ACTIONS OF *HARMONIA AXYRIDIS* (COLEOPTERA: COCCINELLIDAE) ALKALOIDS ON VERTEBRATE AND INVERTEBRATE NICOTINIC ACETYLCHOLINE RECEPTORS

BY

ROHIT NARENDRA PATEL, BSc.

Thesis submitted to the University Of Nottingham for the degree of Master of Research

DECEMBER 2012

Abstract

The productivity of livestock and crops worldwide is dramatically reduced by pest organisms and pesticides are an important front-line control. Current pesticides raise environmental concerns and are threatened by the development of resistance so there is a great need for alternatives.

Previous work has shown that defensive alkaloids secreted by the Harlequin ladybird beetle, *Harmonia axyridis* (*H. axyridis*), act on insect and human nicotinic acetylcholine receptors (nAChRs). Selectivity for insect nAChRs, which are proven pesticide targets, was demonstrated so the alkaloids show promise as leads for the development of novel pesticides. Our aim was to ascertain whether these alkaloids had any anthelmintic effects by examining the effects on the pharynx and body wall neuromuscular systems of the nematode worm, *Caenorhabditis elegans*. Subsequent analysis of mutant strains was used to identify a possible target. Following this we aimed to demonstrate the effects on different vertebrate and invertebrate nAChRs directly using the *Xenopus laevis* oocyte expression system.

The alkaloid extract of *Harmonia axyridis* inhibited the rate of pharyngeal pumping after 1 h (IC_{50} = 1.087 mg/ml) and 24 h (IC_{50} = 0.406 mg/ml). Body wall movement was also affected after 2 h (IC_{50} = 0.64 mg/ml), 4 h (IC_{50} = 0.63 mg/ml) and 6 h (IC_{50} = 0.58 mg/ml). Examination of mutant strains revealed the alkaloids may be acting through EAT-18 and ACR-16. This was confirmed by electrophysiological recordings of ACR-16 nAChRs expressed in *Xenopus* oocytes (IC_{50} = $9.83x10^{-6}$M) but no selectivity is apparent when compared with human α7 nAChRs (IC_{50} = $9.09x10^{-6}$M). Selectivity does appear to exist with a hybrid *Drosophila*/chicken nAChR (IC_{50} = $3.16x10^{-7}$M).

This confirms the nAChR target of the alkaloids and that the selectivity is due to action at the nAChR. The action of alkaloids on nematodes *in vivo* and on nematode and insect nAChRs provides the potential for the alkaloids to serve as lead compounds for novel pesticides.

Acknowledgements

I would like to thank my supervisors Dr Ian Duce and Dr Ian Mellor for all of their support and guidance throughout the year. I would also like to thank David Richards, Mark Burton, Hamid Kachel, John Grzeskowiak, Saleh Alhewairini and everyone else in the Electrophysiology and *C. elegans* labs for all of their help.

I would like to express my gratitude to my parents and family for their encouragement and backing throughout my education; without which this work would not have been possible.

I would also like to express my gratitude to Professor David Sattelle, Dr Kristin Lees, Dr Behrooz Esmaeili and Dr Frederick Partridge at the University of Manchester for their help and generosity during my visit and for providing me with material essential for current and future work.

Table of Contents

1. Introduction

1.1 The global impact of pest organisms

The global population is estimated to reach 9 billion in 2050 and the Food Organisation of the United Nations (FAO) forecasts that global food production will need to increase by 70% in 2050. In order to boost production, the health and productivity of 3.3 billion livestock and 16 billion poultry (Evans and Chapple, 2002) needs to be maintained and improved in order to cope with the increasing demand. Infections by parasitic nematodes (helminths) are important limiting factors for this and anti-helminthic (anthelmintic) therapies are an important line of defence. It is estimated that approximately £83 million was spent in 2011 on anthelmintic drugs for food-producing animals to combat these infections in the UK alone (NOAH, 2011). In 2001 the worldwide market was estimated at US$ 11.050 billion including companion animals and the US accounted for 33% of the market (US$ 3.665 billion) (Evans and Chapple, 2002).

Humans in developing countries are also at a great risk of infection by parasitic helminths. Recent estimates suggest that the soil-transmitted helminths (STHs) *Ascaris lumbricoides* affect over 1 billion people, *Trichuris trichuria* affect over 795 million and the hookworms *Ancylostoma duodenale* and *Necator americanus* affect over 740 million (Hotez et al., 2006). Worms can live for years in the human gastro-intestinal tract and cause a variety of symptoms, the most important being the stunting of growth and cognitive deficits (Bethony et al., 2006). The number of deaths is low compared to the number of people infected. 27,000 people are estimated to die annually from infections by STHs and schistosomes but this figure could be increased depending on different estimates (Hotez et al., 2006). The preferred measurement of the burden of STHs and schistosomes is by assessing the

disability adjusted life years (DALYs) that the infection causes. This is a measure of the years lost due to contraction of the disease and comparison of DALYs caused by the major infectious diseases shows that STHs are comparable to tuberculosis and malaria (Chan, 1997).

The main strategy for treatment of STHs is mass drug administration. This involves the use of three main classes of anthelmintic drugs: benzimidazoles (β-tubulin ligands), avermectins (glutamate-gated chloride channel modulators) and nicotinic acetylcholine receptor agonists. Due to the widespread use of drugs within these classes, resistance has developed. Each anthelmintic drug introduced since Phenothiazine in the 1950s has seen resistance develop within a few years of introduction (Kaplan, 2004). This has been the case with current anthelmintics and populations of STHs resistant to all of the three main classes of drugs have been reported in Scotland (Bartley et al., 2004) and New Zealand (Wrigley et al., 2006; Sutherland et al., 2008). Spreading of resistance to other regions is very likely so there is a great need for new anthelmintics to replace the current batch before resistance becomes widespread.

