the most widely used insecticide on the planet. The purpose of this study was to examine the behavioural and biochemical effects of chronic *in utero* and early postnatal IMD exposure. Our treatment regimen entailed chronic exposure whereby pregnant mice were infused with 0.5mg/kg/day of IMD via a subcutaneous osmotic mini-pump from gestational day 3 to postnatal day 21. Beginning on postnatal day 42, the offspring were studied in a series of behavioural tests assessing locomotor activity, anxiety, social dominance and aggression, sensorimotor gating, and depression, while postmortem biochemical analyses included tests that observed sperm abnormalities, immune response abnormalities, and abnormalities in whole blood composition. A decrease in body weight, increased motor activity, enhanced social dominance, decreased depressive-like behaviours, and a decrease in visible social aggression was observed, demonstrating the induction of abnormal behaviours in mice after *in utero* and early postnatal exposure to IMD.

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Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

5CSRT	5 Choice Serial Reaction Time Task
ADHD	Attention Deficit/Hyperactivity Disorder
ANOVA	Analysis of Variance
AOX	Aldehyde Oxidase
CNS	Central Nervous System
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
GD	Gestational Day
IMD	Imidacloprid
nAChR	Nicotinic Acetylcholine Receptor
NOAEL	No Observed Adverse Effect Level
PBS	Phosphate Buffered Saline
PND	Postnatal Day
PNE	Prenatal Nicotine Exposure

1. Introduction

1.1 Neonicotinoids

Neonicotinoid pesticides are among the most effective and most prevalent insecticides on the planet (Jeschke et al., 2011). As the fastest growing class of insecticides, neonicotinoids have gained their popularity, in part, as a result of their versatility. The seven neonicotinoid pesticides currently on the market (acetamiprid, clothianidin, imidacloprid, nitenpyram, nithiazine, thiacloprid and thiamethoxam) are used to control sucking pests on a diverse range of major crops including (but not limited to) vegetables, pome and stone fruits, citrus, rice, cotton, corn, potato, sugar beet, oilseed rape, and soybean (Jeschke et al., 2011). Neonicotinoid pesticides are also diverse in the way by which they can be applied to plants. Foliar sprays, seed treatments and soil application are currently the primary methods of application, while application methods such as irrigation water in drip or drench systems for vegetables or floating box systems for tobacco seedlings have recently gained popularity (Jeschke et al., 2011).

1.1.1. Neonicotinoid Pharmacology

In addition to versatility, the true quality that distinguishes neonicotinoids from other pesticides is the mode of action by which they elicit their insecticidal effects. Neonicotinoids were designed to be structurally similar to the potent and well-known toxin nicotine, and therefore act on the central nervous system (CNS) as agonists of nicotinic acetylcholine receptors (nAChRs) (See Appendix Fig. 1)(Casida and Durkin, 2013). nAChRs are ligand-gated cation channels which are responsible for excitatory neurotransmission (Tomizawa and Casida, 2005). While the structure and diversity of insect nAChRs is not completely clear (as only a few insect nAChR subunit genes have been identified, while some remain classified as

candidate genes), it is known that the vertebrate nAChR ion channels consist of various subtypes assembled in combinations of α, ß, γ and δ subunits, with each subtype having variable sensitivities to nAChR agonists (Albuquerque et al., 2009, Tomizawa and Casida, 2005). Neuronal nAChR subtypes expressed in the brain of vertebrates are assembled in combinations of α2-α10 and ß2-ß4, with the α4ß2 and α7 subtypes being the two most abundant (Tomizawa and Casida, 2005). nAChR agonist activity in insects and vertebrates results in many cellular modifications such as a rapid change in cell membrane potential (due to an influx of Na^{+} and Ca^{++} ions), an increase in the number of nAChRs in the synaptic cleft, apoptosis of neurons, and proliferation of glia. Each of these effects, alone and combined, can lead to significant detrimental effects (Smith et al., 2010).

It is important to note that neonicotinoids affect insects and vertebrates differently (Tomizawa and Casida, 2003). Neonicotinoids are highly potent agonists of insect nAChRs and are said to have low toxicity to mammals (Tomizawa and Casida, 2005). It is suggested that selectivity for insect nAChRs is due to their protonation status at physiological pH; neonicotinoids are not protonated at physiological pH, and are therefore electronegative in charge, which leads to neonicotinoid binding at a unique cationic subsite on insect nAChRs with higher affinity (IC_{50} = 4.6 nM and EC_{50} = 0.86-1 μM for imidacloprid) than at the anionic subsite on vertebrate/mammalian nAChRs (α4ß2 IC_{50} = 2600 nM and EC_{50} = 70 μM for imidacloprid) (Tomizawa and Casida, 2003, Bal et al., 2010). However, it must also be noted that the α4ß2 affinities described for imidacloprid are for the parent compound only. It has been suggested that imine metabolites from neonicotinoids are more toxic to mammals than the parent compound, and have affinities at the α4ß2 receptor that are comparable to or greater than that of nicotine (Tomizaea and Casida, 2003). One study supports this claim, as they found in M10 (melanoma) cells expressing α4ß2 nAChRs, that imidacloprid and thiacloprid upregulated α4ß2 nAChRs by five- to eightfold while the imine derivatives upregulated α4ß2 nAChRs by

eightfold (Tomizawa and Casida, 2000). As such, the metabolites of neonicotinoids must be investigated further.

1.1.2 Human Exposures

While it has been widely assumed that the nAChRs agonist activity of neonicotinoids is selective for insects, and has little to no effect in mammals, the validity of this statement has recently come into question (Badgujar et al., 2013). Increasing evidence suggests neonicotinoid pesticides are a potential health risk for humans, with one research group claiming neonicotinoids "can cause heart, kidney, and other organ damages along with gastrointestinal irritation, neurological symptoms and even death when ingested along with alcohol" (Badgujar et al., 2013). As such, it is imperative to further investigate the detrimental off-target effects of neonicotinoids.

Studies on human exposures to neonicotinoids are scarce. Due to the high water solubility of neonicotinoids, it is now common to find neonicotinoid contamination in groundwater and surface water in areas where these pesticides are used (see Gibbons et al., 2014). The Texas Poison Center Network reports that between 2000-2012, there were "1142 neonicotinoid exposure cases reported, with less than 2% reported as intentional exposure cases" (Forrester, 2014). The highest rate of reported exposure was in urban counties where exposure resulted from domestic use of neonicotinoids in gardens or as flea control for pets (Forrester, 2014). Due to the potential for exposure in ways that are not direct (i.e. through contamination) it is likely that many cases of human exposure to neonicotinoids are unreported. Studies have demonstrated that residual concentrations of neonicotinoids are found on crops often consumed by humans, including apples, grapes, broccoli, bell peppers and

cauliflower (Chen et al., 2014). According to the Canadian Food Inspection Agency, the most persistent neonicotinoid found on crops was imidacloprid (Public Health Ontario, 2014).

1.1.3 Policy Surrounding Neonicotinoids

Neonicotinoids are currently approved for use without restriction in both Canada and the United States. Over ten years ago, Health Canada's Pest Management Regulatory Agency approved use of these pesticides after a human and environmental risk assessment and value assessment was completed. It was believed that neonicotinoids offered a safer alternative to the insecticides in use at the time, and thus neonicotinoids were approved for use as seed treatments, foliar sprays and soil applications, for a wide variety of crops. However, in the spring of 2012, Health Canada received a large number of honeybee mortality reports from Alberta, Manitoba, Saskatchewan, Nova Scotia, Quebec and Ontario (Health Canada, 2013). After investigation, it was found that the bee mortalities coincided with the planting of neonicotinoid treated corn seeds (Health Canada, 2013). Consequentially, the Pest Management Regulatory Agency declared that the use of neonicotinoid treated corn and soybean seeds was not sustainable (Health Canada, 2013). Despite the concerns outlined in this report, unrestricted neonicotinoid use continues within Canada and the United States today.

In Europe, the policy surrounding neonicotinoid use is quite different. In 2013, the European Food Safety Authority published a study stating that some neonicotinoids pose an unacceptably high risk to bees (European Food Safety Authority, 2013). In response to the study, the European Commission recommended a restriction of neonicotinoid use across the European Union. In a vote in the spring of 2013, 15 of the 27 European Union members (at the time of the vote) voted to restrict the use of three neonicotinoids (imidacloprid, clothianidin and thiamethoxam) for two years. A review of this restriction is being conducted in 2016.

1.2 Imidacloprid

N-{1-[(6-chloro-3-pyridyl) methyl]- 4,5-dihydroimidazol-2-yl} nitramide, more commonly known as imidacloprid (IMD), was the first neonicotinoid insecticide to be produced and is currently the most widely used insecticide (Abbo et al., 2016). IMD is a member of the chloronicotinyl nitroguanidine chemical family and was designed to structurally mimic nicotine (Gervais et al., 2010). Ultimately, IMD was produced with the intention of generating a compound that is persistent and has high systemic toxicity against insects, with limited mammalian toxicity (Kumar et al., 2013). Based on the limited animal studies completed to date, the World Health Organization has classified IMD as "moderately toxic" or Class II. Furthermore, IMD is said to be very low in toxicity through dermal exposure, moderately toxic through ingestion, and highly toxic through aerosol inhalation (Kumar et al., 2013).

IMD is persistent in the environment. In a 2005 study, where IMD concentrations in soils from a broad range of climates were analyzed, it was noted that 91% of all analyzed soil samples contained measurable concentrations of IMD (>0.1µg/kg), despite the fact that only 15% of the analyzed soil samples had been treated with IMD less than 365 days prior to the study (Bonmatin et al., 2005). IMD was detected in 100% of soils that were seeded with IMD-treated seeds less than 365 days before analysis, while IMD was detected in 97% of soils that were seeded with IMD-treated seeds 1-2 years before analysis (Bonmatin et al., 2005). The calculated soil half-life of IMD, which is dependent on many factors (i.e. soil type, moisture, pH, etc.) ranges from 100 to 1230 days following application (Baskaran et al., 1999). When IMD is metabolized in soil, the major metabolites formed include imidacloprid urea, 6-chloronicotinic acid, and 6-hydroxynicotinic acid (which subsequently forms carbon dioxide) (Rouchaud et al., 1996)

The absorption, mode of action and metabolism of IMD in mammals has been studied. Upon exposure, IMD is rapidly absorbed (92%) and distributed with a peak plasma concentration within 2.5 hours (Kumar et al., 2013). In mammals, IMD binds irreversibly to postsynaptic nAChRs located in the central nervous system and at neuromuscular junctions, preferentially at the α4ß2 nAChR subunit (Kumar et al., 2013). By binding to postsynaptic nAChRs, IMD acts as an agonist and subsequently leads to persistent receptor activation (Kumar et al., 2013). This receptor activation is described as persistent, as acetylcholine esterase activity at the synapse cannot degrade IMD (Kumar et al., 2013). As a result of persistent receptor activation, the endogenous nAChR ligand acetylcholine accumulates, as it no longer has a vacant binding site available for binding at the receptor. This irreversible binding of IMD at the receptor results in the receptor remaining opened, blocking nerve impulses (which require both opening and closing of the receptor) (Kara et al., 2015).

IMD metabolism in animals is rapid and primarily occurs in the liver by CYP450 enzymes and Aldehyde Oxidase through two major pathways: 1) oxidative cleavage, to 6-chloronicotinic acid and imidazolidine, and 2) hydroxylation, to 5-hydroxyimidacloprid and olefin derivatives; also produced (through a less significant metabolic pathway) is a nitrosoimine metabolite (see Appendix Fig. 1). The 5-hydroxyimidaclopid and nitrosoimine IMD metabolites both contain an imine group and are therefore described by some as "imine derivatives of IMD". These imine neonicotinoid metabolites, as mentioned above, are highly potent at the mammalian nAChR, with affinities comparable to nicotine (Tomizaea and Casida, 2003). After metabolism, IMD is then excreted, with only 10%-16% of a dose being excreted as unchanged IMD (Kumar et al., 2013).

1.2.1 Imidacloprid and Insect Studies

The vast majority of the scientific literature surrounding IMD pertains to the effects of IMD exposure in insects, particularly *Apis mellifera* (the western honeybee). One study found that sublethal doses of IMD (0.02 ppm, 0.1 ppm, 0.2 ppm and 0.4 ppm) decreased the viability of sperm (collected from the spermetheca of queen *A. mellifera*) by 50%, seven days post-treatment (Chiamanee et al., 2016). In the same study, a low dose of IMD (0.02 ppm) suppressed the expression of genes related to antioxidation, immunity and development in queen honeybees, one day after treatment.

Interesting observations were made in a study that assessed the effects of a sublethal IMD exposure on worker *Apis mellifera* learning, memory and brain metabolism. Using olfactory learning performance in a proboscis extension reflex testing procedure, it was found that oral IMD treatment (12 ng per bee) impaired olfactory learning, as shown by a decrease in both acquisition and retention of the task (Decourtye et al., 2004). It is interesting to note that the findings of Decourtye et al. were further supported by a 2012 study, which found that IMD treatment during the larval stage of development (0.04 ng/larva) led to impairments in olfactory associative behaviour in the mature adult bees (Yang et al., 2012). Using carbon monoxide histochemistry to map brain metabolism, Decourtye et al. found that oral IMD treatment (12 ng per bee) increased oxidative metabolism in the mushroom body calyces, a brain region known to contribute to memory-related neural plasticity (Decourtye et al., 2004). Another study found that sublethal IMD doses also reduced microglomerular density in this same brain region, providing evidence that the mushroom body is an important brain region in mediating the effects of IMD on neurodevelopment (Peng et al., 2015). The findings of these studies are of importance as memory and learning obviously are imperative for the fitness of the honeybee.

In another study, foraging behaviour in worker honeybees exposed to sublethal IMD doses was investigated. By measuring the elapsed time between visits to the same feeding site, it was found that when bees were treated with IMD at a dose greater than or equal to 50 µg/L, the time between visits increased; this delay in "return visits" was found to be concentration-dependent (Yang et al., 2008). At doses of 1200 µg of IMD/L, more severe foraging abnormalities were observed, as some honeybees went missing, while some returned the next day (a delay that is several hours longer than the 300-second delay observed in untreated honeybees) (Yang et al., 2008).

In addition to honeybees, bumble bees (*Bombus terrestris*) are also very important pollinators, whose populations have declined in recent years (Laycock et al., 2012). In a study that observed the reproductive effects of IMD exposure on queenless microcolonies of bumble bees, it was found that IMD doses in the range of 1 µg/L are capable of reducing brood production by 33%, thus a decline in population (Laycock et al., 2012).

The literature surrounding IMD effects in non-bee insects is plentiful, and tends to show similar results to those previously mentioned in honey and bumble bees. For example, in a study observing the effects of a sublethal IMD exposure on the fecundity, longevity and enzyme activity of two aphid species (*Sitobion avenae* and *Rhopalosiphum padi*), it was found that if exposure to IMD was sustained over three generations, fecundity and longevity are both significantly decreased (Lu et al., 2016). Furthermore, in a study observing the effects of a sublethal (1.25 µg/mL) IMD exposure on foraging and competition behaviour in two ant species (*Lasius niger* and *Lasius flavus*), it was found that this exposure to IMD causes a decrease in foraging, a decrease in activity, and an increase in aggression (Thiel et al., 2016).

To summarize, when looking at the scientific literature surrounding the effects of IMD on insect biochemistry and behaviour, substantial evidence exists to suggest that sublethal IMD exposure leads to a wide array of effects, most notably a decrease in insect foraging behaviour, a

decrease in fecundity and reproduction, a decrease in longevity, impairments in immunity, and impairments in memory and learning.

1.2.2 Imidacloprid and Animal Studies

While the literature on the effects of IMD exposure in insects is quite extensive, very few studies regarding the effects of IMD exposure (acute or chronic) on mice and/or rats have been published to date. In a study by Badgujar et al., 2013, the effects of a chronic 28-day oral IMD exposure, at a dose of 10 mg/kg, 5 mg/kg and 2.5 mg/kg, in 4-6 week old mice were investigated. At the 10 mg/kg dose they observed a suppressed cell-mediated immune response, as well as histopathological alterations in the spleen and liver (Badgujar et al., 2013). The 5 mg/kg dose also appeared to impair the cell-mediated immune response, while the low dose had no observable effect (Badgujar et al., 2013).

A study conducted on Wistar rats that were orally exposed to 10 mg/kg, 30 mg/kg and 90 mg/kg of IMD per day, from gestational day 6 (GD6) to postnatal day 21 (PND21), or GD6 to PND42, sought to evaluate cell-mediated and non-specific immunity (Gawade et al., 2013). In this study, through the use of a delayed-type hypersensitivity assessment (to evaluate cell-mediated immunity) and phagocytic index assessment (to evaluate non-specific immunity), it was found that IMD caused age-dependent adverse effects on immune system development, which was worsened by continuous exposure throughout development, ultimately leading to a compromised immune system (Gawade et al., 2013). This study also confirmed the aforementioned findings of Badjugar et al., 2013.

Another study on the effects of IMD exposure in animals was done by Bal et al., 2012, in which they exposed adult male rats to an oral dose of 0.5 mg/kg, 2 mg/kg or 8 mg/kg of IMD for 90 days, all of which are at or below the No Observed Adverse Effect Level (NOAEL) for

rats. In this study, the 8 mg/kg dose led to deterioration in sperm motility, a decrease in epidydimal sperm concentration, and an increase in abnormal sperm morphology (Bal et al., 2012a). The 2 mg/kg dose also led to a decrease in epidydimal sperm concentration, while the 0.5 mg/kg dose had no observable effect (Bal et al., 2012a). In another study done by this same research group, using the same dosing regimen on 7-day-old rats, it was reported that IMD exposure induced DNA damage through oxidative stress in the reproductive organs of male rats (Bal et al., 2012b). The findings of Bal et al., 2012, were further supported by a 2015 study that exposed Swiss albino mice to 22 mg/kg, 11 mg/kg and 5.5 mg/kg of IMD, for 7, 14 and 28 days, where it was observed that all doses (5.5 mg/kg, 11 mg/kg and 22 mg/kg) of IMD induced significant sperm head abnormalities after 14 and 28 days of IMD exposure (Bagri et al., 2015).

In one of the few animal studies in the neonicotinoid field published to date that contains a behavioural component, researchers documented the behavioural and pathological effects of a single 337 mg/kg dose of IMD in pregnant Sprague-Dawley rats on GD9 (Abou-Donia et al., 2008). Specifically, Abou-Donia et al. assessed sensorimotor performance (using the inclined plane test, the beam-walking test and forepaw grip testing) and pathological alterations in the brain (using glial fibrillary acidic protein immunostaining) in the offspring of the exposed mothers, and found that the IMD treatment led to significant sensorimotor impairments at PND30, and an increase in glial fibrillary acidic protein immunostaining in the motor cortex layer III, Cornus Ammonis 1 (CA1), Cornus Ammonis 3 (CA3), and the dentate gyrus subfield of the hippocampus (Abou-Donia et al., 2008).