The productivity of crops worldwide is limited to a major extent by pest insects that feed on crops and insecticides have been used since the 1800s to control the impact of insects on crop yield (Casida and Quistad, 1998). Since 1945, the use of organic synthetic insecticides with selectivity for pest organisms and with reduced environmental impact has greatly improved agricultural productivity (Zhang et al., 2011). Use of insecticides in the control of vector borne diseases carried by insects such as malaria and Chagas disease has also proved valuable (Zaim and Guillet, 2002). In 2005, worldwide sales of insecticides totalled US$ 7.798 billion accounting for 25% of global pesticide sales (Zhang et al., 2011). The neonicotinoid family of

organic synthetic insecticides was first introduced in 1991 with the release of Imidacloprid (Bai et al., 1991). They alone accounted for sales of US$ 1.56 billion in 2006 which represented a 17% share of the global insecticide market (Jeschke and Nauen, 2008) as they proved to be resilient to the development of resistance (Nauen and Denholm, 2005). However, resistance to neonicotinoids has been reported (Nauen and Denholm, 2005) and concerns about their possible role in colony collapse disorder in honeybee populations has risen (Girolami et al., 2009, Henry et al., 2012, Whitehorn et al., 2012). Resistance to the organic synthetic insecticides DDT and the pyrethroid family have also been reported for crop pests (Gunning et al., 1984, Cahill et al., 1995, Guedes et al., 1995) and disease vectors (Martinez-Torres et al., 1998, Brengues et al., 2003). Alternative insecticides and measures to combat resistance are greatly needed as considerable economic losses are envisioned if pesticides are no longer used (Webster, 1999).

1.2 Natural compounds as pesticides

An effective approach for the creation of new pesticides has been to take advantage of natural compounds that exhibit pesticidal activity. One of the most successful examples of this is the discovery of the insecticidal activity of natural pyrethrins from plant species of the genus *Chrysanthemum*. These were unstable in direct sunlight and led to the creation of more stable synthetic analogues called pyrethroids (Elliott, 1976). A fermentation product of the actinomycete *Streptomyces avermitilitis* called avermectin B_1 was found to have potent anthelmintic effects against parasitic nematodes of clinical and veterinary importance. This was used to create the drug ivermectin which is effective against a range of different pest organisms (Campbell, 1985). Fermentation products from another actinomycete, *Saccharopolyspora spinosa*, were also found to exhibit insecticidal activity and have been used to

create the insecticide spinosad (Sparks et al., 2001). The discovery of the insecticidal activity of nereistoxin from the salivary glands of the annelid worm *Lumbriconeireis heteropoda* also led to the use of its synthetic analog, Cartap, as a commercial insecticide (Sattelle et al., 1985). It is clear that discovering novel chemistries from natural sources can provide an effective route for the discovery of new pesticides.

Spinosad and Cartap both target the nicotinic acetylcholine receptor (nAChR) (Sattelle et al., 1985, Sparks et al., 2001), a ligand-gated ion channel (LGIC) located in the nervous systems of the pest organisms. As acetylcholine (ACh) is the major excitatory neurotransmitter in the insect brain (Breer and Sattelle, 1987) nAChRs have successfully been exploited as a target for insecticides (Bai et al., 1991, Matsuda et al., 2009). Parasitic worm nAChRs have also proved a fruitful target for anthelmintic drugs since the 1970s (Lewis et al., 1980, Harrow and Gration, 1985) and have continued to prove fruitful more recently due to significant diversity of nematode nAChR subunits (Kaminsky et al., 2008, Hu et al., 2009, Robertson et al., 2002, Zinser et al., 2002).

Many natural toxins and venoms target the nAChR. Amongst the first to be discovered were the snake venoms α-bungarotoxin (α-BgTX) and the plant extract d-tubocurarine. While not providing a source for new pesiticides, they have proven invaluable tools for characterising the nAChR (Changeux et al., 1970, Mishina et al., 1985, Takahashi et al., 1985) therefore enhancing our understanding of the receptor. The selectivity of α-BgTX for a subtype of nAChR has also allowed differentiation between the various subtypes (Boulter et al., 1986, Boulter et al., 1987, Keyser et al., 1993). Screens of extracts from Chinese medicinal herbs and local wild plants have yielded alkaloids that exhibit activity against insect nAChRs (Liu et al., 2008), 5α -cardenolides from

the plant *Kanahia ianiflora* target muscle-type nAChRs (Clarkson et al., 2006) and a variety of wasp and ant toxins have also been shown to target the nAChR (Zalat et al., 2005). The venom of the solitary digger wasp *Philanthus triangulum* contains a component named PhTX that targets nAChRs, NMDA and AMPA receptors (Rozental et al., 1989). PhTX has served as a lead compound for the creation of synthetic analogues that also inhibit human nAChRs (Mellor et al., 2003, Brier et al., 2003). Thus, there is a great wealth of natural compounds that exhibit activity through targeting nAChRs and discovery of these could pave the way for the creation of novel pesticides.

1.3 Ion channels and the action potential

Neurons are cells specialised for the transmission of electrical signals throughout the body. Various types of neurons exist but they can all be broadly characterised as consisting of a cell body, axon, dendrites and synaptic bulbs (Kandel et al., 2000). Transmembrane proteins known as ion channels and pumps in neurons allow the generation and carrying of these electrical signals. This was first demonstrated by Hodgkin & Huxley in the squid giant axon by measuring sodium ion (Na^+) and potassium ion (K^+) conductances (Hodgkin and Huxley, 1952). A pump protein which exchanges 3 intracellular Na^+ for 2 extracellular K^+ (Na^+/K^+ pump) creates a larger extracellular concentration of Na^+ and a larger intracellular concentration of K^+ so concentration gradients are created. K^+ flows out of leak ion channels selective for K^+ by diffusion down the concentration gradient and anions attempt to move but the membrane prevents their movement out of the cell. Anions inside the cell and K^+ outside the cell are attracted to the membrane as ions are attracted to areas of opposite charge. When K^+ ions reach equilibrium, a membrane potential develops because of the difference in charge across the membrane. The membrane potential of a neuron in this

state is referred to as a resting membrane potential which can range from -40mV to -80mV depending on the type of neuron and organism (Kandel et al., 2000).

A neuron can be stimulated by the activation of LGICs (Changeux and Edelstein, 1998) or sensory receptors (Sachs, 1991). This results in the opening of Na^+ selective ion channels which causes an influx of Na^+ ions into the neuron. The increase of Na^+ causes an increase in the membrane potential called depolarisation. Voltage-sensitive Na^+ channels (VGSCs) followed by voltage-sensitive K^+ (VGKCs) channels shift from a closed to an open state in response to the change in membrane potential and further depolarisation occurs. Depolarisation allows the opening of adjacent VGSCs and so depolarisation occurs in neighbouring areas which allows the propagation of the electrical signal down the axon. The VGSCs then shift from an open to an inactive state at the maximum membrane potential where further opening cannot occur till a shift to a closed state. VGKCs continue to be open allowing K^+ to continue to flow out of the neuron causing a decrease in membrane potential called repolarisation. Hyperpolarisation then occurs as the decrease overshoots the desired resting membrane potential value and then the VGKCs become inactivated. Another action potential cannot be generated when in a hyperpolarised state. The Na^+/K^+ pump then restores the membrane potential to the resting membrane potential (Fig. 1.3.1) (Hodgkin and Huxley, 1952, Kandel et al., 2000).

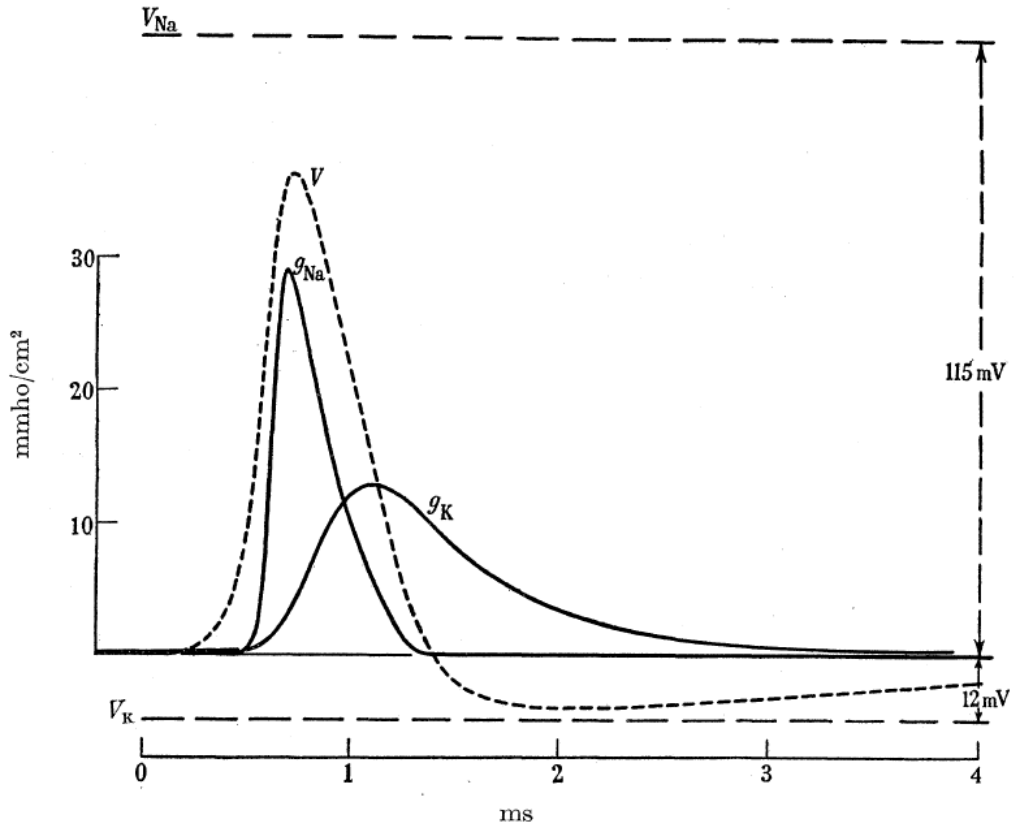


Fig. 1.3.1 – The changes in membrane potential and Na^+/K^+ conductance caused by the action potential. The depolarization, repolarization and hyperpolarization phases are shown along with the ion conductance indicating when the ion channels open and close in relation to the membrane potential. Taken from (Hodgkin and Huxley, 1952).

At the end of the axon an action potential reaches the synaptic terminal. This causes the conversion of the action potential (electrical) signal into a neurotransmitter (chemical) signal. The action potential stimulates the opening of calcium-selective ion channels which causes an influx of calcium ions and release of synaptic vesicles. The neurotransmitter diffuses across the synaptic cleft and binds to LGICs on the post-synaptic terminal. This causes the opening of the LGICs and the post-synaptic membrane becomes permeable to anions or cations depending on the LGIC selectivity. LGICs selective for anions cause inhibitory effects as the flow of anions into the cell causes a decrease in membrane potential and so hyperpolarisation. If selective for cations, depolarisation occurs as cations (predominantly Na^+)

flow into the neuron and cause a decrease in membrane potential. This effect is excitatory and may result in an action potential (Kandel et al., 2000).

The ability of neurons to generate action potentials is vital for a variety of functions and behaviours and interference in the functioning of LGICs has provided a potent mode of action for pesticides (Bai et al., 1991, Sparks et al., 2001, Raymond Delpech et al., 2003, Levandoski et al., 2003, Seo et al., 2009).