The literature on the effects of IMD on avian health is more extensive compared to studies on mammals. One study showed that IMD-treated seed ingestion produced a lethal effect on adult partridge (Lopez-Anita et al., 2015). One year-old red-legged partridges were fed seeds treated with 40-200 mL of IMD per 100 kg of seed (representing 80% to 100% the recommended application rate for cereal seed coating) and the effects of IMD on partridge

survival, body condition, oxidative stress biomarkers, plasma biochemistry, carotenoid-based coloration, t-cell mediated immune response and reproduction were examined (Lopez-Anita et al., 2015). The high dose has a 100% rate of lethality, with mortality occurring faster in females than in males, while the low dose had no effect on mortality (Lopez-Anita et al., 2015). The low dose reduced glucose, magnesium and lactate dehydrogenase, increased blood superoxide dismutase activity, produced changes in carotenoid-based integument colouration, and had negative effects on reproduction and immunity (Lopez-Anita et al., 2015). These results indicate that normal use of IMD has significantly detrimental effects on avian health.

In addition, another study observed the effects of neonicotinoids (specifically IMD) on insectivorous bird populations (Hallmann et al., 2014). In this study conducted in the Netherlands, it was found that in areas where IMD surface water concentrations exceeded 20 ng/L, the bird populations declined by 3.5% annually (Hallmann et al., 2014). Additional analyses found that this decline appeared only after the initiation of IMD use (in the mid 1990's) (Hallmann et al., 2014). Taken with the results of Lopez-Anita et al., 2015, these results suggest that the impact of IMD on the environment is greater than previously reported, and the off-target effects on vertebrates requires substantial investigation.

To summarize, the literature published to date surrounding the effects of IMD on non-insect species is relatively scarce, yet it does provide some evidence suggesting IMD exposure may lead to impairments in immunity, reproduction (specifically sperm morphology), and may also lead to oxidative stress in birds and mammals. In addition, IMD exposure may also lead to sensorimotor impairments. To date there are no published studies that have assessed the behavioural effects of a chronic *in utero* and early postnatal exposure to a low dose of IMD in animals.

1.3 Developmental Effects of Nicotinic Acetylcholine Receptor Agonist Exposure

Nicotine is the definitive nicotinic acetylcholine receptor agonist. The negative effects of prenatal nicotine exposure (PNE) on human and animal health, and specifically on brain development, are well documented. As an exogenous nAChR agonist, nicotine acts on the nAChRs in the CNS, ultimately leading to receptor desensitization. Nicotine readily crosses the placenta and has the ability to enter fetal circulation (and subsequently the fetal CNS) where it evokes its effects by binding to the nAChRs and disrupting the activity of the endogenous nAChR ligand, acetylcholine (Ekblad et al., 2014). Researchers have found that a correct balance in acetylcholine activity at the nAChRs, and the subsequent balance in the nAChR signaling cascades that control downstream events such as gene expression, cell proliferation, differentiation and apoptosis, is of great importance throughout fetal brain development. Nicotine can cause imbalances in acetylcholine activity, and perturbation of subsequent cell signaling, ultimately leading to detrimental effects on fetal brain development (Tiesler and Heinrich, 2014).

Tests on animals that have been prenatally exposed to nicotine have revealed a wide range of behavioural abnormalities. One study assessed the effects prenatal exposure to cigarette smoke on activity and aggression (Yochum et al., 2014). Mice were exposed to cigarette smoke from GD4 to PND0 for four hours per day, five days per week, at a dose that is equivalent to less than one pack of cigarettes per day, and found that prenatal cigarette smoke led to increased hyperactivity and increased aggression in males (but not females), as indicated by an increase in the total distance travelled in the motor activity test, and an increase in attacks and a decrease in latency to attack in the resident intruder test (Yochum et al., 2014). In

addition, a significant decrease in brain-derived neurotrophic factor mRNA and protein was reported (Yochum et al., 2014).

The findings of Yochum et al., 2014 were supported by a study that observed the effects of chronic PNE on locomotor activity in C57BL/6 mice (Pauly et al., 2004). In this study, pregnant mice were exposed to a 200 µg/ml nicotine/drinking water solution, and locomotor activity was assessed in the offspring (Pauly et al., 2004). A significant increase in locomotor activity was observed in nicotine treated males only, as indicated by an increase in total distance travelled in the open field test (Pauly et al., 2004).

Another study observed the effects of PNE and gestational nicotine exposure on emotional behaviours in C57BL/6J mice (Alkam et al., 2013). Pregnant mice were exposed to 200 µg/ml of nicotine in drinking water, from GD0-GD13, GD14 to PD0, GD0 to PD0, GD14 to PD7, GD0 to PD7 or PD0 to PD7 (Alkam et al., 2013). Between PND28 and PND36, the mice were assessed in several behavioural tests, including the light and dark box test to measure anxiety, marble burying activity test to measure repetitive behaviours and anxiety, novelty-suppressed feeding test to measure anxiety, sociability and social novelty preference test to observe social interactions, social avoidance tube test to measure social dominance/avoidance, and elevated plus maze to measure anxiety (Alkam et al., 2013). The results indicated that treatment of male mice during largest treatment time-window (GD0-PND7) resulted in significant impairments in marble burying behavior (more marbles buried), sociability (a decreased preference for novel mice), an increase in social avoidance and a decrease in the time spent in the open arms of the elevated plus maze (Alkam et al., 2013). In the female mice treated from GD0 to PND7, no significant effects of gestational and PNE on emotional behaviour were observed (Alkam et al., 2013).

A functional magnetic resonance imaging study in human subjects assessed the effects of PNE on verbal working memory (Longo et al., 2014). Twelve young adults with PNE and

thirteen non-exposed controls performed a 2-Back working memory task while blood oxygen level-dependent responses were examined through functional magnetic resonance imaging (Longo et al., 2014). This study concluded that PNE contributes to altered neural functioning during verbal working memory, therefore identifying a long-term detrimental effect of PNE (Longo et al., 2014).

The effect PNE has on the risk for offspring attention deficit / hyperactivity disorder (ADHD) is well studied. The link between maternal smoking and ADHD in children was postulated in a 1996 study, which assessed the association between ADHD and maternal smoking using human male subjects aged 6-12 that were both normal and diagnosed with ADHD (Milberger et al., 1996). This study concluded that maternal smoking during pregnancy was a risk factor for ADHD (Milberger et al., 1996). This same research group extended and improved upon their findings from 1996 in a 1998 study that investigated the role of maternal smoking during pregnancy in the etiology of ADHD (Milberger et al., 1998). Using siblings of ADHD and non-ADHD probands (to provide the study with high- and low-risk subjects), and controlling for sibling ADHD status, socioeconomic status, parental IQ and parental ADHD status, Milberger et al. ultimately reached the same conclusion, as they found a positive association between maternal smoking during pregnancy and ADHD (Milberger et al., 1998).

Subsequently, a 2014 Danish National Birth Cohort study observed the effects of PNE on the risk of ADHD in children (Zhu et al., 2014). This study included 84,803 children, and concluded that maternal and paternal smoking during pregnancy was associated with an increased risk of ADHD (Zhu et al., 2014). This result replicated the findings of a 2012 study that observed the effects of maternal and paternal smoking during pregnancy and risk of ADHD symptoms in 8,324 offspring from the United Kingdom Avon Longitudinal Study of Parents and Children (Langley et al., 2012). This study evaluated the associations between offspring ADHD and maternal and paternal smoking during pregnancy (which were assessed using regression

analyses) and concluded that maternal and paternal smoking during pregnancy were indeed associated with offspring ADHD symptoms (Langley et al., 2012).

The link between PNE and ADHD was further supported by a study in mice, which found that PNE at doses of 0.1mg/kg resulted in hyperactivity, decreases in cingulate cortex volume and radial thickness and decreased dopamine turnover in the frontal cortex, closely mimicking the human ADHD phenotype (Zhu et al., 2012). While the aforementioned studies have established a strong link between PNE and ADHD risk, it has been difficult to prove direct causation (Langley at al., 2012, Zhu et al., 2014). To prove direct causation, a mechanism through which PNE elicits the observed lasting effects on behavior (and thus, ADHD) must be elucidated. One recent study suggested the possibility that there is an underlying epigenetic mechanism that mediates the persistent effects of prenatal nicotine exposure on neuronal structure and resulting behaviour (Jung et al., 2016).

2. Hypotheses, Rationale and Objectives

To the best of our knowledge, no studies to date have been conducted to assess how neonicotinoid pesticides may affect rodents following chronic *in utero* and early postnatal exposure. In light of the substantial body of knowledge on how nicotine elicits its detrimental effects on the CNS during development following *in utero* and early postnatal exposure, and knowing that neonicotinoid pesticides (which are found on fruits and vegetables that are consumed by humans) are nicotine analogs which act in a similar manner, it is imperative to conduct more thorough testing to examine the effects of neonicotinoid pesticide exposure on brain development in mammals (Sanchez-Hernandez et al., 2014). Because imidacloprid is the most widely used neonicotinoid insecticide, we chose to study imidacloprid in the context of chronic exposure in mice throughout development.

I hypothesize that chronic *in utero* and early postnatal exposure to a relatively low dose of imidacloprid will induce abnormal behaviours in the offspring and will also cause lasting detrimental effects on the reproductive and immune systems. The objectives for this study are as follows:

Objective 1: To treat pregnant female mice with imidacloprid from early gestation to postnatal day 21, through osmotic mini-pump infusion.

Rationale: An osmotic pump was implanted as close as possible to GD0 (without disturbing the mating and fertilization process) to ensure the mice were exposed to IMD during the most crucial time of neurodevelopment (prior to PND21). The osmotic pump allowed for the delivery of IMD to the pregnant female mice at a low chronic dose, without the use of repeated injections (and thus repeated handling), both of which are known to be highly stressful for the pregnant mice.

Objective 2: To study the behaviour of the adult mouse offspring using behavioural tests, including tests measuring locomotor activity, anxiety, aggression, depression, sensorimotor gating, attention and cognition.

Rationale: There are currently no published studies on the behavioural effects of a chronic *in utero* and early postnatal exposure to a low dose of IMD. We selected a battery of behavioural tests that assessed behaviours that are commonly affected by chronic prenatal exposure to other nAChR agonists (i.e. nicotine).

Objective 3: To perform selected postmortem analyses on the adult mouse offspring. These included tests measuring cell-mediated immunity, sperm morphology and haematology.

Rationale: The delayed type hypersensitivity assessment and haematology analyses (which measured immune cell concentrations in whole blood samples) were selected to assess if *in utero* and early postnatal exposure to IMD had an effect on immunity. The sperm head abnormality assay was selected to assess the effects of IMD on sperm morphology.

3. Experimental Procedures

3.1 Animals and Treatments

All animal experiments were carried out in accordance with the guidelines set by the Canadian Council on Animal Care and were approved by the University of Toronto Animal Care Committee. Figure 1 shows the experimental organization and timelines of all experiments. In Study 1, female C57BL/6 mice (Charles River) were implanted with an osmotic mini-pump (Alzet Osmotic Pumps: Model 2006, 200 µL reservoir, 0.15 µL/hour flow rate, 42 days duration) containing 0.75 mg/kg/day of imidacloprid (PESTANAL 37894, Sigma-Aldrich) dissolved in 20% dimethyl sulfoxide (DMSO, Sigma Aldrich), 20% DMSO alone, or sterile water alone, thus generating three treatment groups. IMD was dissolved in various concentrations of DMSO, and it was found that at DMSO concentrations of less than 15%, IMD would precipitate out of solution. Therefore, 20% DMSO was selected to ensure IMD would remain well dissolved. Seven days following osmotic mini-pump implantation, the implanted females were mated with untreated C57BL/6 males. The status of the vaginal plug was assessed each day at 9 am and 4 pm; the day the vaginal plug was present was marked GD0. The offspring were caged with their mother until weaning on PND21. After weaning, which included ear tagging for identification, the offspring were sex-matched and housed with siblings in groups of 2-4 offspring per cage. All mice were left undisturbed, with the exception of a biweekly cage cleaning, and behavioural testing. All behavioural tests were performed between 9 am and 5 pm, beginning on PND42. All mice were naïve to each behavioural test, and were tested in each test only once.

In Study 2, 3 and 4, untreated female CD-1 mice (Charles River) were mated with untreated male CD-1 mice. The status of the vaginal plug was assessed each day at 9 am and 4

pm; the day the vaginal plug was present was marked GD0. Between gestational GD3-6, the pregnant female CD-1 mice were implanted with an osmotic mini-pump (Alzet Osmotic Pumps: Model 2006, 200 µL reservoir, 0.15 µL/hour flow rate, 42 days duration) containing 0.5 mg/kg/day of IMD dissolved in 25% DMSO or 25% DMSO alone, thus generating two treatment groups. In Study 2, a water treatment group was added, thus generating three treatment groups. The offspring were caged with their mother until weaning on PND21. After weaning, which included ear tagging for identification, the offspring were sex-matched and housed with siblings in groups of 2-4 offspring per cage. All mice were left undisturbed, with the exception of a biweekly cage cleaning, and behavioural testing. All behavioural tests were performed between 9 am and 5 pm, beginning on PND42. All mice were naïve to each behavioural test, and were tested in each test only once. The behavioural tests were arranged such that the most stressful tests (i.e. the forced swim test) were performed at the end of the behavioural testing, while less stressful tests (i.e. open field test) were performed at the beginning of behavioural testing. Furthermore, the mice only participated in a maximum of five behavioural tests.

In the Pilot Experiment, adult male CD-1 mice were implanted with an osmotic mini-pump (Alzet Osmotic Pumps: Model 2006, 200 µL reservoir, 0.15 µL/hour flow rate, 42 days duration) containing IMD dissolved in 25% DMSO at a dose of 0.5 mg/kg/day, or 25% DMSO alone. Thirty-six days following implantation, the brain, liver, serum, and caudae epididymis were collected from all mice, and a sperm head abnormality assay was performed.

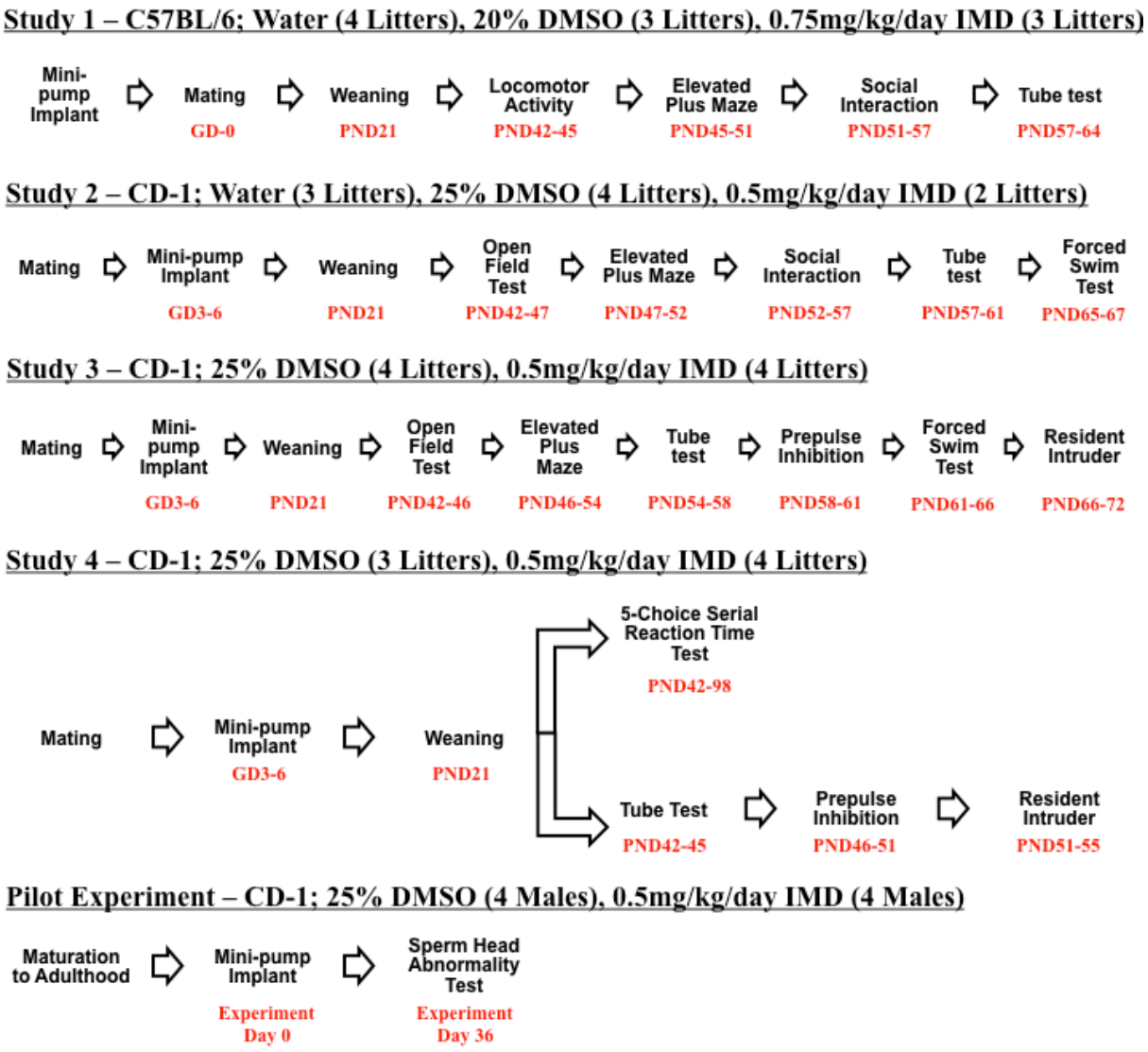


Fig. 1. Study work flow and experimental timelines. Abbreviations: DMSO: Dimethyl Sulfoxide, GD: Gestational Day, IMD: Imidacloprid, PND: Postnatal Day.

	Study 1	Study 2	Study 3	Study 4
Strain of Mice	C57BL/6	CD-1	CD-1	CD-1
Imidacloprid Dosage	0.75mg/kg/day	0.5mg/kg/day	0.5mg/kg/day	0.5mg/kg/day
Offspring Exposure Time	~30 Days	~42 Days	~42 Days	~42 Days
Treatment Groups	Water, N=32 20% DMSO, N=19 Imidacloprid, N=24	Water, N=55 25% DMSO, N=36 Imidacloprid, N=25	25% DMSO, N= 44 Imidacloprid, N= 55	25% DMSO, N= 47 Imidacloprid, N= 55
Total N-Value	75	116	99	102

Table 1. Summary of experimental parameters.