1.4 *Caenorhabditis elegans* as a model parasite

Caenorhabditis elegans (*C. elegans*) is a free-living nematode worm consisting of hermaphrodite and male genders. Sydney Brenner first introduced the worm as a model organism in the 1970s and it has become one of the most well characterised organisms on the planet as the complete genome sequence (Consortium, 1998), nervous system map (White et al., 1986), embryonic (Sulston et al., 1983) and post-embryonic (Sulston and Horvitz, 1977) cell lineages have been determined. Also, the creation of transgenic *C. elegans* lines has been made simple by the technique of micro-injection (Mello et al., 1991). *C. elegans* culture is easy due to its short life-cycle consisting of 4 larval stages lasting 3 days at 20˚C, large number of offspring and ease of culture on Petri dishes containing an agar growth medium with an *Escherichia coli* (*E. coli*) lawn (Brenner, 1974). Moreover, there is a great similarity in the neuronal and muscular systems in nematodes (Holden-Dye and Walker, 2007) and transparency allows internal structures to be visualised easily so *C. elegans* has use as a model for the related parasitic nematodes.

Key anthelmintic drugs such as ivermectin and levamisole target the nervous system of parasitic nematodes and *C. elegans* has played a major

role in the understanding of these drugs. It was thought that the target in nematodes of the anthelmintic ivermectin was the glutamate-gated chloride channel (GluCl) and this was confirmed by studying the *C. elegans* GluCl. The GluCl was expressed in *Xenopus laevis* oocytes and the inhibitory effects were confirmed by electrophysiological analysis of the oocyte (Arena et al., 1992). For levamisole, different mutant strains of *C. elegans* were examined for levamisole resistance and subsequent genetic analysis revealed the nAChR target (Lewis et al., 1980, Brenner, 1974). More recently the target of the amino-acetonitrile derivative, monepantel, was also discovered as a specific nAChR subunit using a similar method of examining resistant strains of *C. elegans* (Kaminsky et al., 2008). Due to the ease of genetic manipulation in *C. elegans* it is also possible to examine parasitic molecular targets in *C. elegans.* The GluCl of the parasitic nematode *Haemonchus contortus* was expressed in ivermectin resistant *C. elegans* and this was able to rescue sensitivity to ivermectin (Glendinning et al., 2011).

There is great potential for using *C. elegans* as a parasitic nematode model for examining novel and existing anthelmintic compounds that target LGICs; especially if the compound's target is well characterised in *C. elegans.*

1.5 The *Xenopus laevis* oocyte expression system

Oocytes from the African clawed frog, *Xenopus laevis*, can be used to express non-native proteins by injecting foreign DNA or mRNA. They are obtained by ovariectomy of adult females and are immature eggs that are not competent for fertilisation (Bianchi and Driscoll, 2006). Oocytes go through six stages and stages IV-V are used for experiments. These are approximately 1mm in diameter and segmented into an animal pole (black region) and a vegetal pole (white region) (Bianchi and Driscoll, 2006). Expression of non-

native proteins was first achieved by injecting haemoglobin mRNA (Gurdon et al., 1971) and the *Torpedo* acetylcholine receptor was the first transmembrane receptor to be investigated (Sumikawa et al., 1981). Use of this expression system allowed the examination of different subtypes of nAChRs (Takahashi et al., 1985, Boulter et al., 1987) and the role of specific amino acids in receptor function (Mishina et al., 1985).

Recording the electrical response of oocytes injected with the mRNA of LGICs is achieved by using the two electrode voltage clamp (TEVC) technique. A voltage sensing microelectrode and a current microelectrode are inserted into the oocytes in a perfusion chamber. Saline is perfused and a command voltage is set. The voltage sensing microelectrode measures the membrane potential and an amplifier is used to level the membrane potential and a command voltage by injection of current through the current microelectrode. The injected current is used to measure the total membrane current and therefore the changes in current caused by opening of expressed LGICs when ligands and drugs are perfused (Fig. 1.7.1) (Baumgartner et al., 1999). This allows the analysis of the electrical response of a receptor in isolation and can allow the confirmation of the target site of different compounds. This has successfully been used to confirm the target sites of many anthelmintics and insecticides on the functioning of their target receptors. The free living nematode worm *C. elegans* has previously been used as a model for parasitic worms and studies using the oocyte expression system have proven useful for discovering and confirming the targets of ivermectin (Arena et al., 1992). The oocyte expression system also allows the expression of hybrid insect/mammal nAChRs and this has proven useful in confirming the nAChR target of the neonicotinoids (Ihara et al., 2003, Nishiwaki et al., 2003) and nereistoxin (Raymond Delpech et al., 2003).

Expression of mammalian nAChRs in oocytes has also proved useful for examining the effects of levamisole (Levandoski et al., 2003) and morantel (Seo et al., 2009).