3.2 Locomotor Activity and Open Field Tests

Locomotor activity testing was conducted on PND42-45 between the hours of 1 pm and 5 pm. On the day of testing, mice were acclimated to the testing room, which was dimly lit (~40 lux) by two 20 W light fixtures, for 15 minutes. Before each trial, all materials and testing surfaces were thoroughly cleaned with Virox (0.4% hydrogen peroxide). Locomotor activity was assessed using an automated open field monitoring system (Omnitech Electronics, Inc.). The automated monitoring system consisted of a 42 x 42 x 30 cm plexiglass box placed inside a detection system, which emitted 16 x 16 x 16 laser beams, each separated by 2.5 cm. The large chamber was divided diagonally into two 21 x 21 x 21 cm chambers to increase throughput. The test began by placing a mouse in the center of the chamber, free to explore for one 60-minute trial. At the conclusion of the 60-minute trial, the mice were returned to their home caging, and all materials and testing surfaces were thoroughly cleaned with Virox. Fusion software (Omnitech Electronics Inc.) was used to convert the data obtained from the laser beam

breaks, into useful variables including (but not limited to) total distance travelled, rest time, and movement time. The data obtained from the trial was analyzed in one 60-minute bin, and three 20-minute bins. In all tests and analyses, across all studies, data collected from the males and females were analyzed separately and together to allow for the identification of sex-dependent differences. SPSS (IBM) statistical software was used for all statistical analyses. A two-way analysis of variance (two-way ANOVA) with Bonferroni and Tukey post-hoc tests was used for statistical analysis.

The open field test was performed separately from the locomotor activity test (on PND42-47 between the hours of 1 pm and 5 pm) and utilized the same monitoring system and the same lighting as the aforementioned locomotor activity test. However, the entire 42 x 42 x 30 cm plexiglass box was used, and a center zone (a 26 x 26 cm square in the center of the chamber) was virtually defined. The test began with a mouse being placed in the center of the chamber, free to explore for one 20-minute trial. At the conclusion of the 20-minute trial, the mice were returned to their home caging, and all materials and testing surfaces were thoroughly cleaned with Virox. Time spent in the center zone and total distance travelled was recorded. The data obtained from the trial was analyzed in one 20-minute bin, and two 10-minute bins. In Study 2 a two-way ANOVA with Bonferroni and Tukey post-hoc tests was used for statistical analysis. In Study 3, a two-tailed Student's t-test with a 95% confidence interval was used for statistical analysis. Data collection from the open field test in Study 2 was done with the assistance of Charlotte Pidgeon.

3.3 Elevated Plus Maze

The elevated plus maze test was conducted on PND47-54 between the hours of 9 am and 1 pm. On the day of testing, the mice were acclimated to the testing room, which was normally

lit for 15 minutes. Before each trial, all materials and testing surfaces were thoroughly cleaned with Virox. The elevated plus maze is a maze consisting of two opposing open arms, two opposing closed arms, and a center zone; open arms are adjacent to closed arms, and all arms are at a 90º angle to each other. During the test, video recording software (Viewer^2 BIOBSERVE GmbH) recorded the animal's movements. The test began when the mouse was placed in the center zone, facing an open arm. The mouse was left to explore the maze for one 5-minute trial. At the conclusion of the 5-minute trial, the mice were returned to their home caging, and all materials and testing surfaces were thoroughly cleaned with Virox. The time spent in the open arms, closed arms, and center zone was analyzed. In Study 1 and Study 2, a two-way ANOVA with Bonferroni and Tukey post-hoc tests was used as a test for statistical significance. In Study 3, a two-tailed Student's t-test with a 95% confidence interval was used as a test for statistical significance.

3.4 Social Interaction and Social Preference Test

The modified three-chamber paradigm protocol was based on Xuan and Hampson, 2014, with modifications. The social interaction and social preference test took place on PND51-57 between the hours of 1pm and 5pm. On the day of testing, the mice were acclimated to the testing room, which was dimly lit (~40 lux), for 30 minutes. Before each trial, all materials and testing surfaces were thoroughly cleaned with Virox. During the test, a ceiling-mounted video camera and video recording software (Viewer^2 BIOBSERVE GmbH) recorded the animal's movement. Two identical parallel circular zones (diameter: 19.8 cm), equidistant (lengthwise: 7.1 cm, widthwise: 10.5 cm) from all sides of the white plexiglass stadium (61.7 x 40.8 x 23 cm) were virtually defined. An empty wire cage (top diameter: 7.5 cm, bottom diameter: 10 cm, height: 10 cm) was placed in the center of each zone. To prevent the mice from climbing, a

circular disk (diameter: 10 cm) and a 400 mL glass beaker were placed on top of the wire cage. Two removable cardboard dividers were placed in the centre of the stadium to shield both zones.

The test began with a stadium habituation period; the test mouse was placed in the center of the stadium, between the dividers, and the two empty wire cages were placed in their respective positions, at the center of the two virtually defined circular zones. The dividers were subsequently lifted, and the mouse was free to explore the stadium for 10 minutes. At the conclusion of the habituation period, the mouse was returned to the center of the stadium, and the two dividers were put in place. The social interaction test commenced. A stimulus mouse (strain-matched, age-matched and sex-matched mouse) was placed within the wire cage, which was placed in the center of the circular zone defined as the "social" zone, while a roll of green tape was placed within the second wire cage, which was placed in the center of the circular zone defined as the "non-social" zone. The dividers were lifted and the mouse was free to explore the entire stadium for 10 minutes. The time spent in the "social" and "non-social" zones were recorded. At the conclusion of the social interaction test, the mouse was returned to the center of the stadium, and the two dividers were put in place. The social preference test commenced. The stimulus mouse in the "social" zone remained unchanged, while a second stimulus mouse replaced the roll of green tape under the wire cage in the "non-social" zone; this zone was then redefined as the "new social" zone. The dividers were lifted and the mouse was free to explore the entire stadium for 10 minutes. The time spent in the "social" and "new social" zones were recorded. At the conclusion of the test, the mice were returned to their home caging, and all materials and testing surfaces were thoroughly cleaned with Virox.

A two-tailed Student's t-test with a 95% confidence interval was used to compare, within treatment groups, the time spent in the "social" zone and "non-social" zone, and the time spent in the "social" zone and the "new social" zone. To compare the average time spent in each zone between treatment groups, a two-way ANOVA with Bonferroni and Tukey post-hoc tests was

used as a test for statistical significance. The results from this test are not shown, as the test was inconsistent, non-reproducible, and unreliable.

3.5 Tube Test

The tube test took place on PND54-64 between the hours of 1 pm and 5 pm. On the day of testing, the mice were individually caged and acclimated to the testing room, which was normally lit, for 30 minutes. Before each trial all materials and testing surfaces were thoroughly cleaned with Virox and water. The tube test consisted of placing two sex-matched and age-matched mice, from two different treatment groups, at opposite ends of a transparent polyvinyl chloride tube (inner diameter: 2.5 cm, length: 30.5 cm). The mice were released simultaneously, with both mice entering the tube. The test concluded when one mouse placed at least two paws outside the tube, with the mouse remaining inside the tube being declared the winner. After the winner was declared, both mice were returned to their individual cage, and the tube was thoroughly cleaned with Virox followed by water. This procedure was repeated a maximum number of times (5 matchups per mouse), such that the same matchup was never repeated. At the conclusion of the test, all mice were returned to their original home caging, and all materials and testing surfaces were thoroughly cleaned with Virox. The number of wins and winning percentage per treatment group were compared. The tube test utilized the Fisher's Exact test as a test for statistical significance. Data collection from the tube test in Study 4 was done with the assistance of Dr. Yosuke Niibori.

3.6 Pre-pulse Inhibition

The pre-pulse inhibition test took place on PND58-61 between the hours of 9 am and 1 pm. Prior to testing, the animal enclosure (length: 12.7 cm, inner diameter: 3.81 cm) within the

SR-LAB startle response system chamber (San Diego Instruments) was cleaned thoroughly with Virox, and then wiped with 70% ethanol. Three different intensities of pre-pulse sounds (74 dB, 78 dB and 82 dB) and one pulse (120 dB) were used to calculate startle response, average inhibition, and percent inhibition (respective to the pre-pulse sound intensity). The mouse was placed inside the animal enclosure, the chamber was closed, and the trial began. The first 5 minutes of the test consisted of a habituation period, during which the mice were exposed to white noise. After the 5-minute habituation period, the mice were exposed to a randomized pattern of no pulse, 74 dB pre-pulse + 120 dB pulse, 74 dB pre-pulse only, 78 dB pre-pulse + 120 dB pulse, 78 dB pre-pulse alone, 82 dB pre-pulse + 120 dB pulse, 82 dB pre-pulse alone, and 120 dB pulse alone. Each scenario occurred 6 times, thus generating 48 data points per mouse. After the trial was complete, the mice were removed from the animal enclosure and returned to their home cage. A two-tailed Student's t-test with a 95% confidence interval was used as a test for statistical significance. Data collection from the pre-pulse inhibition test in Study 4 was done with the assistance of Dr. Jason Arsenault.

3.7 Forced Swim Test

The forced swim test was conducted on PND61-67 between the hours of 9 am and 1 pm. On the day of testing, the mice were acclimated to the testing room, which was dimly lit (~40 lux), for 30 minutes. A tripod-mounted video camera recorded all mouse activity during the forced swim test. The test began by placing a mouse, tail first, into a 2000 mL beaker filled with 1600 mL of tap water (22-24ºC). The mouse was free to swim in the beaker for one 6-minute trial. At the conclusion of the 6-minute trial, the mice were dried with a towel under a heat lamp, and returned back to their home caging. The water was replaced when it became visibly soiled, and the beaker was cleaned with Virox and water. The final 4 minutes of the 6-

minute video recording were analyzed, and the time spent swimming and the time spent immobile were recorded and compared. In Study 2, a two-way ANOVA with Bonferroni and Tukey post-hoc tests was used as a test for statistical significance. In Study 3, a two-tailed Student's t-test with a 95% confidence interval was used as a test for statistical significance. Data collection from the forced swim test in Study 2 was done with the assistance of Charlotte Pidgeon.

3.8 Resident Intruder Test

The resident intruder test was conducted on male mice on PND66-72 between the hours of 1 pm and 5 pm. Eleven to seventeen days prior to testing (PND55-59), the mice were individually caged. On the day of testing, the mice were acclimated to the testing room (normally lit) for 20 minutes. A video camera recorded the activity of the mice during the test. The test began with the resident cage being placed in front of the video camera, and the age-matched and sex-matched wild type CD-1 intruder mouse being placed in the resident cage. The resident and intruder mice were free to interact for 10 minutes. At the conclusion of the test, the intruder mice were returned to their home caging. The video recording was reviewed, and the number of attacks (count), the duration of attacks (measured in seconds), and the total fight time (measured in seconds) were recorded for both the resident and the intruder. In addition, the latency to the first attack (measured in seconds) was also recorded, for the resident or the intruder (whichever initiated the first attack). A total fight time (measured in seconds) was calculated by adding the duration of attacks by resident to the duration of attacks by intruder. A two-tailed Student's t-test with a 95% confidence interval was used as a test for statistical significance.

3.9 Five Choice Serial Reaction Time task

For the five choice serial reaction time task (5CSRT), we used the Bussey-Saksida Touchscreen Operant Chamber (Campden Instruments, model 80614-20) which consists of a chamber base with perforated floors, trapezoidal walls (dimensions: 46 mm wide at the feeder, 238 mm wide at the screen, and 170 mm Deep for a total of 241.4 cm^2), the touch screen, and a liquid reward feeder (with pump). All of these components were placed within a sound attenuated cubicle. For the liquid reward, we used MILK2GO™ Strawberry Splash strawberry milkshake (Saputo Dairy Products Canada). Each day after testing, the touchscreen was thoroughly cleaned with water, while on Fridays, the touchscreen was thoroughly cleaned with Virox and water. All testing took place between the hours of 1pm and 5pm, Monday to Friday. At the conclusion of daily testing, the mice were returned to their home cage.

The 5CSRT experimental setup began on PND42. On PND42, the mice were weighed and placed on dietary restriction, during which they were fed ~2 pellets per day (and weighed each day) for one week. After the first week, the mice were weighed each day, and the food amount was adjusted such that the mice were kept at a weight that is 90% of the CD-1 target weight (target weight was provided by Charles River, corresponding to mouse age and sex). The mice were fed between 5 pm and 7 pm each day, from Monday to Friday. On Friday, the mice were fed ad libitum until Monday morning at 9:30 am. This dietary restriction and feeding pattern continued for the duration of testing.

The 5CSRT pretraining began on PND49. On PND49, the mice were exposed to the operant chamber for one 10-minute habituation trial. No stimulus or reward was presented during this trial. On PND50, the mice were exposed to the operant chamber for one 20-minute habituation trial. During this trial, the tray-light was turned on, and the reward tray was primed with 150 µL of strawberry milkshake. Once the mouse entered, and subsequently left the food

tray, the tray-light was turned off. A 10 second delay occurred, followed by the tray-light illuminating and 7 μL of reward being delivered. If the mouse was in the reward tray at the end of the 10 second delay, an additional 1 second was added to the delay time. This procedure was repeated until the trial was complete. On PND51, the mice repeated the trial from PND50, but the length of the trial was increased from 20 minutes to 30 minutes. On PND53, the length of the trial was increased from 30 minutes to 40 minutes. The trial on PND53 was repeated each day until 30 trials were completed in 40 minutes.

Once the habituation step concluded, initial touch training commenced. First, the reward tray was primed with 150 μL of strawberry milkshake. During the initial touch training trials, which were 40 minutes in length, a stimulus (a white square) was displayed in one of five window locations, while the other 4 windows were left blank (black). The position was chosen pseudo randomly such that the stimulus would not be displayed in the same position more than 3 consecutive times. After a delay (variable image time, 30 seconds) the image was removed, the tray light was turned on, and food reward (20 μL) was delivered (no touch necessary). After the inter-trial interval (ITI, 0 seconds), which began once the mouse entered the reward tray, another image was displayed. If the mouse touched the illuminated screen, a larger food reward was immediately delivered (three times larger). This sequence was repeated until the mouse completed 30 trials, or 40 minutes had elapsed. Initial touch training was repeated daily until the mouse achieved 30 trials in 40 minutes.

Once the mice had reached the required criteria in the initial touch training, they advanced to must touch training, also known as location discrimination training. Must touch training was identical to initial touch training, with the exception that the mouse had to touch the illuminated window to receive a reward; no reward was delivered if the mouse touched the incorrect window. The ITI was increased to 5 seconds for the must touch training, and the

image remained onscreen until touched. The mice had to complete 20 trials in 40 minutes on two consecutive days before advancing from must touch training to 5CSRT training.

5CSRT training was similar to must touch training with some exceptions. The trial began with the delivery of 7 μL of strawberry milkshake; after collecting this initial reward, the first trial commenced. The ITI of 5 seconds occurred after reward collection, after which stimuli were pseudo-randomly presented in one of the 5 windows of the touch screen (such that each window was illuminated 4 times in 20 trials). The mouse had to respond during the limited hold period (the time the stimulus was displayed on screen, 34 seconds), otherwise the trial would count as an omission. A correct response was rewarded with strawberry milkshake, while an omission or incorrect response, or a premature response, led to a 4 second timeout (during which the house light in the chamber was turned on). After the timeout, or after the correct response, the mouse had to enter the reward tray to begin the next trial. The 5CSRT training session consisted of 120 trials, or 40 minutes. To advance to 5CSRT training session 2, the mice had to achieve >80% accuracy and <20% omissions on two consecutive days.

3.10 Delayed-type Hypersensitivity and Flow Cytometry

To study inflammatory immune responses, a delayed-type hypersensitivity reaction experiment was conducted on or after PND67. For this experiment we used a modified version of the protocol outlined by Kuntsfelt et al., 2004. The experiment began with sensitization; each mouse was sensitized by the topical application of 2% oxazolone (Sigma-Aldrich) solution in acetone/olive oil (4:1 vol/vol) to the shaved abdomen (50 μL) and to each paw (5 μL). Five days after sensitization, the right ear was challenged by a topical application of 20 μL of 1% oxazolone solution, while the left ear was treated with vehicle alone. 24 hours after the 1% oxazolone solution challenge, the mice were sacrificed and the left and right auricular lymph

nodes were collected. The auricular lymph nodes are small in size; therefore, the auricular lymph nodes from 3 age-matched, sex-matched and treatment-matched mice were pooled together for the purpose of increasing our overall cell count.

A single cell suspension was prepared from the pooled lymph nodes by teasing the lymph nodes apart using the plunger of a 3 mL syringe in a petri dish containing 10 mL of staining buffer (eBioscience). The contents of the petri dish were ran through a nylon mesh filter, and centrifuged at 350 x g for 4 minutes at 4ºC. The pellet was resuspended in 5 mL of staining buffer, and a cell count was performed using a haemocytometer (Hausser Scientific). Cells were then incubated for 20 minutes in the dark at 4ºC with a Mouse T Lymphocyte Subset Antibody Cocktail (with Isotype Control; PE-Cy7 CD3e, PE CD4, and FITC CD8; BD Pharmingen); the incubation was done at a ratio of 1.0×10^6 cells in 20 µL of antibody cocktail. At the conclusion of the incubation period, 2 mL of staining buffer was added to the sample. The sample was then centrifuged at 350 x g for 5 minutes at 4ºC. The supernatant was discarded, and this procedure was repeated an additional two times, for a total of three washes. The final pellet was resuspended in 500 µL of staining buffer and ran on a 3 laser (488/633/405 nm), 8-parameter analyzer flow cytometer (BD FACS Canto II, BD Biosciences). The samples were analyzed until a cell count of at least 8000 cells was achieved. Flowjo single cell analysis software (FlowJo, LLC) was used to quantify the populations of CD3+, CD8+ and CD4+ t-cells.

3.11 Haematology Assay

Whole blood (25 µL) was collected in Ethylenediaminetetraacetic acid (EDTA) coated collection tubes (microvette CB300 K2 EDTA, Sarstedt) from male and female mice from all treatment groups through cardiac puncture. The whole blood samples were delivered to Zorana Berberovic at The Toronto Centre for Phenogenomics. A Hemavet Hematology Analyzer

(950FS; Drew Scientific) was used to measure the concentration of neutrophils, lymphocytes, monocytes, basophils and eosinophils. The mean values of the aforementioned parameters were calculated, and a two-tailed Student's t-test with a 95% confidence interval was used as a test for statistical significance.

3.12 Sperm Head Abnormality Assay

A sperm head abnormality assay was performed to measure spermatogenic damage that results from chronic imidacloprid exposure. For this experiment we used the sperm head abnormality assay protocol outlined by Bagri et al., 2014. After 36 days of exposure to 0.5 mg/kg/day of imidacloprid dissolved in 25% DMSO, or 25% DMSO alone, the adult male mice were sacrificed and the caudae epididymides were dissected and collected in 3 mL of phosphate-buffered saline (PBS) in a petri dish. After cutting the caudae epididymides into several pieces, the suspension was gently pipetted 6 times through a fine silk mesh filter to remove debris, leaving the sperm cells in the filtrate. The sperm cells were stained using 50 µL of 1% Eosin-Y (Sigma-Aldrich) in 500 µL of filtrate, followed by agitation for 5 minutes. Slides were prepared by spreading 2 drops of stained solution on a clean slide by three passes of another slide. The sperm cell slides were examined by light microscopy at 100X magnification. For each animal, 200 sperm cells were assessed for morphological abnormalities. Abnormal sperm cells were defined as those containing a banana-shaped head, prism-shaped head, blunt-hooked head, curved hooked head, wrong-angled hook head, apical-hooked head, hookless head, amorphous head, or a pin head. A two-tailed Student's t-test with a 95% confidence interval was used as a test for statistical significance.

3.13 Statistical Analysis

All data collected from the males and females were analyzed separately and together to allow for the identification of sex-dependent differences. SPSS (IBM) statistical software was used for all statistical analyses. With the exception of the tube test, in experiments where two treatment groups were present, a two-tailed Student's t-test was used as a test for statistical significance. In experiments where three treatment groups were present, a two-way ANOVA with Bonferroni and Tukey post-hoc tests was used as a test for statistical significance.

TESTS	Study 1	Study 2	Study 3	Study 4
Locomotor Activity OR Open Field Test	YES	YES	YES	NO
Elevated Plus Maze	YES	YES	YES	NO
Social Interaction	YES	YES	NO	NO
Tube Test	YES	YES	YES	YES
Prepulse Inhibition	NO	NO	YES	YES
Forced Swim Test	NO	YES	YES	NO
Resident Intruder	NO	NO	YES	YES

Table 2. Overview of behavioural testing. YES indicates that the test was included in the study, while NO indicates that the test was not included in the study.

4. Results

4.1 Study 1

The purpose of Study 1 was to establish whether chronic *in utero* and early postnatal exposure to IMD leads to any lasting perturbations in behaviour. To observe the behaviour of the adult offspring following the aforementioned exposure to IMD, we devised a behavioural testing regimen that consisted of carefully selected behavioural tests, which covered a broad range of behaviours (Table 2). The results from Study 1, conducted on C57BL/6 mice, are shown in Figure 2.

In the locomotor activity test, which measures baseline motor activity, no significant difference in the total distance travelled was observed between treatment groups (Fig. 2A). In the elevated plus maze, which measures explorative behavior and anxiety, a significant decrease in the time spent in the closed arms of the maze was observed in the IMD treatment group males and females combined, compared to both water and DMSO treatment groups (Fig. 2B). In the tube test, which measures social dominance, a significant increase in winning percentage in the IMD treatment group was observed in both males, and males and females combined, when matched up against the DMSO treatment group (Fig. 2C).

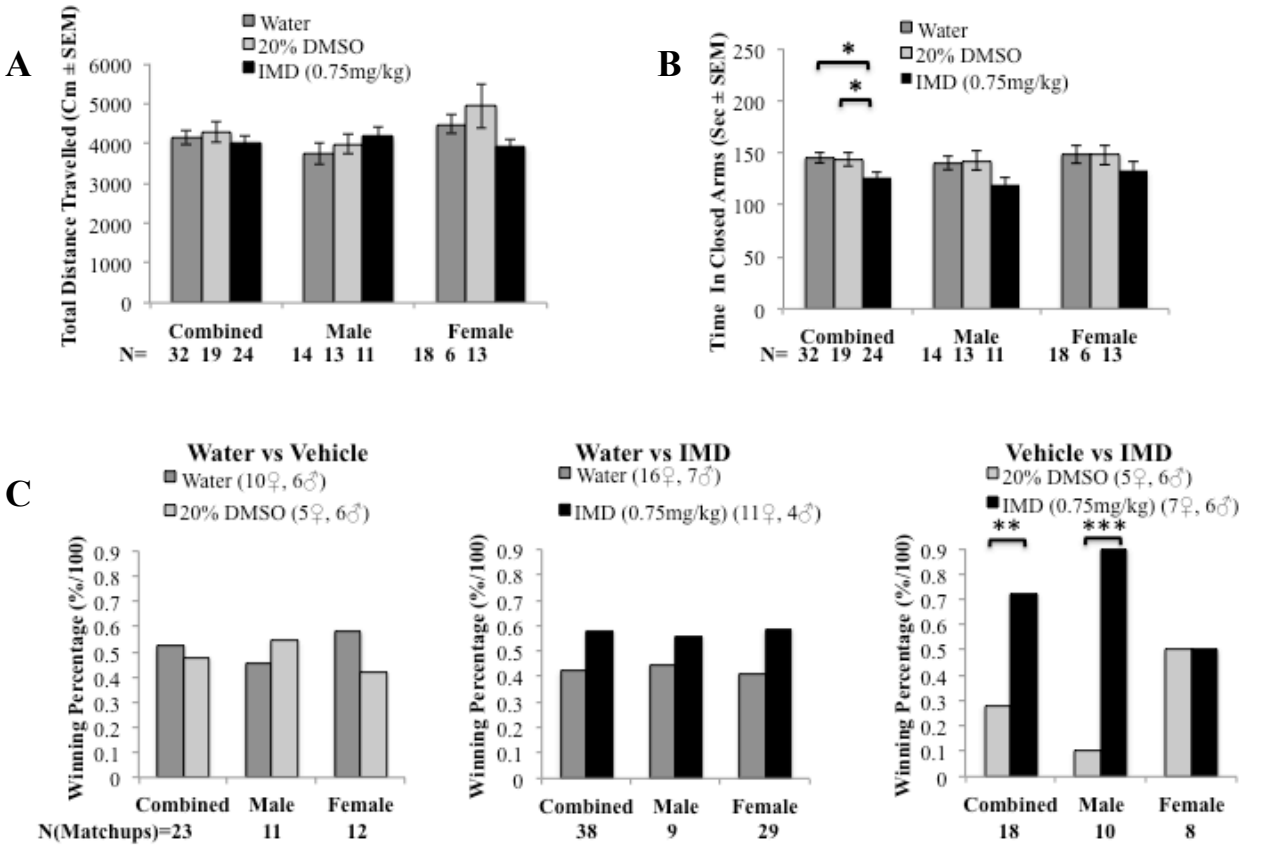


Fig. 2. Results from Study 1. Study 1 was conducted on C57BL/6 mice. Control mice were treated with water or 25% DMSO, while test mice were treated with 0.75 mg/kg of IMD. **(A)** Total distance travelled during the locomotor activity testing. The test consists of one 40-minute trial. Each column represents the mean ± SEM. N = number of mice in each group. Two-way ANOVA with Bonferroni post-hoc test. **(B)** Elevated plus maze closed arm analysis. Each column represents the mean ± SEM. N=number of mice in each group. Two-way ANOVA with Bonferroni post-hoc test, *$p<0.05$. **(C)** Tube test analysis. Each column represents the mean winning percentage, calculated as the number of wins divided by the total number of matchups. No mice were used in more than 5 matchups. N = number of matchups between treatment groups. The number of males and females used in each treatment group are displayed in the legends within the figure panels. Fisher's exact test, **$p<0.01$, ***$p<0.001$.

4.2 Body Weight

Body weight was measured on PND42-46 as a static measure of overall health. In Study 3, a significant decrease in body weight in the IMD treatment group was observed in males and females, compared to the DMSO treatment group (Fig. 3A). In Study 4 no significant difference in body weight was observed between treatment groups regardless of sex (Fig. 3B). When the results from Study 3 and Study 4 were compiled, a significant decrease in body weight in the IMD treatment group was observed in males and females compared to the DMSO treatment group (Fig. 3C).

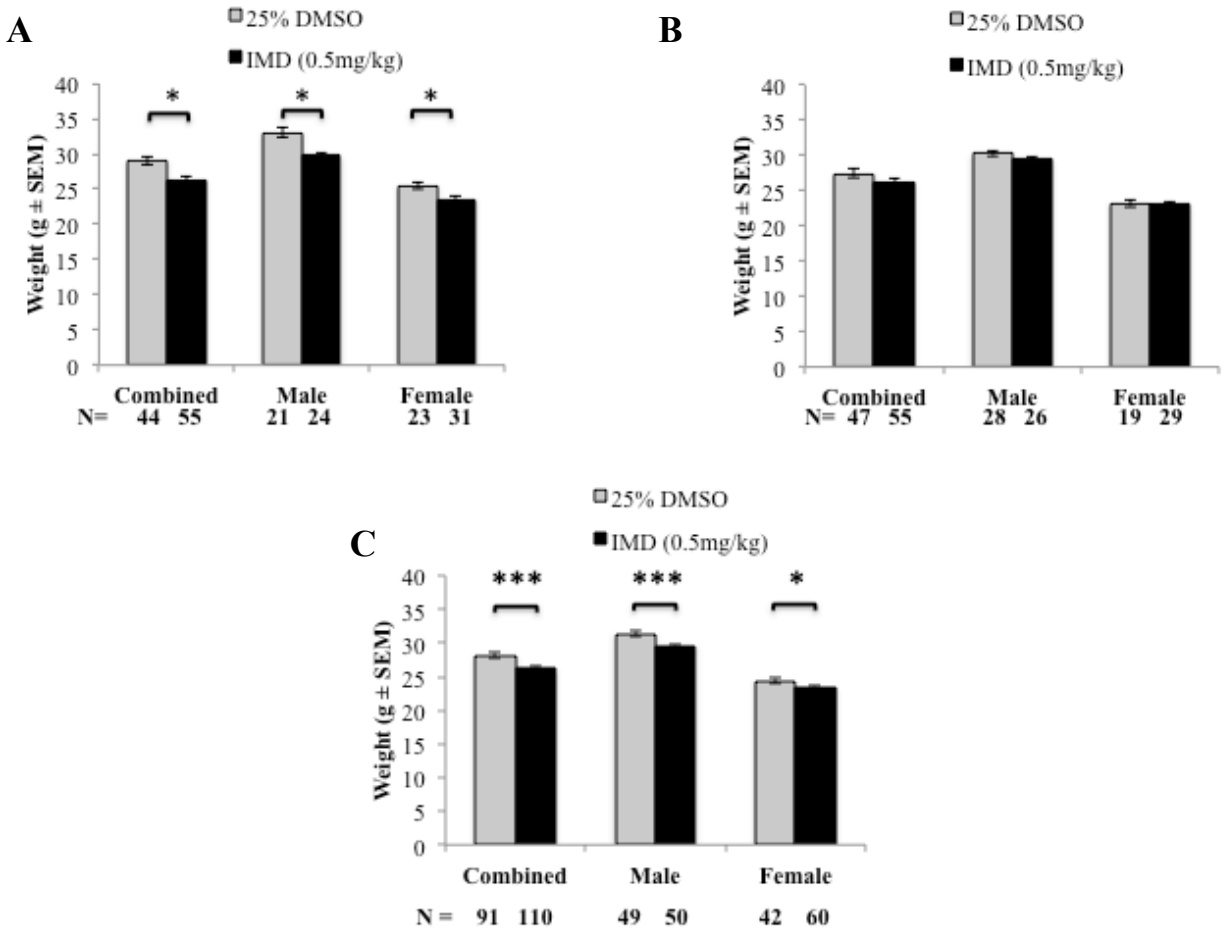


Fig. 3. Body weight analysis. The mice were weighed on PND42-46. **(A)** Body weight analysis of mice from Study 3. **(B)** Body weight analysis of mice from Study 4. **(C)**

Compilation of body weight analyses from Study 3 and Study 4. Each column represents the mean ± SEM. N = number of mice in each group. Two-tailed Student's t-test, *$p<0.05$, ***$p<0.001$.

4.3 Motor Activity

The open field test was used to analyze both the baseline motor activity (reflected in the total distance travelled) and the general anxiety level (reflected in the time spent in the centre zone) of the adult offspring. In Study 2, a significant decrease in the total distance travelled by the DMSO and IMD treatment groups was observed in female mice compared to the water treatment group (Fig. 4A). In Study 2, the zone data collected during the open field test showed no significant differences between treatment groups, regardless of sex (Fig. 4B). In Study 3, a significant increase in the total distance travelled in the males of the IMD treatment group was observed, compared to the DMSO treatment group (Fig. 4C). In Study 3, the zone data collected during the open field test showed no significant differences between treatment groups, regardless of sex (Fig. 4D). When the results from Study 2 and Study 3 were compiled, a significant increase in total distance travelled was observed in the IMD-treated male mice, and in the IMD-treated male and female mice combined, compared to the DMSO treatment group (Fig. 4E). No significant differences were observed when the zone data from Study 2 and Study 3 were compiled (Fig. 4F).

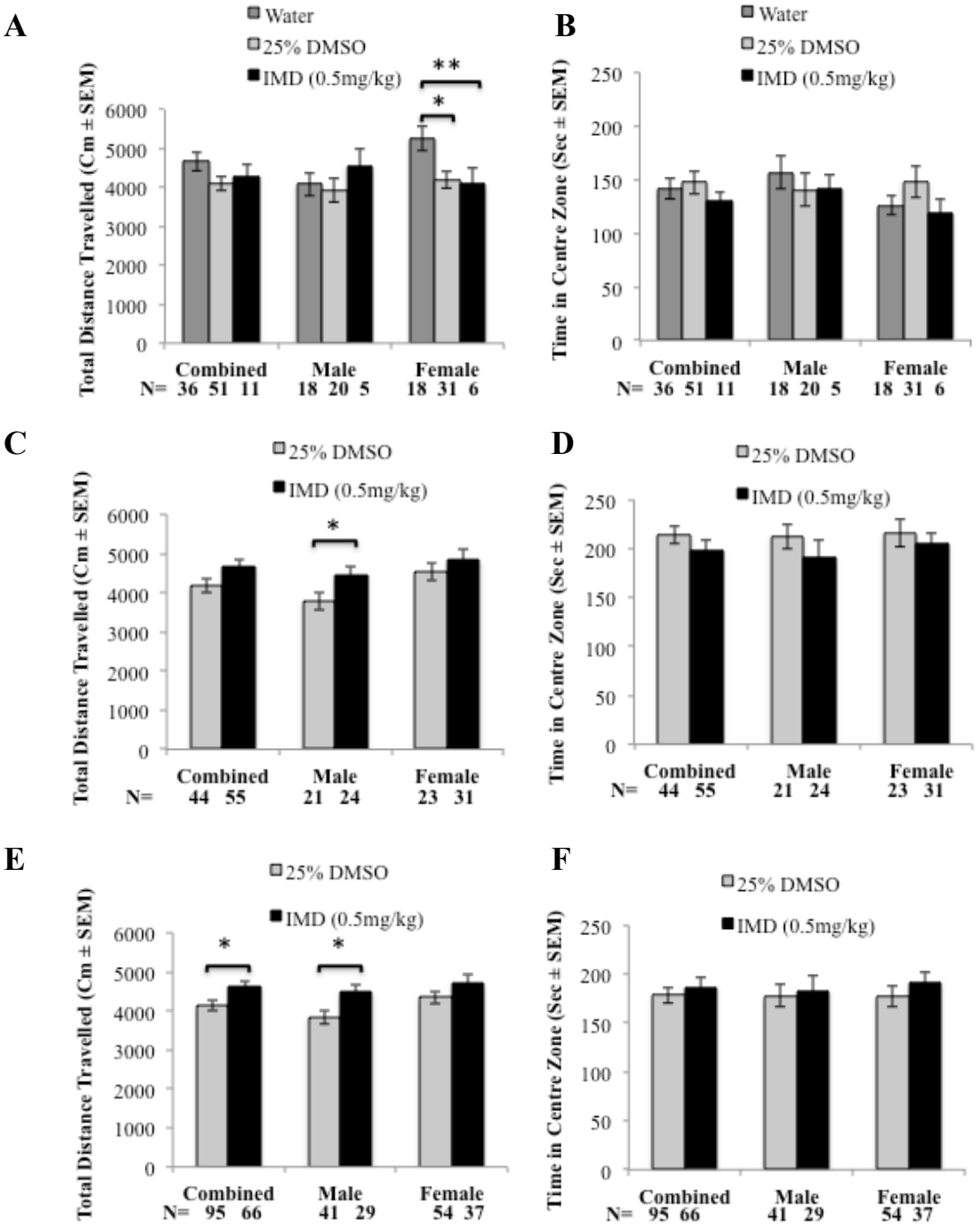


Fig. 4. Open field test analysis. (A) Motor activity analysis from Study 2. **(B)** Zone analysis from Study 2. **(C)** Motor activity analysis from Study 3. **(D)** Zone analysis from Study 3. **(E)** Compilation of motor activity analyses from Study 2 and Study 3. **(F)** Compilation of zone analyses from Study 2 and Study 3. In both studies, this test consisted of one 20-minute trial. Each column represents the mean ± SEM. N = number of mice in each group. Study 2; two-way

ANOVA with Bonferroni post-hoc test, *p<0.05, **p<0.01. Study 3 and compilation; two-tailed Student's t-test, *p<0.05.

4.4 Anxiety and Explorative Behaviour

The elevated plus maze is a well-established behavioural test used to assess anxiety and explorative behaviour. In the open arm analysis of Study 2, a significant decrease in the time spent in the open arms was observed in both males and females combined, in both the DMSO and IMD treatment groups, compared to the water treatment group. A similar result was observed in the females, as a significant decrease in the time spent in the open arms in both the DMSO and IMD treatment groups was observed, compared to the water treatment group (Fig. 5A).

In the closed arm analysis of Study 2, a significant increase in the time spent in the closed arms was observed in both males and females combined, in both the DMSO and IMD treatment groups, compared to the water treatment group. There was a significant increase in the time spent in the closed arms in the DMSO treatment group in the males, compared to the water treatment group. In the females, a significant increase in the time spent in the closed arms was observed in both the DMSO treatment group and IMD treatment group, compared to the water treatment group. In addition, also in the females, a significant increase in the time spent in the closed arms was observed in the IMD treatment group compared to the DMSO treatment group (Fig. 5B).

The results from Study 3 showed no statistically significant differences between treatment groups, regardless of sex (Fig. 5C, Fig. 5D). When the results from Study 2 and Study 3 were combined, no significant differences were observed between treatment groups in both the open and closed arm analyses (Fig. 4E, Fig. 5F).