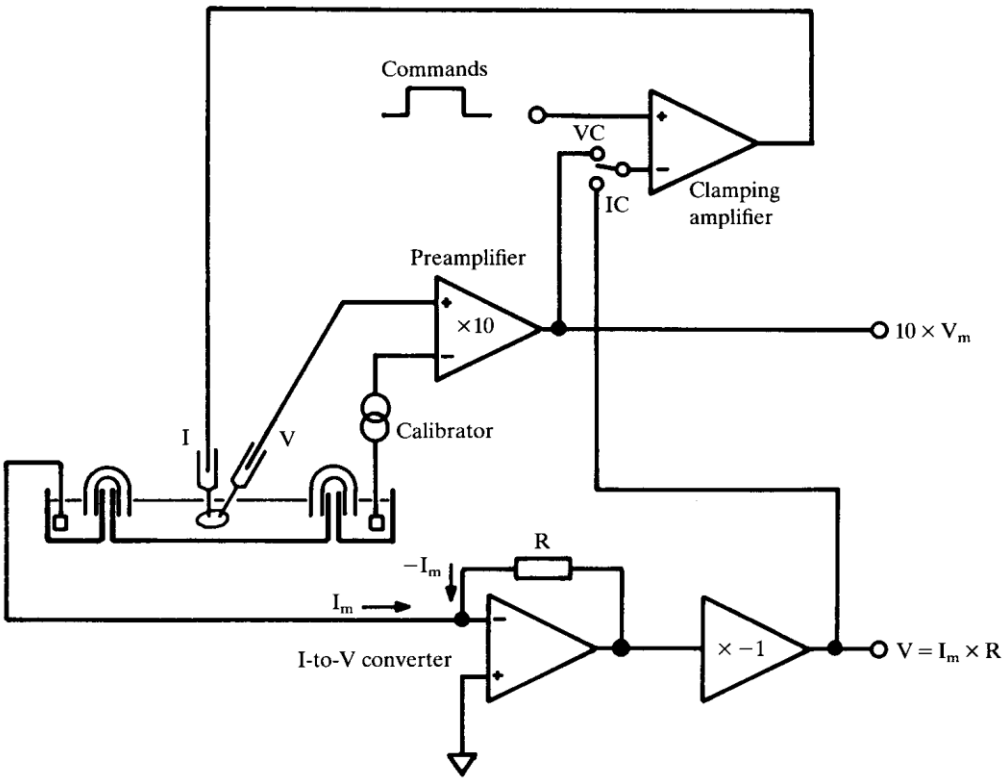


Fig 1.7.1 – The circuit used for the two-electrode voltage clamp (TEVC) technique. The voltage microelectrode (V) measures the membrane potential of the oocyte and this is referred to a command potential. If different, current is injected using the current microelectrode (I) to bring the membrane potential to the command voltage. Taken from (Halliwell et al., 1987).

The alkaloid extract from the Harlequin ladybird *Harmonia axyridis* (Coleoptera: Coccinellidae) and a synthetic form of hippodamine from the convergent ladybird *Hippodamia convergens* (Rejzek et al., 2005) have been examined for their effects on the nAChR response of TE671 human muscle cells and locust neurons using patch-clamp electrophysiology. The response was reduced in both cases and a greater reduction of the nAChR response in locust neurons was observed so it is thought the alkaloids are selective for

insect over mammalian neurons (Richards, 2011). As there is selectivity for the proposed insect nAChR target and compounds targeting the nAChR have become successful anthelmintics, there is a potential for these alkaloids to also have anthelmintic effects. *C. elegans* provides the ideal model to investigate this and the *Xenopus* oocyte expression system provides an ideal route for investigating the effect of the alkaloids directly on the nAChR

1.6 The nicotinic acetylcholine receptor

LGICs can be classed into two broad categories: the Cys-loop family and the NMDA/AMPA family. ACh receptors belong to the Cys-loop family (Changeux and Edelstein, 1998). A pharmacologically distinct class of ACh receptor are the nicotinic acetylcholine receptors (nAChRs) that are selectively sensitive to nicotine and are divided into muscle and neuronal types (Bursztajn and Gershon, 1977).

In 1906, experiments with nicotine on muscle contraction in fowl led to the conclusion that a "receptive substance" combined with nicotine to elicit a contraction (Changeux et al., 1984). Later, a protein "which presents (in solution) several characteristic properties of the cholinergic receptor macromolecule" was isolated from the electric organ from the electric eel, *Electrophorus electricus* (Changeux et al., 1970). The snake venom α-BgTX irreversibly blocks the actions of cholinergic agonists on membrane proteins and was found to block: the depolarisation of electric organ membrane, the binding of radiolabelled decamethonium (cholinergic agonist) to the electric organ membrane and binding to the isolated protein. This confirmed that the isolated protein was a membrane bound acetylcholine receptor and was the first characterised nAChR (Changeux et al., 1970).

Studies on the nicotinic acetylcholine receptors of the electric rays of the genus *Torpedo* have contributed the most to our understanding of the structure and function of nAChRs. The *Torpedo californica* nAChR was found to be composed of 5 subunits consisting of 2α, β, γ and δ subunits (Reynolds and Karlin, 1978) and that these subunits form a barrel surrounding a central pore (Karlin et al., 1983). The β subunit was found to be unable to form ACh binding sites when being flanked by α subunits and so the arrangement of αγαβδ is the most likely (Karlin et al., 1983). Analysis of the mRNA of the α subunit revealed a mature protein containing 437 amino acids and 4 regions containing approximately 20 uncharged amino acid residues flanked by charged residues indicating the α subunit has 4 transmembrane domains. These were thought to be α-helices and 3 of the helices are linked by short polypeptide sequences so a compact core should form (Devillers-Thiery et al., 1983). cDNA of β, γ and δ subunits were cloned and found to have homology and several conserved regions with α subunits so all subunits were proposed to contain 4 transmembrane domains (Fig. 1.4.1) (Claudio et al., 1983, Noda et al., 1983b, Noda et al., 1983a). *Torpedo* electric organ mRNA extract was injected into *Xenopus laevis* oocytes and functional ACh receptors were observed to form (Barnard et al., 1982). Later, *Torpedo* α, β, γ and δ subunit cDNA was used to produce mRNA that was injected into *Xenopus* oocytes and ACh responses that were reversibly blocked by d-tubocurarine were observed confirming that all four subunits were required for functional receptors (Takahashi et al., 1985). Multiple α and β subunits were later identified that were transcribed in different regions of the rat central nervous system that were not blocked by α-BgTX which blocks muscle-type nAChRs indicating a pharmacologically distinct family of neuronal-type nAChRs (Boulter et al., 1986, Boulter et al., 1987). Later α2- α9 and β2- β4 subunits were identified (Changeux and Edelstein, 1998) and injection of cRNA into

Xenopus oocytes revealed that α7 subunits could form functional receptors when injected alone (Raymond et al., 2000) but α3 and α4 required co-injection of β2 suggesting that homomeric and heteromeric neuronal-type nAChRs existed (Boulter et al., 1987). Analysis of chick neuronal nAChR subunits α4 and non-α1 revealed that a stoichiometry of 2α and 3 non-α existed in functional heteromeric neuronal-type nAChRs similar to muscle-type (Fig. 1.4.1) (Cooper et al., 1991). Neuronal nAChRs in the chick retina were discovered to bind α-BgTX indicating further subtypes exist (Keyser et al., 1993).