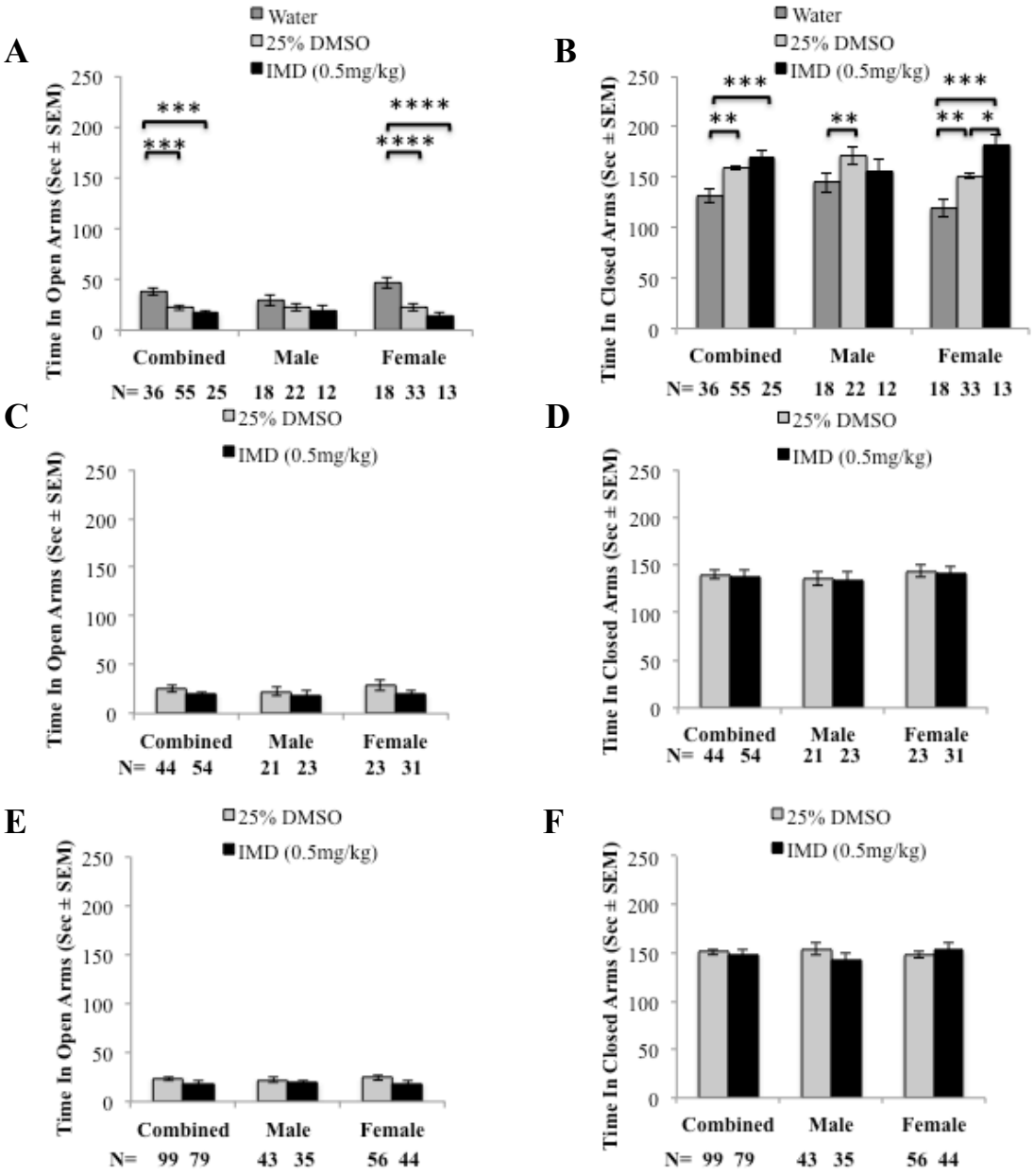


Fig. 5. Elevated plus maze analysis. (A) Open arm analysis from Study 2. **(B)** Closed arm analysis from Study 2. **(C)** Open arm analysis from Study 3. **(D)** Closed arm analysis from Study 3. **(E)** Compilation of open arm analyses from Study 2 and Study 3. **(F)** Compilation of closed arm analyses from Study 2 and Study 3. In both studies, this test consisted of one 5-min. trial. Each column represents the mean ± SEM. N = number of mice in each group. Study 2;

two-way ANOVA with Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Study 3 and compilation; two-tailed Student's t-test.

4.5 Social Dominance

The tube test is a test used to evaluate social dominance in treated mice. In Study 2, when the DMSO treatment group was tested against the water treatment group, in both males and females combined, a significant decrease in the winning percentage of the DMSO treatment group was observed, compared to the water treatment group. In Study 2 males alone, a significant decrease in the winning percentage of the DMSO treatment group was observed, compared to the water treatment group. No significant results were observed in the females alone, yet an opposite trend was observed compared to the male and combined sex cohorts, as the DMSO treatment group had a greater winning percentage (though not statistically significant) than the water treatment group (Fig. 6A).

In Study 2, when IMD-treated mice were matched against water treated mice, both males and females, there was a significant increase in the winning percentage of the IMD treatment group (at variable levels of statistical significance), compared to the water treatment group (Fig. 6B). Also in Study 2, when IMD was tested against DMSO, in both males and females combined, no significant difference between treatment group winning percentage was observed. However, in males, a significant increase in winning in the IMD treatment group was observed compared to the DMSO treatment group. In the females, a significant decrease in winning in the IMD treatment group was observed, compared to the DMSO treatment group (Fig. 6C).

In Study 3, when DMSO was matched against IMD, a significant increase in winning in the IMD treatment group was observed in the males, females, and combined males and females, compared the DMSO treatment group (Fig. 6D).

In Study 4, the females, and the females and males combined, showed a non-significant trend towards more wins in the IMD-treated mice (Fig. 6E). However, when the tube test results from Study 2, Study 3 and Study 4 are combined, a significant increase in winning percentage in the IMD treatment group was observed in males, females and combined sexes, compared to the DMSO treatment group (Fig. 6F)

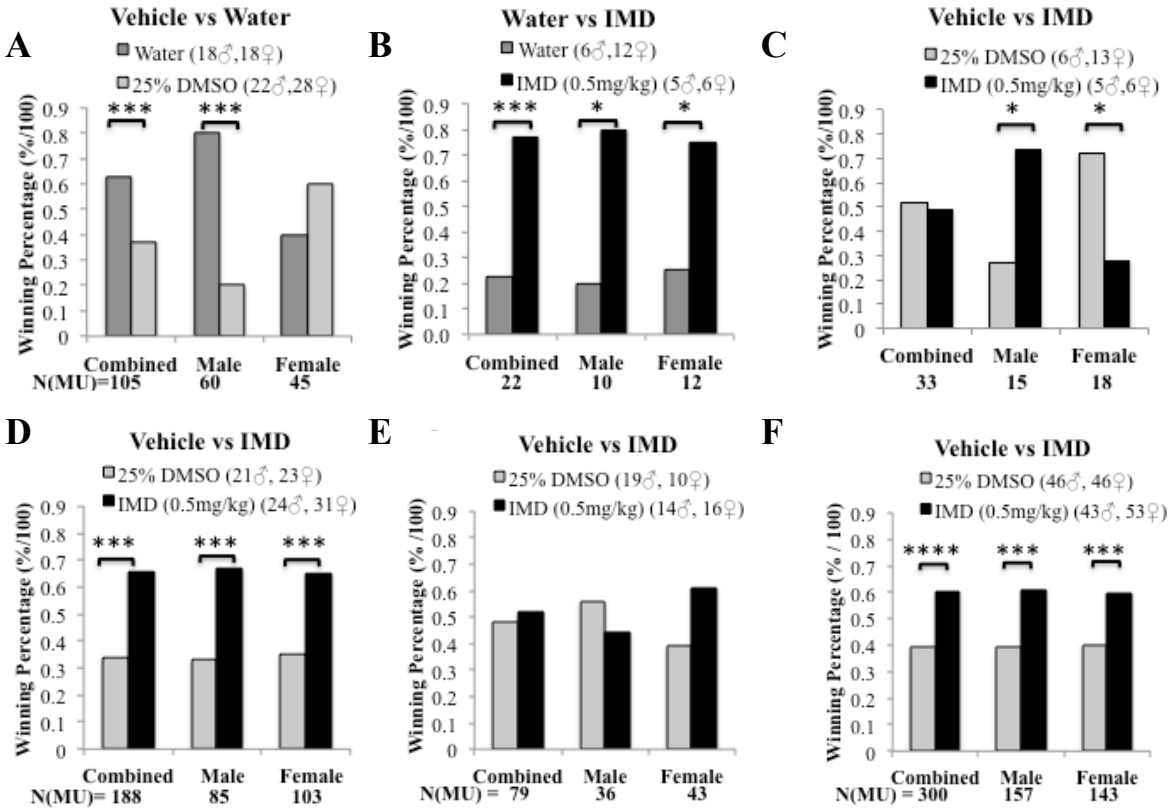


Fig. 6. Tube test analysis. (A-C) Tube test analyses from Study 2. **(D)** Tube test analysis from Study 3. **(E)** Tube test analysis from Study 4. **(F)** Compilation of tube test analyses from Study 2, 3 and 4. No mice were used in more than 5 matchups. The data are presented as the winning percentage, calculated as the number of wins divided by the total number of matchups. N (MU) = number of matchups between the treatment groups. The number of males and females in each treatment group are displayed in the legends within the figure panels. Fisher's exact test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.6 Sensorimotor Gating

The pre-pulse inhibition test is a test used to provide an operational measure of sensorimotor gating. In Study 3, no significant difference between treatment groups was observed, in both the startle response and the average pre-pulse inhibition analyses (Fig. 7A, Fig. 7B). While there appears to be various trends present, statistical significance was not achieved due to a high degree of variability. In Study 4, no significant difference between treatment groups was observed, in both the startle response and the average pre-pulse inhibition analyses (Fig. 7C, Fig. 7D). When the results from Study 3 and Study 4 were compiled, no significant difference between treatment groups was observed, in both the startle response and the average pre-pulse inhibition analyses (Fig. 7E, Fig. 7F).

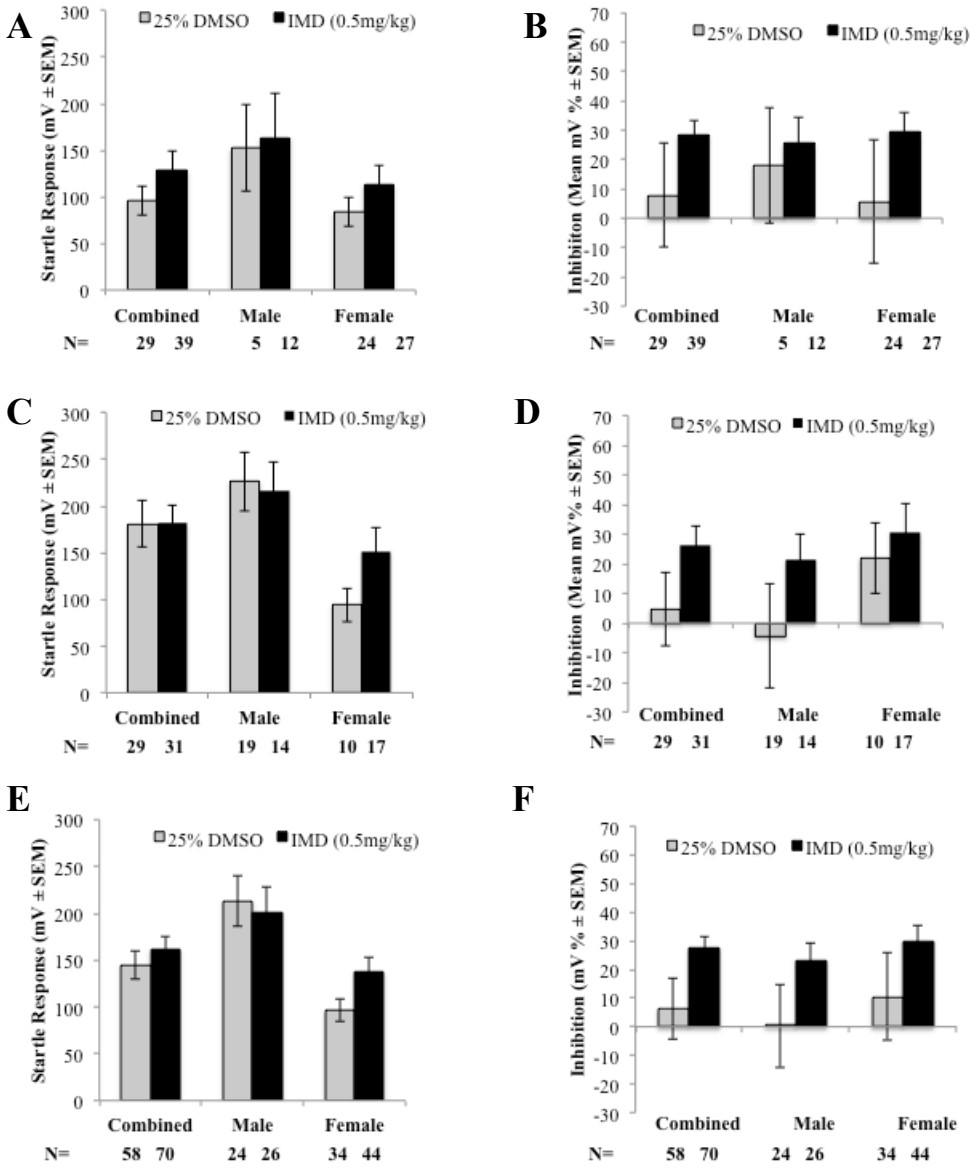


Fig. 7. Pre-pulse inhibition analysis. (A) Startle response analysis from Study 3. **(B)** Average pre-pulse inhibition analysis from Study 3. **(C)** Startle response analysis from Study 4. **(D)** Average pre-pulse inhibition analysis from Study 4. **(E)** Compilation of startle response analyses from Study 3 and 4. **(F)** Compilation of average pre-pulse inhibition analyses from Study 3 and 4. Each column represents the mean ± SEM. N = number of mice in each group. There were no outliers in Study 3 or Study 4, as determined by Grubbs' test. Two-tailed Student's t-test.

4.7 Depressive-like Behaviour

The forced swim test is a test of behavioural despair, which ultimately evaluates depressive-like behaviours. In Study 2, in both males and females combined and the females alone, a significant decrease in the time spent immobile in the IMD treatment group compared to the DMSO treatment group was observed (Fig. 8A). In Study 3, in both males and females combined and the females alone, a significant decrease in the time spent immobile in the IMD treatment group compared to the DMSO treatment group was observed (Fig. 8B). When the results from Study 2 and Study 3 were combined, a significant decrease in the time spent immobile in the IMD treatment group was observed in males alone, females alone, and combined males and females, compared to the DMSO treatment group (Fig. 8C).

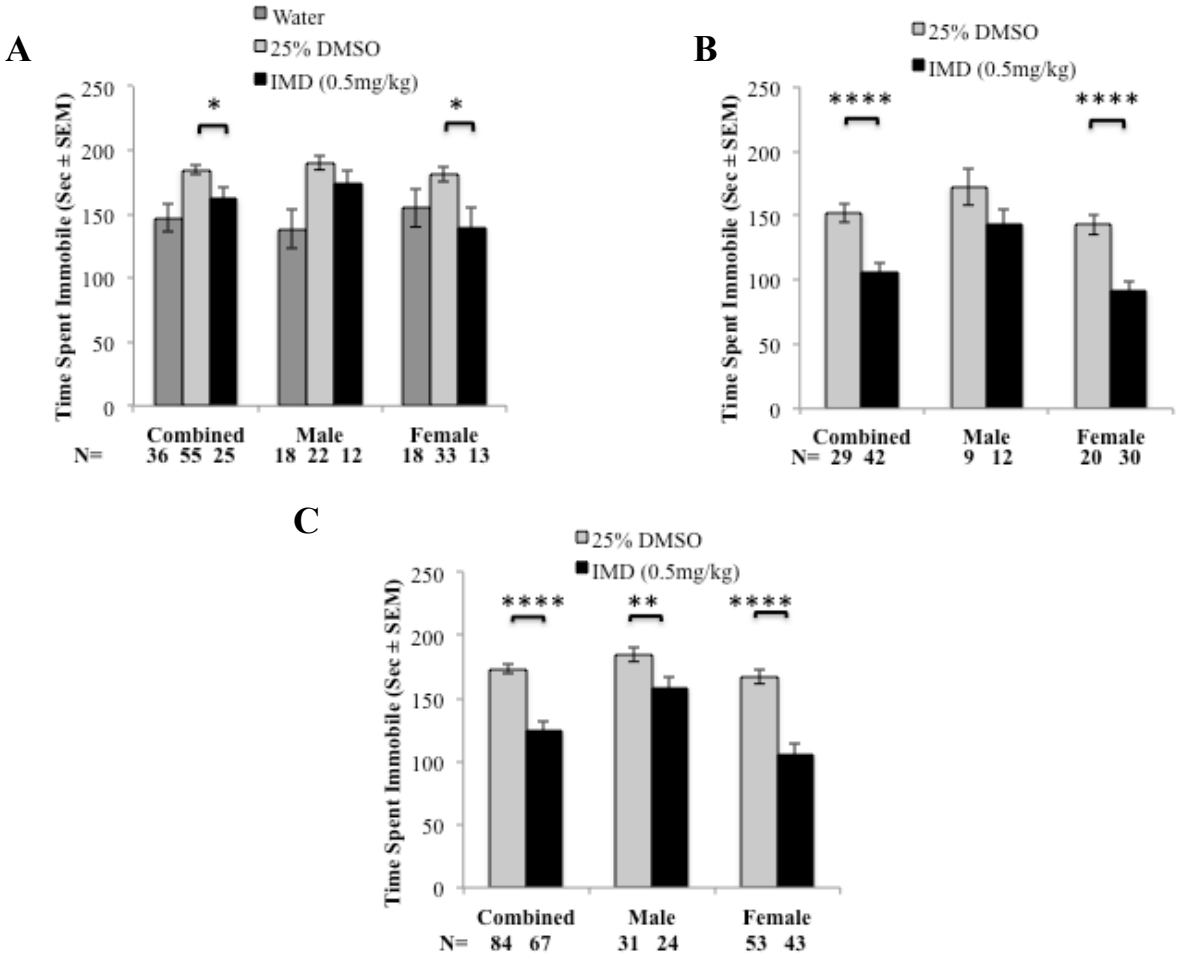


Fig. 8. Forced swim test analysis. (A) Forced swim test analysis from Study 2. **(B)** Forced swim test analysis from Study 3. **(C)** Compilation of forced swim test analyses from Study 2 and 3. This test consisted of one 4-min. trial. Each column represents the mean ± SEM. N = number of mice in each group. Study 2; two-way ANOVA with Bonferroni post-hoc test, *p<0.05, **p<0.01, ****p<0.0001. Study 3 and Compilation; two-tailed Student's T- test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