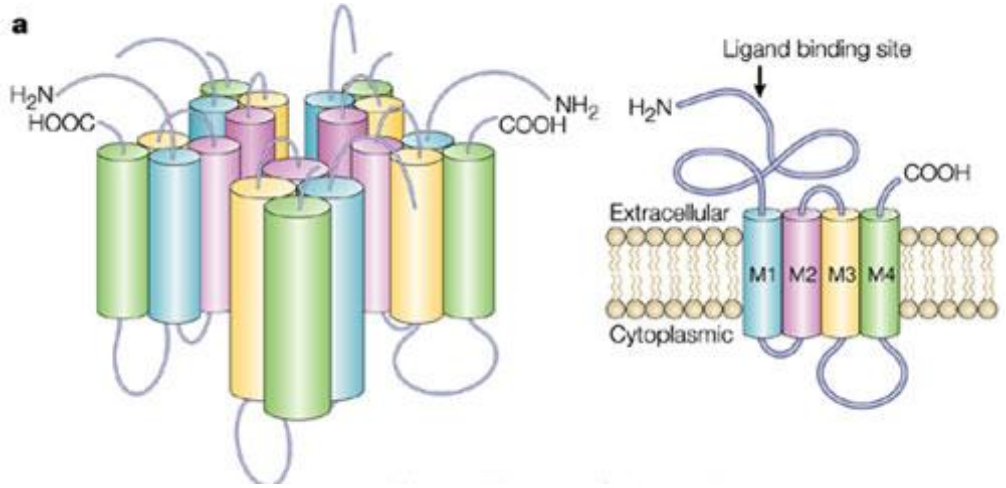


Fig 1.4.1 – The pentameric structure of a neuronal nAChR is shown with a composition of 2 α and 3 non-α subunits (left). The orientation of the N and C-terminals of α subunits is also shown in a 2D diagram of the subunit in the membrane (right). Taken from (Laviolette and van der Kooy, 2004).

The binding of ACh elicits a number of conformational changes that results in the transient opening of the channel which allows cations to pass through the cell membrane. A disulphide bond on the N-terminal of α subunits is implicated in the binding of acetylcholine and is created by the interaction of two adjacent cysteine residues (Cys192 & Cys193) that are conserved in α subunits only (Kao and Karlin, 1986). The bond is readily reducible and this causes conformational changes in the receptor which allows affinity labelling

agents to bind. Use of the affinity label [^{3}H]MBTA revealed the two cysteine residues to be extracellular and close to the binding site of ACh. Cys192, Cys193 and 4 other residues were also found to be conserved across species boundaries (Karlin, 1993). Conversion of Cys128 and Cys142 in α subunits to serine residues abolished both the ACh response and α-BgTX binding indicating the major role these cysteine residues play in the binding and response caused by ACh (Mishina et al., 1985). Cys128 and Cys142 also form disulphide bonds and are conserved in beta, gamma and delta subunits in an extracellular region (Kao and Karlin, 1986).

Acetylcholine binding protein (AChBP) is a soluble protein found in the snail *Lymnaea stagnalis* most similar to the α7 subtype of nAChRs (Smit et al., 2001) that is the same length as the N-terminal sequences of pentameric LGICs (approx. 210 amino acids), has 15-28% sequence similarity to all LGICs (Celie et al., 2004), contains almost all residues conserved in the nAChR family, binds known nAChR ligands and forms cavities lined by residues known to be involved in ligand binding (Brejc et al., 2001). Studies have revealed that the AChBP ACh binding site is formed by a series of loops from the principal face of one subunit and β-sheets from the complementary face of an adjacent subunit (Fig. 1.4.2). This binding site contains a double cysteine motif conserved in α subunits of nAChRs. Also, a Cys123-Cys136 disulphide bond exists which is important for the packing of β-sheets and explains the importance of Cys128-Cys142 in *Torpedo* nAChRs (Brejc et al., 2001). An analysis of AChBP binding to nicotine, HEPES and carbamylcholine confirmed the binding of ligands to the interface between subunits by making contact with loops A, B and C on the principle face of a AChBP subunit. When HEPES is bound, loop C was found to make significant backbone movements and all side chains involved in binding were rearranged to accommodate

binding. The Lys139 residue moved and formed a hydrogen bond with Tyr185 confirming previous indications that Tyr190 (the equivalent of Tyr185) in α subunits is critical for ACh binding (Celie et al., 2004).

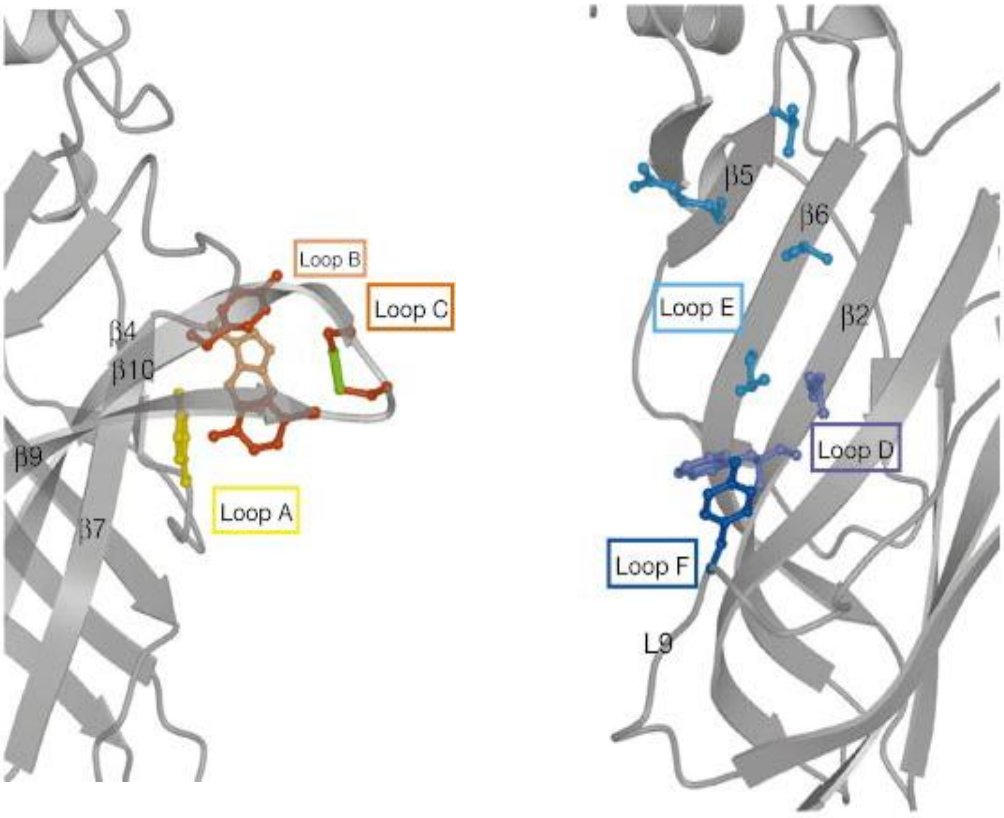


Fig. 1.4.2 – The acetylcholine (ACh) binding site of acetylcholine binding protein (AChBP) showing the binding residues on the principle face (left) and binding residues on the complementary face (right). Adapted from (Brejc et al., 2001).

1.7 Nicotinic acetylcholine receptors in nematodes & insects

The characterisation of nematode nAChRs began when Sydney Brenner selected and identified mutants of the free living nematode worm *Caenorhabditis elegans* (*C. elegans*) based on their resistance to the nAChR agonist tetramisole (contains levamisole as the active ingredient). Levamisole affected the movement of the worms causing paralysis and the selected levamisole-resistant mutants exhibited an uncoordinated movement phenotype. The mutated genes were identified as *unc-63*, *unc-38*, *unc-29*, *lev-1* and *lev-8* (Brenner, 1974). These were later cloned and revealed to be nAChR subunits (Fleming et al., 1997, Culetto et al., 2004, Towers et al., 2005) and were required along with the proteins RIC-3, UNC-50 and UNC-74 to produce a functional levamisole-sensitive ACh receptor in *Xenopus* oocytes (Boulin et al., 2008).

This receptor is expressed in the body wall muscle and functions at the neuromuscular junction along with a γ-aminobutyric acid (GABA) receptor and another pharmacologically distinct ACh receptor sensitive to nicotine but insensitive to levamisole (Richmond and Jorgensen, 1999). This nicotine-sensitive receptor was first isolated by the screening of a *C. elegans* cDNA library with part of an avian α5 cDNA and a cDNA dubbed Ce21 was found to contain the cysteine residue pair characteristic of nAChR α subunits. Ce21 aligned best with vertebrate α7 subunits, shared 47% identity and formed functional ACh receptors sensitive to nicotine and insensitive to levamisole when injected into *Xenopus* oocytes (Ballivet et al., 1996). This was later labelled *acr-16* and knockouts reduce the response to ACh but not to levamisole when recording from the body wall muscle. Double mutants of *acr-16* and *unc-63* caused the abolition of the ACh response confirming that *acr-16* was responsible for a large part of the ACh response that is not caused by

the levamisole-sensitive receptor (Touroutine et al., 2005).

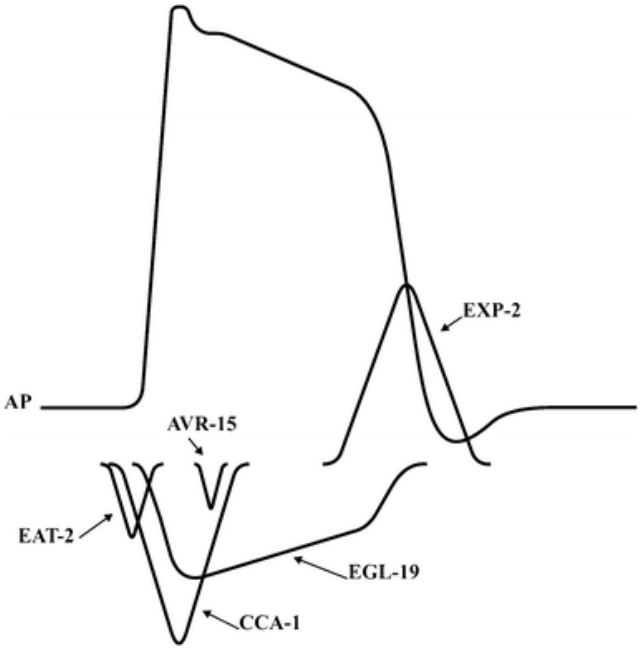


Fig. 1.5.1 – The different ion channels underlying the action potential in the *C. elegans* pharynx muscle. EAT-2 containing nAChRs are thought to intiate the action potential by activating CCA-1 T-type calcium channels. EGL-19 L-type calcium channels cause the plateau and EXP-2 potassium channels are thought to cause repolarization. AVR-15 is a glutamate-gated chloride channel. Taken from (Franks et al., 2002).

The pharynx is a neuromuscular pump consisting of the corpus, isthmus and terminal bulb which allows *C. elegans* to feed (Fig. 1.5.2). The MC motor neuron is responsible for rapid pumping of the pharynx in response to a food stimulus (Fig 1.5.2) (Avery and Horvitz, 1989). The MC neuron was found to use ACh as a neurotransmitter, be inhibited by d-tubocurarine and stimulated by nicotine. Therefore, a nAChR in the pharynx was proposed to govern the rate of pumping. Mutations in the genes *eat-2* and *eat-18* were also found to be required for MC transmission and were thought to be nAChR subunits (Raizen et al., 1995). The EAT-2 protein was later located at the MC synapse in the post-synaptic region on the pharynx muscle and was revealed to encode a non-α nAChR subunit. A small transmembrane protein encoded

by *eat-18* and is required for α-BgTX binding and so could also be a component of a nAChR (McKay et al., 2004). It is thought that the activation of EAT-2 containing nAChRs causes an initial depolarisation which activates further voltage-sensitive ion channels and allows a pharyngeal muscle action potential to develop (Fig. 1.5.1) (Franks et al., 2002). *C. elegans* is thought to contain at least 27 nAChR subunits compared to 17 in mammals and birds so a great diversity exists (Jones and Sattelle, 2004).