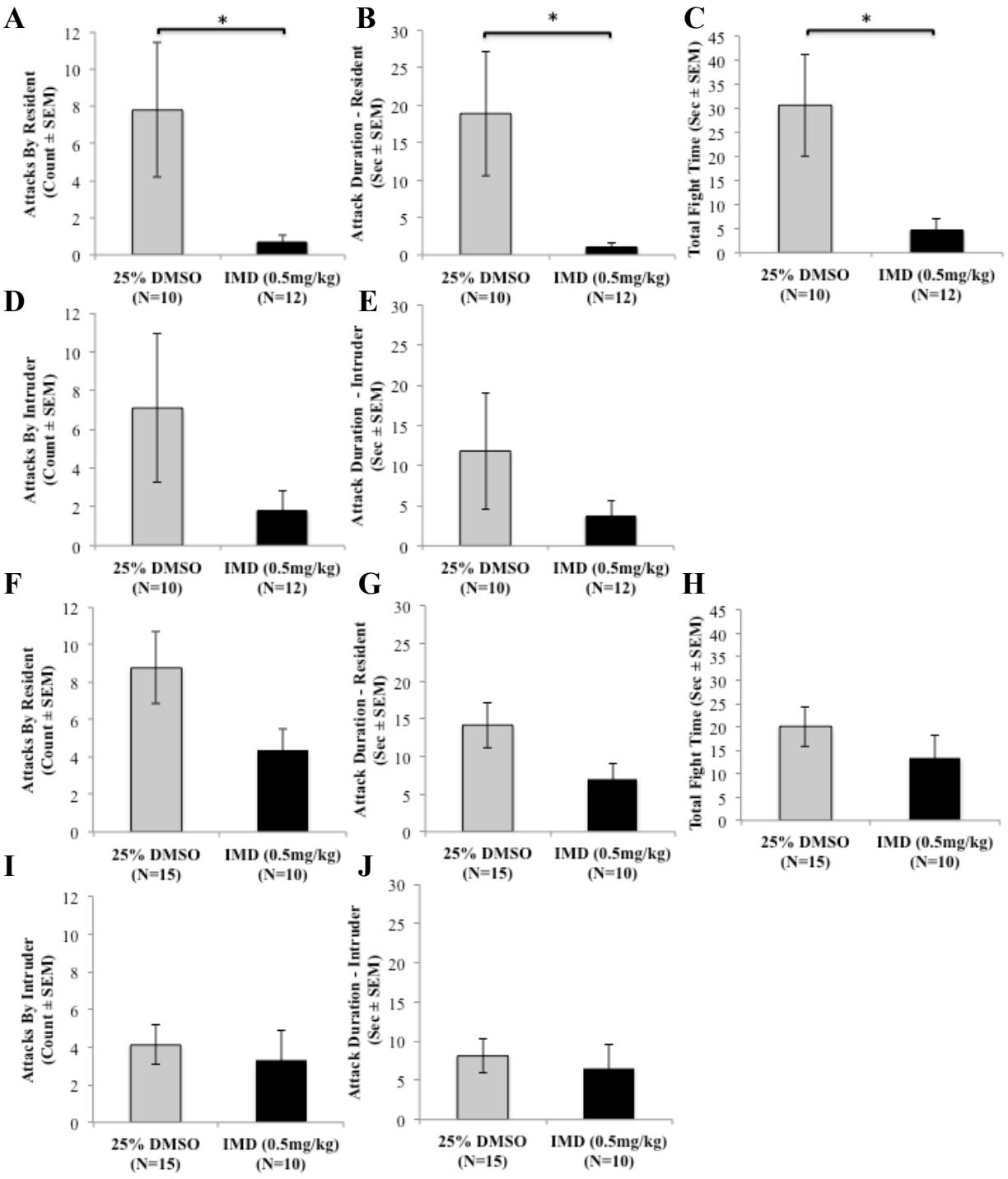
4.8 Social Aggression and Dominance

The resident intruder test was employed to evaluate social aggression and dominance. Two mice, both from the DMSO treatment group, were excluded from the analysis as they

exhibited abnormal hyperactive behavior, and did not engage in any social interaction. In Study 3, a significant decrease in the number of attacks by the IMD treatment group residents was observed, compared to the DMSO treatment group (Fig. 9A). In addition, the duration of the attacks by resident was significantly decreased in the IMD treatment group residents, compared to the DMSO treatment group (Fig. 9B). A significant decrease in total fight time was also observed in the IMD treatment group, compared to the DMSO treatment group (Fig. 9C). The numbers of attacks by the intruder, and the duration of the attacks by the intruder, were decreased in the IMD treatment group compared to the DMSO treatment group, but this parameter was not statistically significant (Fig. 9D, Fig. 9E). The latency to attack by resident or the latency to attack by the intruder were recorded and analyzed (i.e. the latency was recorded for the mouse (resident OR intruder) that initiated the first attack); however, the results are not shown, as the numbers of mice per treatment group were not sufficient for analysis (Experimental parameter and number of mice per treatment group: Latency to Attack by Resident, $N_{DMSO} = 4$, $N_{IMD} = 0$, Latency to Attack by Intruder $N_{DMSO} = 1$, $N_{IMD} = 3$).

In the Study 4 resident intruder test analysis, the attacks by resident, the duration of attacks by resident, and the total fight time, appear to be decreased in the IMD treatment group residents, compared to the DMSO treatment group, but these observations were not statistically significant (Fig. 9F, Fig. 9G, Fig. 9H). In addition, no significant differences in the number of attacks by intruder, or duration of attacks by intruder, were observed between treatment groups (Fig. 9I, Fig. 9K). Once again, the latency to attack by resident, and by intruder, were recorded and analyzed, but the results are not shown, as the numbers of mice per treatment group were not sufficient for analysis (Experimental parameter and number of mice per treatment group: Latency to Attack by Resident $N_{DMSO} = 11$, $N_{IMD} = 7$, Latency to Attack by Intruder $N_{DMSO} = 2$, $N_{IMD} = 2$).

When the results from Study 3 and Study 4 were combined, a significant decrease the number of attacks by the IMD treatment group residents compared to the DMSO treatment group was observed (Fig. 9K). Furthermore, the duration of the attacks by resident was significantly decreased in the IMD treatment group residents, compared to the DMSO treatment group (Fig. 9L). In addition, a significant decrease in total fight time was also observed in the IMD treatment group, compared to the DMSO treatment group (Fig. 9M). No significant differences in the number of attacks by intruder, or duration of attacks by intruder were observed between treatment groups (Fig. 9N, Fig. 9O). The latency to attack by resident, and by intruder, were compiled and analyzed, but the results are not shown, as the numbers of mice per treatment group remained insufficient for analysis, even after compilation (Experimental parameter and number of mice per treatment group: Latency to Attack by Resident $N_{DMSO} = 15$, $N_{IMD} = 7$, Latency to Attack by Intruder $N_{DMSO} = 3$, $N_{IMD} = 5$).


A
Attacks By Resident (Count ± SEM)
*
25% DMSO (N=10)
IMD (0.5mg/kg) (N=12)
B
Attack Duration - Resident (Sec ± SEM)
*
25% DMSO (N=10)
IMD (0.5mg/kg) (N=12)
C
Total Fight Time (Sec ± SEM)
*
25% DMSO (N=10)
IMD (0.5mg/kg) (N=12)
D
Attacks By Intruder (Count ± SEM)
25% DMSO (N=10)
IMD (0.5mg/kg) (N=12)
E
Attack Duration - Intruder (Sec ± SEM)
25% DMSO (N=10)
IMD (0.5mg/kg) (N=12)
F
Attacks By Resident (Count ± SEM)
25% DMSO (N=15)
IMD (0.5mg/kg) (N=10)
G
Attack Duration - Resident (Sec ± SEM)
25% DMSO (N=15)
IMD (0.5mg/kg) (N=10)
H
Total Fight Time (Sec ± SEM)
25% DMSO (N=15)
IMD (0.5mg/kg) (N=10)
I
Attacks By Intruder (Count ± SEM)
25% DMSO (N=15)
IMD (0.5mg/kg) (N=10)
J
Attack Duration - Intruder (Sec ± SEM)
25% DMSO (N=15)
IMD (0.5mg/kg) (N=10)

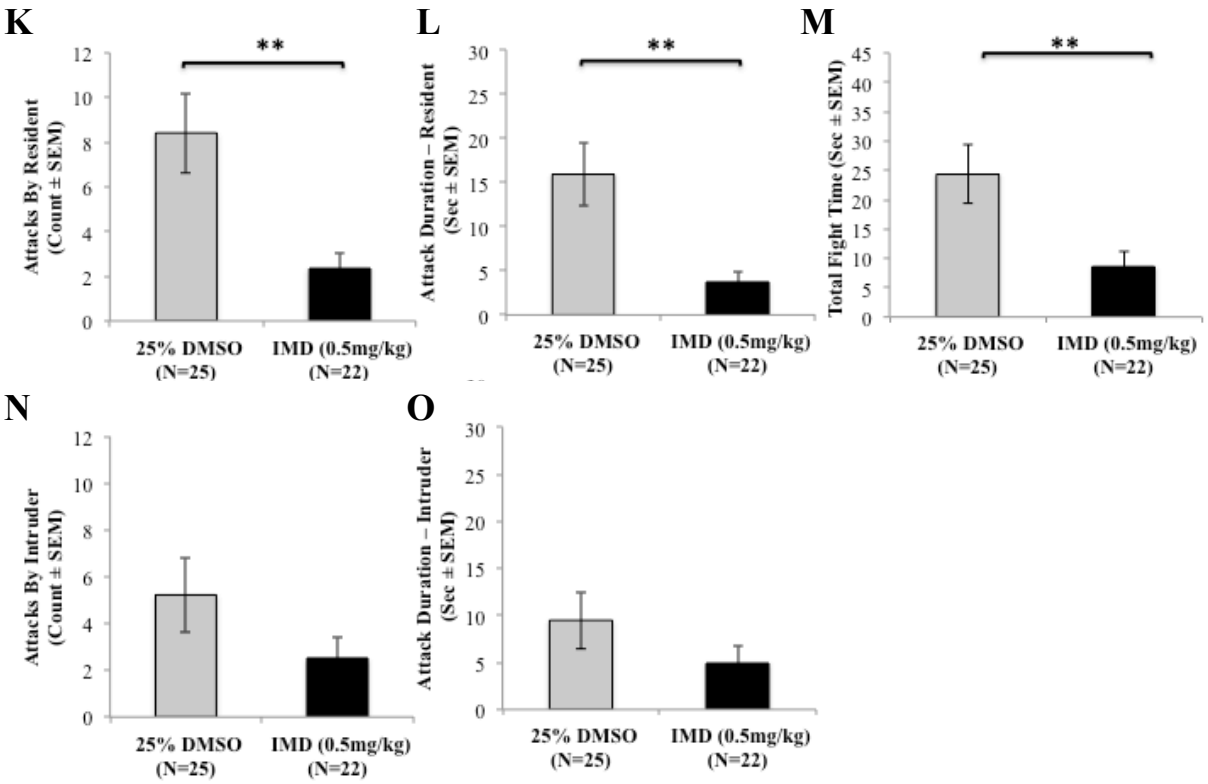


Fig. 9. Resident intruder test analysis. (A-E) Resident intruder analysis from Study 3. **(F-J)** Resident intruder analysis from Study 4. **(K-O)** Compilation of resident intruder analyses from Study 3 and Study 4. This test was conducted on male mice only. Each resident mouse was socially isolated for 10-12 days prior to testing. The intruder for this test was an age and sex-matched naïve wild-type CD-1 male. Each column represents the mean ± SEM. N = number of mice in each group. Two-tailed Student's t-test, *p<0.05.

4.9 Attention Performance

The 5-choice serial reaction time task (5CSRT) is a test that is commonly used to evaluate attention performance. Unfortunately, the execution of this test was unsuccessful as the mice were unable to advance beyond pretraining (see Discussion for further explanation).

4.10 Delayed-type Hypersensitivity - Cell-Mediated Immunity

To assess cell-mediated immunity in response to an immune stimulus, we used a delayed-type hypersensitivity assessment. To induce an inflammatory response, 1% oxazolone (irritant) was applied to the right ear of the mouse, while vehicle (acetone/olive oil 4:1vol/vol) was applied to the left ear. The auricular lymph nodes were collected 24 hours later, and the cells within the lymph nodes were analyzed using flow cytometry. The immune stimulation had no significant effect on CD4+ or CD8+ T-cell populations (Fig. 10). Furthermore, no significant differences in CD4+ and CD8+ T-cell populations were observed between treatment groups, with or without an immune stimulus (Fig. 10).

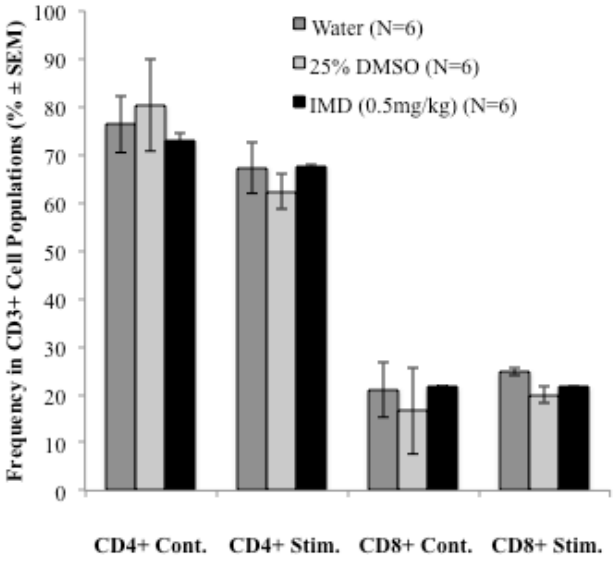


Fig. 10. Delayed-type hypersensitivity analysis from Study 3. Auricular lymph nodes were collected 24 hours after oxazolone challenge (N=3 males per treatment group). Lymphocytes were mechanically released and incubated with a mouse T lymphocyte subset anybody cocktail (PE-Cy7 CD3e, PE CD4, FITC CD8). Flow cytometry was used to analyze at least 8000 cells per treatment group. Each column represents the mean ± SEM. N=number of mice in each group. Two-way ANOVA with Bonferroni post-hoc test. Cont.: Non-stimulated control, Stim.: Oxazolone stimulated.

4.11 Haematology

A haematological analysis was used to measure static general health of the mothers. In Study 2, no significant difference in immune cell concentration was observed between the treatment groups (Fig. 11A). In Study 4, no significant difference in immune cell concentration was observed between the treatment groups (Fig. 11B). When the results from Study 2 and Study 4 were compiled, no significant difference in immune cell concentration was observed between the treatment groups (Fig. 11C).

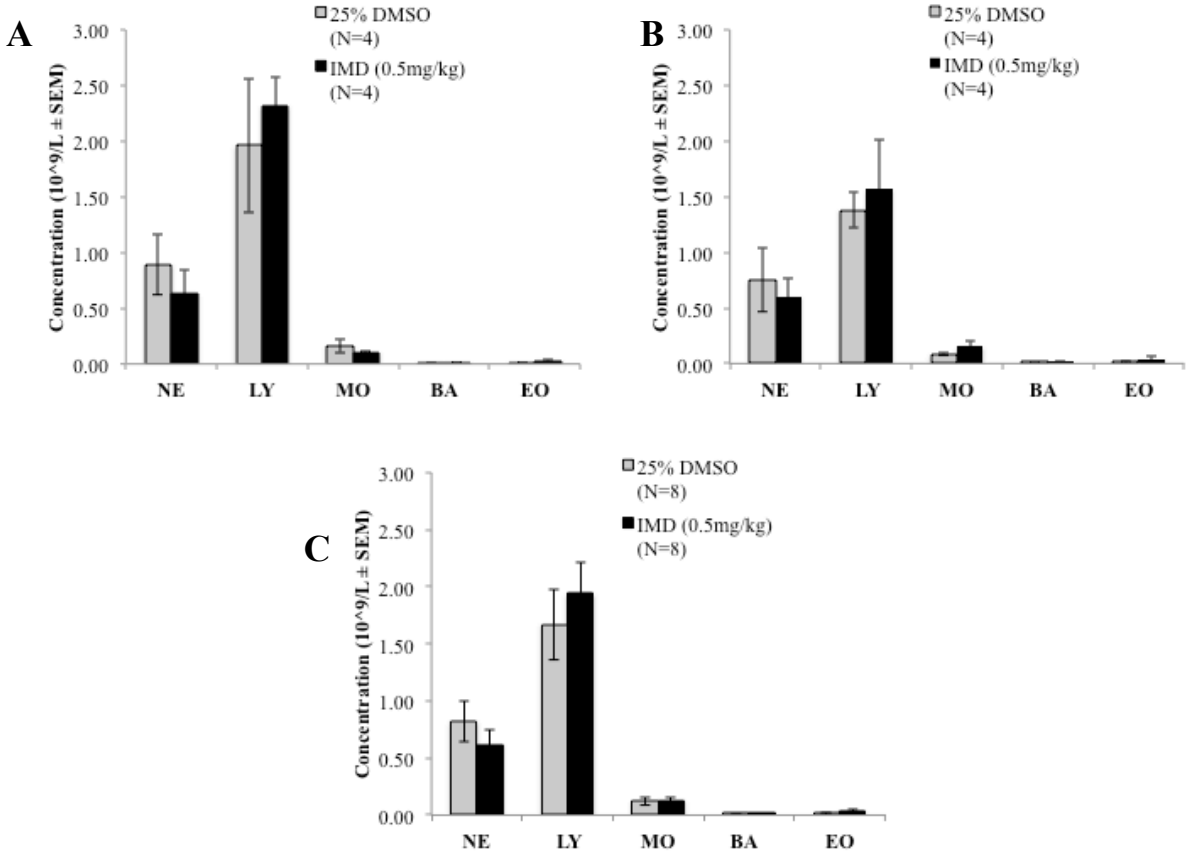


Fig. 11. Haematology analysis from the mothers of Study 2 and Study 4. (A) Haematology analysis from mothers of Study 2. **(B)** Haematology analysis from mothers of Study 4. **(C)** Compilation of haematology analyses from Study 2 and 4. Whole blood samples were collected in EDTA coated tubes, and taken to the Toronto Centre for Phenogenomics. Each column represents the mean ± SEM. N=number of mice in each group. Two-tailed Student's t-

test. BA: Basophils, DMSO: Dimethyl Sulfoxide, EO: Eosinophils, IMD: Imidacloprid, LY: Lymphocytes, MO: Monocytes, NE: Neutrophils.

4.12 Pilot Experiment – Sperm Head Abnormality

The purpose of Pilot Experiment 2 was to establish whether a chronic low-dose exposure to 0.5mg/kg of IMD has any lasting effect on spermatozoon morphology, through the use of a sperm head abnormality assay. There was no significant difference observed in the percentage of normal cells counted, and thus the percentage of abnormal cells counted, between the DMSO and IMD treatment groups (Fig. 12).

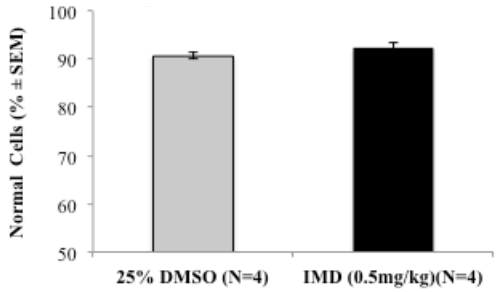


Fig. 12. Sperm head abnormality assay results from Pilot Experiment. The male mice were exposed to 0.5mg/kg of IMD or 25% DMSO for 36 days, after which the sperm cells were collected and observed for abnormalities. Abnormal sperm cells were defined as those containing a banana-shaped head, prism-shaped head, blunt-hooked head, curved hooked head, wrong-angled hook head, apical-hooked head, hookless head, amorphous head, or a pin head. The data are portrayed as the mean percentage of normal cells observed in the 200-cell population, ± SEM. N=number of mice in each group. Two-tailed Student's t-test. DMSO: Dimethyl Sulfoxide, IMD: Imidacloprid.

TESTS	Study 1	Study 2	Study 3	Study 4
Motor Activity (Distance Travelled)	NS	NS	+ (Male)	N/A
Elevated Plus Maze (Closed Arm Time)	- (Combined)	+ (Female)	NS	N/A
Social Interaction	NS	NS	N/A	N/A
Tube Test (Winning %)	+ (Combined) + (Male)	+ (Male) - (Female)	+ (Combined) + (Male) + (Female)	NS
Prepulse Inhibition (Average Inhibition)	N/A	N/A	NS	NS
Forced Swim Test (Immobility Time)	N/A	- (Combined) - (Female)	- (Combined) - (Female)	N/A
Resident Intruder (Attack Count)	N/A	N/A	-	-

Table 3. Summary of findings from behavioural testing. A plus sign (+) indicates a significant increase in the measured variable, while a minus sign (-) indicates a significant decrease in the measured variable. NS: Non-significant, N/A: Not-applicable.

5. Discussion

5.1 Study Design and General Points

The primary goal of Study 1 was to obtain preliminary data that would allow us to gain a general understanding of the potential effects of a prenatal and early postnatal exposure to IMD. Prior to Study 1, very little data existed in the scientific literature with regards to prenatal and early postnatal IMD exposure. As a result, Study 1 served as an important starting point for establishing the orientation of the project and for providing insight into which measurement parameters should be further investigated, and which parameters should be discarded. Using the scientific literature surrounding prenatal and early postnatal exposure to nicotine, we designed Study 1 to capture as many different relevant behavioural features as possible, while ensuring that the mice were not over-tested.

In selecting the doses for our studies, we sought to use a dose that was lower than the doses typically used in the current neonicotinoid literature (which had an average chronic dose of between 5 mg/kg and 10 mg/kg). As such, the doses of IMD we selected for both Study 1 (0.75 mg/kg) and Studies 2, 3 and 4 (0.5 mg/kg) are considered (relatively) to be low doses. While the average dosage of neonicotinoids to which humans are routinely exposed to has not been established, one Japanese study that performed biological monitoring of human exposure to neonicotinoids using urine samples estimated (based on their results) that the average daily intake of neonicotinoids was 0.53-3.66 μg/day to a maximum of 64.5 μg/day (Harada et al., 2016). Therefore, while the doses used in this study are low relative to those used in animal studies published to date, it must be noted that the tissue concentrations present in the offspring of the treated mothers used here, are in all likelihood higher than the levels of neonicotinoids that humans may be exposed to from ingesting contaminated food and water. Nevertheless,

future studies should seek to assess the relationship between low-dose neonicotinoids administered to animals, and the levels of the parent drugs and active metabolites achieved in the tissues.

In Study 1, we implanted C57BL/6 mice with an osmotic pump that delivered 0.75 mg/kg of IMD or 20% DMSO. Prenatal and early postnatal exposure to IMD appeared to have no observable effect on locomotor activity (Fig. 2A). However, IMD exposure may have led to a decrease in anxiety, as the IMD-treated mice spent more time in the open arms of the elevated plus maze compared to DMSO and water treated mice (Fig 2B). In addition, it appeared as though IMD exposure increased aggression, as an increase in tube test winning was observed in both IMD-treated males and IMD-treated males and females combined, compared to the DMSO and water control treatment groups (Fig. 2C).

Several critical changes in study design were made in Study 2. The first change made was the strain of mice used. In Study 1, we used C57BL/6mice, while in Studies 2-4 we used the CD-1 strain. The first reason for this change was due to the fact that there is extensive literature on the behaviour of CD-1 mice, especially within the nicotine literature; as such, the way nAChR agonists affect CD-1 mice is well established (Lewis et al., 2015, Brioni et al., 1993, Lobina 2011). The second reason for the mouse strain change was because the CD-1, as an outbred strain, most closely replicates the inter-individual variability observed in humans (Aldinger et al., 2009). A third reason for changing to the CD-1 strain was due to the large litter size produced by CD-1 mice. This allowed us to have a greater number of test subjects, improving the validity and reducing the variability of our results. Finally, the fourth reason for the change to the CD-1 strain was due to the way by which CD-1 mice metabolize neonicotinoids. According to Swenson and Casida, 2013, neonicotinoids are metabolized through reduction by aldehyde oxidase (AOX), and oxidation by CYP450 enzymes (Swenson and Casida, 2013). In a 2004 study, Vila et al. found that CD-1 mice expressed high levels of

AOX homologues in both the skin and liver, and therefore are capable of metabolizing IMD effectively (Vila et al., 2004). As a result, the CD-1 mice provide an animal model that is more representative of humans.

The second critical change we made in Study 2 was the IMD dosage and the DMSO concentration used. In Study 1, we used an IMD dosage of 0.75 mg/kg and a 20% DMSO vehicle, while in Study 2 we used an IMD dosage of 0.5 mg/kg and a 25% DMSO vehicle. The reason for this is due to the solubility of IMD, and is directly related to the use of CD-1 mice. CD-1 mice are heavier than C57BL/6 mice, with the average weight of CD-1 mice at 5 weeks of age ranging from ~21 g (females) to ~31g (males), compared to ~15 g (females) to ~22 g (males) for C57BL/6 mice (information from Charles River Laboratories). As such, the amount of IMD that would need to be inserted into the fixed-volume (200 μL) osmotic mini-pump to deliver the 0.75 mg/kg dosage to a heavier CD-1 mouse would be larger than that required for C57BL/6 mice. Unfortunately, a dosage of 0.75 mg/kg of IMD in 20% DMSO for a 30 g pregnant female (6.25 μg/μL required) was beyond the solubility limits of IMD. Therefore, we reduced the dosage to 0.5mg/kg, and as an extra precaution, increased the DMSO concentration to 25% to ensure that the IMD would not precipitate out of solution once injected into the pump.

Small changes were made from study to study, all with the purpose of improving our experiments and refining our results. In particular, between studies, we added and removed certain behavioural tests. The changes we made in the behavioural testing regimen were based on the results from the previous experiments; for example, if the results from a previous study were not reproducible and/or not statistically significant, that behavioural test was replaced with a test that was more likely to provide definitive success in supporting (or disproving) our hypothesis. To summarize, a major strength of this project is the study design. We used a wide array of biochemical and behavioural tests, across four large studies, using approximately 400

mice in total, all with the goal of identifying the significant and reproducible effects of an *in utero* and early postnatal exposure to IMD.

Previous studies have established that neonicotinoids can pass through intestinal mucosa, the blood-brain barrier, and the placenta, thereby entering adult and fetal tissues (Abou-Donia et al., 2008; Taira, 2014). In an attempt to determine IMD concentrations in the tissues of treated CD-1 mothers, we have established a collaboration with Dr. Rafael Mateo at the Instituto de Investigacion en Recursos Cinegeticos in Spain. Dr. Mateo's laboratory will measure IMD levels in brains and livers of treated (via osmotic mini-pump) mice using liquid chromatography/mass spectrometry. We are currently waiting for these results.

One limitation of our study was the use of DMSO as the vehicle for IMD delivery. Unfortunately, due to the insolubility of IMD in water at high concentrations, DMSO was the most suitable option for this study. In using DMSO as the vehicle for this project, the potential effects of DMSO must be recognized. One study, which assessed the effects of organic solvents on the activities of cytochrome P450 enzymes, found that DMSO (at a concentration of 2% (v/v)) led to a significant inhibition in the activities of CYP2C9, CYP2C19, and CYP2E1 (Easterbrook et al., 2001). This is of great importance to this project, as cytochrome P450 enzymes are responsible for the metabolism of IMD (see Introduction).

If the DMSO vehicle in this study inhibited the CYP450 enzymes that are responsible for the metabolism of IMD, it is possible that the results of this project are not a complete representation of the true effects of IMD. For example, it is established that both the IMD parent compound and the IMD metabolites are active at the nAChR and thus can have toxic effects. Therefore, if the mice in this study were unable to metabolize IMD (and therefore did not produce toxic IMD metabolites), it is possible that the effects of IMD observed in this study are an underestimation of the true effects of IMD, and account for the effects of the IMD parent compound only. If this was indeed the case, one may predict that the effects of IMD on

neurodevelopment may have been stronger than what was observed in this study, if a different vehicle had been used and toxic metabolites had thus been produced.

5.2 Non-Significant and/or Non-Reproducible Results

The elevated plus maze is one of the most widely used tests for measuring anxiety-like behavior in animals (Komada et al., 2008). Across the four studies completed in this project, there was no obvious reproducible pattern gleaned from the results of the elevated plus maze. A brief review of the literature surrounding the influence of PNE on the elevated plus maze provides insight into the high degree of variability that exists in the results of this particular behavioural test. A 2014 study that assessed the effects of PNE on anxiety found that PNE led to no significant effects in the elevated plus maze, consistent with our results (Santiago and Huffman, 2014). However, a 2016 study observed a significant increase in anxiety as a result of PNE, as shown by a significant decrease in the time spent in the open arms of the elevated plus maze (Aoyama et al., 2016). Finally, a 1998 study observed an increase in the time spent in the open arms of the elevated plus maze following PNE, therefore implying that PNE reduces anxiety, thus refuting the findings of Aoyama et al (Ajarem and Ahmad, 1998). These inconsistencies in the literature suggest that both nicotine and neonicotinoids might not have a strong influence on anxiety, at least as measured in the tests employed in these studies.

Pre-pulse inhibition is a neurological phenomenon that provides an operational measure of sensorimotor gating. The pre-pulse inhibition test analyses showed no statistically significant differences between treatment groups in startle response or average pre-pulse inhibition (Fig. 7). Due to the high degree of variability in the results, it was difficult to confidently identify the presence of any data trends. Previous studies have shown PNE causes a decrease in pre-pulse inhibition (specifically in female rats, (Popke et al., 1997)), however, our results did not support

this trend. Further investigation into the effects of IMD exposure on pre-pulse inhibition might provide clarification.

In addition, further investigation into the effects of IMD exposure on attention performance is also required. Previous studies in humans and laboratory animals have observed that PNE results in an increased likelihood for ADHD, though direct causation has been difficult to prove thus far (Tiesler and Heinrich, 2014, Zhu et al., 2014). As such, with knowledge of this potential correlation between PNE and ADHD, we felt that it would be informative to investigate a potential correlation between *in utero* and early postnatal IMD exposure and ADHD as well. We attempted to investigate this relationship through the use of the 5CSRT, however our efforts were unsuccessful. The mice in our study successfully completed habituation and pretraining, but for reasons largely unknown were unable to acquire the 5CSRT task in 5CSRT training phase of this test. Some studies suggest poor vision may contribute to a lack of success for CD-1 mice in repetitive continuous performance tasks (Kim et al., 2015). Several modifications in our experiment were made (such as increasing time, decreasing criteria, temporarily increasing dietary restriction) in an attempt improve the likelihood of successful task acquisition, but these attempts to encourage advancement beyond the 5CSRT training phase were unsuccessful. Therefore, a decision was made to terminate the experiment after four weeks of unsatisfactory results, as the mice were not reaching the training advancement criteria of >80% accuracy and <20% omissions. In summary, we were unable to obtain useful data from this experiment.

Based on previous studies, we expected IMD exposure to cause a deficit in cell-mediated immunity (and thus variations in haematology) (Badgujar et al., 2013 and Gawade et al., 2013). In addition, we also expected to observe a significant increase in sperm head abnormalities in the IMD-treated male mice of the Pilot Experiment, as was observed by Bagri et al., 2015. However, the biochemical assays performed in this thesis project failed to show statistically

significant differences in all biochemical parameters, between treatment groups. One explanation for the lack of a treatment effect in these biochemical analyses is due to the low dosage of IMD that we employed. The studies that provided the foundation for our hypotheses employed dosages higher than our dose of 0.5mg/kg and, in looking across all aforementioned studies, the lowest dose with an observable effect in these studies was 5mg/kg, which appeared to cause a deficit in cell-mediated immunity (Badgujar et al., 2013). We hypothesized that chronic exposure to a low dose of IMD throughout development would ultimately lead to lasting biochemical perturbations in immunity and sperm head abnormality, and the results obtained in this project appear to disprove this part of our hypothesis.

5.3 Significant and Reproducible Results

5.3.1 Body Weight

When assessing the overall health of laboratory mice, one of the primary indicators of poor health is a variation from normal body weight (Foltz et al., 1999). While body weight alone is usually not sufficient to identify sick individuals, it provides a crude measurement that is both easy to collect and may identify perturbations in animal welfare that can be further investigated (Burkholder et al., 2012). Importantly, body weight can also have a significant influence on the outcome of several behavioural tests, which will be further discussed in subsequent sections. For example, in a test of social dominance and aggression (i.e. tube test), whereby a small mouse is matched against a much larger mouse, we would expect the larger mouse to assert greater dominance. Therefore, the body weight analysis also aids in drawing accurate conclusions from behavioural data.

In Study 3, a significant decrease in body weight was observed in IMD-treated males, females, and males and females combined, compared to the DMSO treatment group (Fig. 3A).

In Study 4, while a trend was present, no statistically significant decrease in body weight was observed (Fig. 3B). However, when the results from Study 3 and Study 4 were compiled, a significant decrease in body weight was observed in all of the IMD-treated mice, compared to the DMSO treatment group (Fig. 3C).

The results from Study 3, and the combined results from Study 3 and Study 4, are supported by the findings of a 2014 study, in which a significant decrease in body weight was observed in adult male albino mice that were treated with a 15 mg/kg dose of IMD for 15 days (Arfat et al., 2014). However, one difference that exists between our findings and the findings of Arfat et al., is that in their study there was no significant treatment effect on body weight observed in mice treated with a lower dose of IMD (5 mg/kg and 10 mg/kg) (Arfat et al., 2014). One potential explanation for this discrepancy is the timing of treatment; the mice in our studies were exposed *in utero* and throughout early postnatal life, during a very critical time in development, whereas Arfat et al. exposed male mice for a shorter time, and during adulthood. It is well documented that perinatal condition can influence adult well-being and also that the fetal environment can have an impact on adult body weight (Eide, 2005, Slotkin et al., 2016). Supporting this is the fact that during prenatal development, the fetus would largely rely on the placental barrier for protection from toxins, while adult male mice would be well equipped to metabolize and effectively clear systemic IMD. Therefore, it is plausible that a smaller dose (such as the one used in our study) would have a much greater impact on a fetus than on an adult mouse, and a larger dose would be required in adult mice to observe the same effect.

To conclude, *in utero* and early postnatal exposure to IMD led to a decrease in body weight, compared to DMSO-treated mice. While this decrease was statistically significant, it was not a large weight difference, and the weight of the IMD-treated mice remained within the range of healthy CD-1 mice.

5.3.2 Motor Activity

In addition to body weight, another significant result observed in our project was the effect of IMD on motor activity. Motor activity is an important parameter to assess in behavioural experiments, as (like body weight) motor activity can strongly influence the results of other behavioural tests, potentially resulting in false positives and/or false negatives, and thus incorrect conclusions. For example, due to activity levels alone, a hyperactive mouse is more likely to explore all arms of an elevated plus maze, while a hypoactive mouse is more likely to remain in one arm of the maze. Therefore, one may incorrectly conclude that the hypoactive mouse has increased levels of anxiety (as it spent more time in the one closed arm of the maze and did not explore the remaining arms due to hypoactivity), while also concluding that the hyperactive mouse has lower levels of anxiety (as it spent more time in all arms of the maze, including the open arms). As such, motor activity was used in this study as both an experimental parameter, and a tool for understanding the outcomes of the other behavioural analyses.

A significant increase in the total distance travelled by the IMD-treated males was observed in Study 3, and when the results of Study 2 and Study 3 were combined, compared to the DMSO treatment group (Fig. 4C, Fig. 4E). A similar trend was also observed when both the males and the females were combined in both Study 3 and in the Study 2 and Study 3 combined results, as IMD-treated mice travelled greater distances compared to the DMSO treatment group; however, this observation was only statistically significant when the results from Study 2 and Study 3 were combined (Fig. 4C, Fig. 4E). In terms of gender effects, the male mice treated with IMD showed a significant elevation of motor activity, while female mice showed a less robust (non-significant trend).

The scientific community is in agreement that PNE increases the likelihood for adults to display hyperactivity. As such, our findings are well supported by the literature on PNE and hyperactivity. One study found that oral administration of a 0.1 mg/ml dose of nicotine to C57BL/6 mice, starting 3 weeks prior to mating and continuing throughout gestation, results in hyperactivity at both PND42 and PND60, thus supporting our findings (Zhu et al., 2012). Our findings are further supported by a 2012 study that observed a significant increase in locomotor activity in rats prenatally exposed to 0.06 mg/ml of nicotine (Schneider et al., 2012).

The effects of PNE on hyperactivity in humans is often studied in the context of ADHD. As mentioned, the literature surrounding PNE and ADHD in humans is extensive as well. Several studies have found a link between PNE and ADHD (Milberger et al., 1996, Milberger et al., 1998, Zhu et al., 2014, Langley et al., 2012). Therefore, our findings that *in utero* and early postnatal exposure to IMD led to an increase in adult motor activity is supported by the nicotine literature.

5.3.3 Depressive-like Behaviour as Measured by the Forced Swim Test

The forced swim test is a test of behavioural despair that is often used to evaluate the efficacy of antidepressant medications (Can et al., 2012). The time spent swimming (and the time spent immobile) is recorded during the 6-minute trial, with a larger time spent immobile (and a lower time spent swimming) being indicative of increased depressive-like behaviour. The forced swim test was administered only in Studies 2 and 3. In Study 2 and Study 3 a significant decrease in immobility time was observed in IMD-treated females, and males and females combined, compared to DMSO-treated mice; this same trend was observed in the IMD-treated males, but the trend was not statistically significant (Fig. 8a and Fig. 8b). In the combined results from Study 2 and Study 3, a significant decrease in immobility time was

observed in IMD-treated mice compared to DMSO-treated mice, regardless of sex (Fig. 8C). Therefore, based on our observations, it appears as though *in utero* and early postnatal exposure to IMD results in a reduction in depressive-like behaviours compared to vehicle-treated mice.

The effects of neonicotinoids on depression and depressive behaviour has not been studied. Moreover, the literature on PNE and depression is generally inconsistent. One study in humans reported that prenatal exposure to maternal smoking leads to an increased rate of depression and a higher risk for lower levels of happiness (Menezes et al., 2013). This finding is further supported by a longitudinal study that found prenatal smoking exposure leads to an increased rate of internalizing behaviour (Ashford et al., 2008). However, a conflicting study observed no association between maternal smoking and emotional/internalizing problems (like depression) (Brion et al., 2010). Therefore, at this time, the literature is not in agreement as to whether prenatal exposure to nAChR agonists influences depressive behaviours.

Like the tube test, several factors may have influenced the outcome of the forced swim test analyses. The first factor is the swimming ability of the mice. Swimming ability may be influenced by factors such as body weight and baseline motor activity. For example, body weight influences buoyancy; the heavier mice might be expected to be more buoyant, depending on the nature of the higher body weights (extra muscle vs. fat tissue), which could result in an increase in the time spent immobile/floating. In addition, baseline motor activity may also influence swimming ability, as hyperactive mice may be more inclined or able to swim greater distances (or for a longer duration). The influence of body weight and hyperactivity provide a potential explanation of our forced swim test results, as the IMD-treated mice were significantly lighter and hyperactive and thus spent more time swimming (indicative of a decrease in depressive behaviours) while the DMSO-treated mice, which were heavier and less active, spent more time immobile.

A second factor that may influence the outcome of the forced swim test results is swimming endurance. While the effect of PNE on adult swimming endurance is unknown, one study examining the effect of acute nicotine exposure on swimming endurance in adult rats observed a significant dose-dependent decrease in swimming endurance as a result of nicotine exposure (Temocin et al., 1993). This effect of nicotine on swimming endurance is the opposite of what we observed, as the IMD-treated mice displayed greater swimming endurance. Furthermore, on PND61-67, the mice in our study were no longer being exposed to IMD, unless there was IMD bioaccumulation (and at this time, there is no evidence to suggest that there was IMD bioaccumulation in our test subjects). Therefore it is unlikely that IMD treatment had an impact on swimming endurance.

To conclude, *in utero* and early postnatal exposure to IMD led to a significant decrease in depressive-like behaviours compared to DMSO-treated mice; however, this result may have been influenced by both body weight and motor activity.

5.3.4 Social Dominance as Assessed by the Tube Test

The tube test is a rudimentary behavioural test used to assess social dominance (Gholizadeh et al., 2014). Social dominance is defined as an intergroup theory of social hierarchy, whereby an animal is "ranked" higher than another (Weissbrod et al., 2013). Social dominance and social hierarchy are important parameters to measure as they both strongly influence animal survival, and provide insight into the way by which a group of animals interact. Therefore, we used the tube test to gain insight into the effect *in utero* and early postnatal IMD exposure has on social interactions within mice. With no treatment effect, one would expect an equal number of dominant and an equal number of submissive mice in a treatment group. However, if impairments in social dominance are present as a result of a

treatment effect, one would expect to see an increase in the number of submissive mice within the experimental treatment group. Likewise, if enhancements in social dominance are present as a result of a treatment effect, one would expect to see an increase in the number of dominant mice within the experimental treatment group.

In Study 2, a significant increase in tube test winning in IMD-treated males was observed compared to both water and DMSO treatment groups (Fig. 6B and Fig. 6C). The finding of enhanced male social dominance as a result of IMD treatment was replicated in Study 3, which had a larger number of test subjects and matchups, further solidifying our finding from Study 2 (Fig. 6D). Also in Study 3, a significant increase in tube test winning in IMD-treated females, as well as IMD-treated males and females combined, was also observed (Fig. 6D). Unfortunately, Study 4 did not replicate the findings of Study 2 and Study 3, although a trend towards an increase in winning was present in IMD-treated females and males and females combined in Study 4 (Fig. 6E). When the results from Study 2, Study 3 and Study 4 were combined, a highly significant increase in winning was observed in IMD-treated males, females, and males and females combined, compared to the DMSO treatment group (Fig. 6F). We conclude that pre- and early postnatal exposure to IMD enhances social dominance, at least in the context of the tube test. The literature on the effects of PNE (or exposure to other nAChR agonists) on social dominance (or on sociability as a whole) is essentially non-existent, thereby identifying an area that requires further research (Ross et al., 2015). Therefore, the literature does not provide guidance to refute or support our findings at this time.

A number of factors may have influenced the observed tube test results. First, one must take into consideration the body weight of the test subjects. As noted, IMD-treated mice (males and females) were slightly (but significantly) lighter than their DMSO-treated counterparts, therefore one might expect them to be more submissive. This was not the case, as the lighter IMD-treated mice had enhanced social dominance. Therefore, body weight likely did not

influence the tube test results. However, if weight did have an influence on the tube test results, the influence would favour the heavier DMSO-treated mice (enhancing their winning percentage based on weight alone), and would have led to an underestimation of the social dominance observed in the IMD-treated mice. The body weight difference was not visibly noticeable, and therefore it is more likely that body weight did not have a strong influence on the tube test.

A second factor that may have influenced the results of the tube test is baseline motor activity. The hyperactivity observed in IMD-treated mice may have resulted in an increase in tube test winning in the IMD treatment group compared to the DMSO treatment group, as the hyperactive mice may have been more inclined to continually push forward in the tube (thus causing the DMSO-treated mice to exit the tube and "lose"). It is also possible that the hyperactivity observed in the IMD-treated mice may have resulted in a decrease in winning in the IMD treatment group compared to the DMSO treatment group, as the hyperactive mice may have been more inclined to reverse and escape from the constraints of the small tube. The latter may be more plausible as the mice would likely take the path of least resistance out of the tube, enabling them to be more active. Therefore, it is possible that the hyperactivity of the IMD-treated mice might have led to a decrease in winning, and that the IMD-treated mice may have won even more had they not been hyperactive.

An interesting anecdotal observation was made during the tube testing, which unfortunately was not quantified. The observation is best described as a repeated avoidance behavior that was observed in one treatment group across all 4 studies. The mouse displaying the avoidance behaviour would promptly reverse out of the tube during the tube test, thus resulting in a loss. The (traditionally observed) pushing of the submissive mouse out of the tube by the dominant mouse was not frequently observed. Interestingly, it was noted that the DMSO-treated mice were the treatment group that exhibited this avoidance behavior,

specifically when they were matched against the IMD-treated mice. One could hypothesize that this avoidance by the DMSO-treated mice is a result of dominance that is enforced by the IMD-treated mice through non-visual means such as scent and/or vocalization. A 2008 study supports the hypothesis that scent is responsible for this non-visual dominance, as they state that mice frequently deposit scents that specify their social status (Arakawa et al., 2008). It has also been hypothesized that ultrasonic vocalizations may be responsible for this dominant behaviour, as it has been concluded that differences exist in the vocalizations produced by dominant male mice, when compared to subordinates (Nyby et al., 1976). It is plausible that both scent and ultrasonic vocalizations are responsible for this non-visual dominance portrayed by the IMD-treated mice during the tube test; it would be valuable for future studies assessing the effects of neonicotinoids on social dominance to measure these parameters.

To conclude, in the context of the tube test, *in utero* / early postnatal exposure to IMD leads to a significant enhancement in social dominance in the adult offspring.

5.3.5 Social Aggression and Dominance as Assessed by the Resident Intruder Test

The resident intruder test was employed to further explore the effects of IMD on social behaviour: both the tube test and the resident intruder test provide insight into social interactions and social dominance (Koolhaas et al., 2013). The number of attacks, duration of attacks, and total fight time were recorded, with an increase in attacks, an increase in duration of attacks, and an increase in total fight time being indicative of aggression and socially dominant behaviour. The findings from the resident intruder test are interesting in light of the findings from the tube test. In juxtaposition to the tube test results, one might have predicted more aggression in the IMD-treated mice compared to DMSO-treated mice. However, in both Study 3 and in the

combined Study 3 and Study 4 analysis, the number of attacks by the resident, the duration of attacks by the resident and total fight time were *reduced* in IMD-treated mice compared to the DMSO-treated mice (Fig. 9A-C and Fig. 9K-M). A similar trend was observed in the Study 4 analysis, but statistical significance was not achieved (Fig. 9F-H). The traditional interpretation of these resident intruder results suggests IMD-treated mice displayed decreased social dominance and aggression.

As noted above, the literature surrounding PNE and social dominance is sparse. The literature on PNE and social aggression is slightly more plentiful, but this is still an area that requires further investigation. In one study, male mice exposed to prenatal maternal smoking exhibited enhanced aggression and social dominance in adult offspring, as there was in increase in the number of attacks and a decrease in latency to attack in the resident intruder test, thus supporting our tube test findings but refuting our resident intruder test results (Yochum et al., 2014). In another study assessing the developmental consequences of prenatal tobacco exposure, it was concluded that prenatal tobacco exposure has a significant direct effect on increased child aggression (Day et al., 2000); these findings were replicated and confirmed by the same group in 2009 (Cornelius and Day, 2009). The conclusion that PNE leads to an increase in adolescent aggression appears to be generally accepted in the field, and no studies published at this time refute this claim.

Like the tube test, similar external factors may influence the results of the resident intruder test as well. The most important factor potentially influencing the resident intruder results is the resident and intruder body weights. Variation in body weight may provide false positives and/or false negatives in the resident intruder analysis, leading to incorrect conclusions. For example, if the resident is much larger than the intruder, it is likely that the resident will not need to fight/attack to assert dominance and the intruder will avoid conflict with the larger counterpart, ultimately leading to a decrease in the number and duration of

attacks (in both the resident and intruder). This would be incorrectly interpreted as a decrease in social aggression and a decrease in social dominance in the resident, when the observed result may be due to the variation in weight alone. On the contrary, if the resident mouse is much smaller than the intruder, it may feel the need to fight more to protect its home cage territory, thus artificially increasing the number and duration of attacks by the resident (interpreted as an increase in social dominance, even if the mouse is ultimately submissive due to size difference). In our study, we used age and sex matched wild-type CD-1 mice as intruders, and the weights of the residents and intruders were recorded. On average, the intruder was slightly heavier than both DMSO-treated and IMD-treated residents. Therefore, if the body weight difference between the resident and the intruder had an effect, the effect would have been equal for both DMSO and IMD treatment groups. As such, it is unlikely that body weight negatively influenced our conclusions.

It could be important to question the traditional interpretation of results from animal behavioural analyses. Upon analyzing the videos from the resident intruder test, like in the tube test, an interesting observation was made once again, bringing traditional interpretation of resident intruder results into question. Like in the tube test, a behaviour best described as avoidance was once again observed in some of the intruder mice that were matched with IMD-treated resident mice, however this behaviour was not quantified and is therefore a subjective impression. The proposed avoidance behaviour displayed by the wild-type intruder mouse, which may have been caused by scent and/or vocalization by the resident mice, led to the intruder mouse spending more time distant from the resident mouse which, in turn, may have resulted in a decrease in attacks by resident (and intruder), a decrease in the duration of attacks by resident (and intruder) and a decrease in total fight time compared to the DMSO-treated mice. As a result, this may have contributed to the decreased social dominance and social

aggression in the IMD-treated resident mice, when in fact the social aggression and dominance may have been asserted through different non-visible means.

To conclude, the results from the resident intruder test showed a decrease in both number and duration of attacks by resident, and a decrease in total fight time, in IMD-treated resident mice compared to DMSO-treated resident mice. The inference of this is a reduction in aggressive behavior, which traditionally would be labeled as a decrease in social dominance and aggression, but is opened to interpretation. Based on the results of the tube test and the avoidance behaviour that was observed in both the tube test and the resident intruder test, it is conceivable (though speculative and requiring further investigation) that the IMD-treated mice may indeed exhibit an increase in social dominance and aggression that is asserted through mechanisms not apparent upon visual observation, but perhaps could have been mediated by olfaction and ultrasonic communication.

6. Conclusions

There are an extremely limited number of published studies on neonicotinoids and behavior, especially regarding prenatal exposure. As such, the results of our experiments were compared to the literature on PNE. It is important to note however, that nicotine and neonicotinoids are not identical. For example, nicotine is rapidly metabolized to inactive metabolites (e.g. cotinine), while some neonicotinoids are metabolized to active metabolites (e.g. imine derivatives). In addition, nicotine is much more potent at the nAChR when compared to the neonicotinoid parent compound, binding to the receptor with a much higher affinity. Therefore, it would likely be incorrect to assume that neonicotinoids would have effects that are identical to those of nicotine.

Our study was unique in that there are no similar studies reported in the literature on neonicotinoid exposure in rodents at low doses during fetal and early postnatal development. The primary objective of this thesis research was to elucidate the significant and reproducible behavioural and biochemical perturbations that result from a chronic *in utero* and early postnatal exposure to a low dose of IMD. Our results indicate that a chronic *in utero* and early postnatal exposure to a low dose of IMD does not appear to have an effect on anxiety and explorative behaviours, sensorimotor gating, cell-mediated immunity, haematology, and sperm head abnormality, in the context of the behavioural and biochemical assessments employed in this project. The effect of IMD on sociability and attention performance remain inconclusive at this time.

However, our results do indicate that a chronic *in utero* and early postnatal exposure to a low dose of IMD leads to decreased body weight, elevated motor activity in IMD-treated male mice, an increase in social dominance (observed as an increase in winning in the tube test), a decrease in depressive behaviours (observed as a decrease in the time spent immobile during the

forced swim test), and reduced social aggression (as indicated by a decrease in the number and duration of attacks by IMD-treated residents during the resident intruder test).

Several recent studies have indicated that both humans and wildlife are frequently exposed to neonicotinoids in small doses through the consumption of contaminated fruits, vegetables, vegetation and water (Abou-Donia et al., 2008, Chen et al., 2014, Lopez-Anita et al., 2015). Therefore, it is in nature and society's best interest to establish the deleterious effects of these chemicals on animals. The findings reported here provide evidence that neonicotinoids may have persistent negative effects in mice, which (upon further investigation) may translate to other animals and humans. Collectively, the findings of this research project suggest that the current unrestricted use of neonicotinoids is not sustainable, and may lead to detrimental health effects in the future. As such, with the health of society and wildlife in mind, it is suggested that the use of neonicotinoids be restricted and/or banished.

7. Future Directions

This project provides the scientific community with evidence that suggests *in utero* and early postnatal IMD exposure may have a lasting effect on mammalian health, therefore providing motive for further investigation. Additional behavioural and biochemical analyses, beyond those employed in this project, may identify additional perturbations that result from *in utero* and early postnatal IMD exposure. Specifically, based upon the literature surrounding the effects of PNE, a behavioural study solely dedicated to evaluating whether *in utero* and early postnatal exposure to IMD has an effect on cognition and attention performance is an area of interest. With several published studies (on both animals and humans) strongly suggesting a link between nAChR agonist (nicotine in particular) exposure and ADHD, one may hypothesize that *in utero* and early postnatal exposure to IMD may lead to deficits in attention performance (Yochum et al., 2014, Zhu et al., 2014). The 5CSRT test, which was attempted but unsuccessful in this project, could be informative as it would evaluate both attention performance and impulsivity, while also providing a crude measure of cognition (by evaluating the ability of the treated mice to acquire the task).

In addition, it would also be of value to further investigate the biochemical effects of *in utero* and early postnatal IMD exposure in greater depth. Evaluating biochemical parameters like IMD (and IMD metabolite) bioaccumulation in tissue (through the use of liquid chromatography–mass spectrometry) would be valuable, as this is not currently clear in the literature. Furthermore, evidence suggests that the lasting effects of PNE are due to nicotine-induced epigenetic changes (Jung et al., 2016). It would therefore be interesting to assess the effects of prenatal and early postnatal IMD exposure on epigenetics. Importantly, biochemical analyses will help elucidate the mechanism through which the behavioural perturbations come into fruition.

Finally, upon establishing the effects of neonicotinoids in mice, it may be of value to replicate the results in an animal model that more closely represents humans. Some suggest that rats provide a more human-like animal model than mice, more closely resembling human disease in many cases (Iannaccone and Jacob, 2009). Therefore, it may be of value to replicate the biochemical and behavioural findings in rats. Findings in a more human-like model could influence decisions that go into policy making, which may lead to the restriction or banishment of use of neonicotinoid pesticides, thus creating a safer environment.

The findings of this thesis project suggest that chronic neonicotinoid exposure, even at low doses, may have lasting detrimental effects on animal health. With further investigation and subsequent restriction of the use of neonicotinoids, the findings of this thesis may contribute to the health of society and wildlife as a whole.

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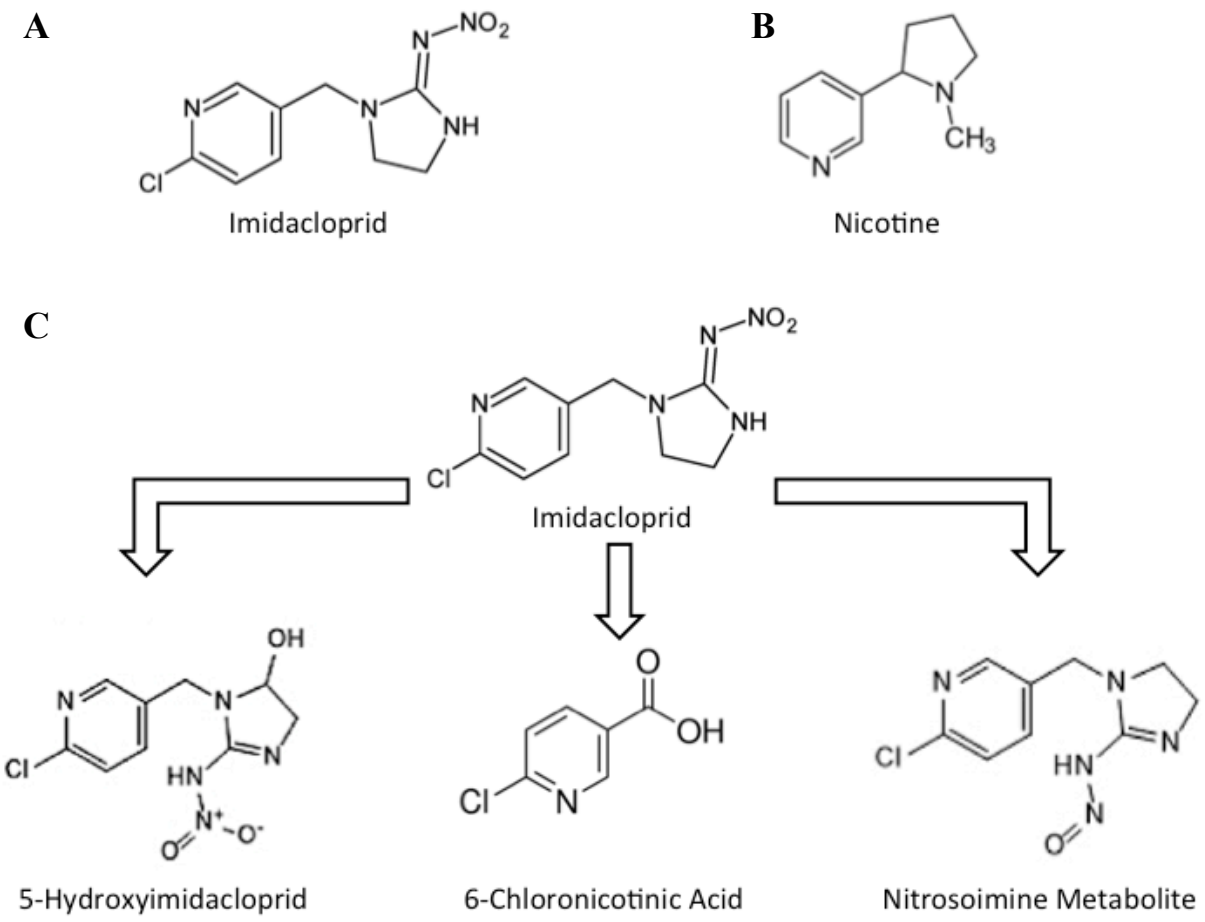
Appendices



Appendix 1. Structures of Imidacloprid, Nicotine, and the Metabolism of Imidacloprid. **(A)** Structure of Imidacloprid. **(B)** Structure of Nicotine. **(C)** Metabolism of Imidacloprid, to 5-Hydroxyimidacloprid, 6-Chloronicotinic acid and Nitrosoimine Metabolites. The structures of Imidacloprid, 5-Hydroxyimidacloprid and Nitrosoimine were obtained from 2015.igem.org, while the structure of nicotine was obtained from Wikimedia.org.