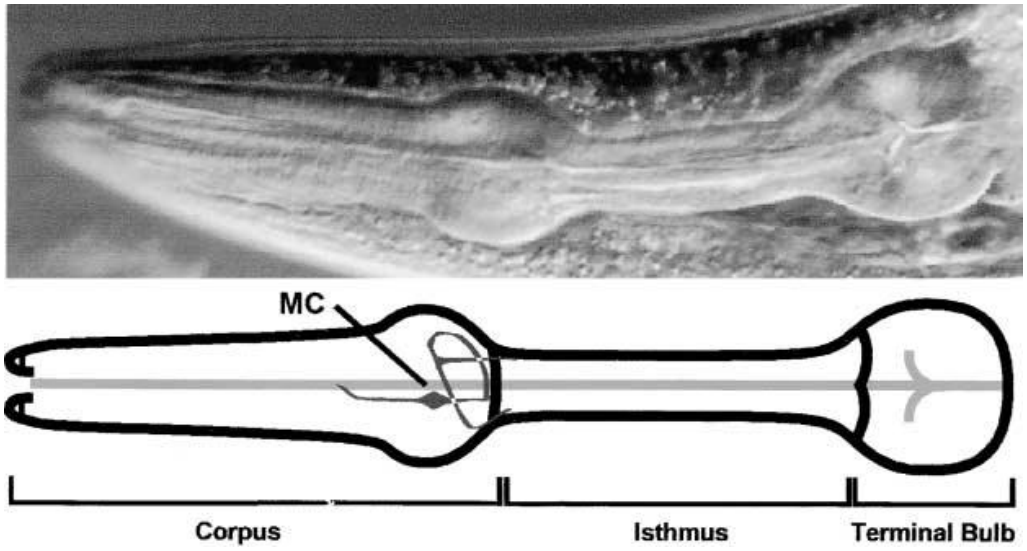


Fig. 1.5.2 – A light microscope image of the *C. elegans* pharynx (top) and a schematic of the pharynx highlighting the corpus, isthmus and terminal bulb regions as well as the location of the MC neuron (bottom) is shown. Taken from (McKay et al., 2004).

Studies on the body wall muscle of the parasitic nematode, *Ascaris suum* (*A. suum*), have revealed 3 subtypes of nAChR that are pharmacologically distinct: Levamisole-sensitive (L-type), bephenium-sensitive (B-type) and nicotine-sensitive (N-type) (Qian et al., 2006). Little is known about the nAChR subunit composition but the subunits in *A. suum* homologous to *C. elegans* UNC-29 and UNC-38 were able to form functional, L-type nAChRs in *Xenopus* oocytes (Williamson et al., 2009). The subunits and associated proteins required to form L-type receptors in *C. elegans* were

found to have homologues in the parasitic nematode, *Haemonchus contortus*. When injected into *Xenopus* oocytes functional L-type receptors were formed (Boulin et al., 2011). Species specific subunits can also exist as the ACR-26 subunit is found in *A. suum* but not in other nematodes including *C. elegans* (Bennett et al., 2012).

ACh is the main excitatory neurontransmitter in insects and nAChRs are abundant in the insect nervous system (Breer and Sattelle, 1987). The nAChR families of various insects are known and appear to express fewer subunits compared to mammals and nematodes (Sattelle et al., 2005, Jones et al., 2005, Jones et al., 2006, Jones et al., 2007, Shao et al., 2007, Dale et al., 2010). Direct recordings of insect neurons have confirmed the presence of nAChRs (Thany and Tricoire-Leignel, 2011) and allowed the investigation of the mode of action of different neonicotinoid insecticides (Bai et al., 1991, Brown et al., 2006). However, insect nAChRs have proved difficult to express in heterologous expression systems such as the *Xenopus* oocyte and therefore are not as well characterised as their nematode and mammalian counterparts. Locust (*Schistocerca gregaria*) alpha L1 subunits have expressed in *Xenopus* oocytes at low levels but the subunit composition of the receptor remains unknown (Amar et al., 1995). Recently, *Drosophila* α5 and α7 subunit cRNA expressed homopentameric nAChRs (Lansdell et al., 2012) and α6, α5 and *ric-3* expressed functional nAChRs (Watson et al., 2010) in *Xenopus* oocytes but robust expression of insect nAChRs remains elusive.

A combination of *Drosophila* α subunits and chick β2 subunits in *Xenopus* oocytes gives robust expression of hybrid nAChRs sensitive to α-BgTX (Bertrand et al., 1994) and this provides a viable alternative to expressing pure insect nAChRs. The hybrid *Drosophila* α2/chicken β2 (*D*α2/β2) has successfully allowed investigation of the neonicotinoid

interaction with nAChRs (Matsuda et al., 1998, Ihara et al., 2003, Shimomura et al., 2006) and gives the potential for its use in discovering novel insecticidal compounds.

1.8 Ladybird beetles

Ladybird beetles (Coleoptera: Coccinellidae) also known as coccinellids are beetles ranging from 1.3-10mm in length belonging to the endopterygota group as they undergo complete metamorphosis consisting of egg, larval, pupal and imaginal (adult) stages (Majerus, 1994). Carl Linnaeus began the classification of coccinellids in the mid-1700s (Gordon, 1985) and now over 4300 species have been identified (Majerus, 1994). They are widespread with large areas of land being suitable for habitation and species have been documented in North America, South America, Europe, Africa, Asia and Australasia (Poutsma et al., 2008).

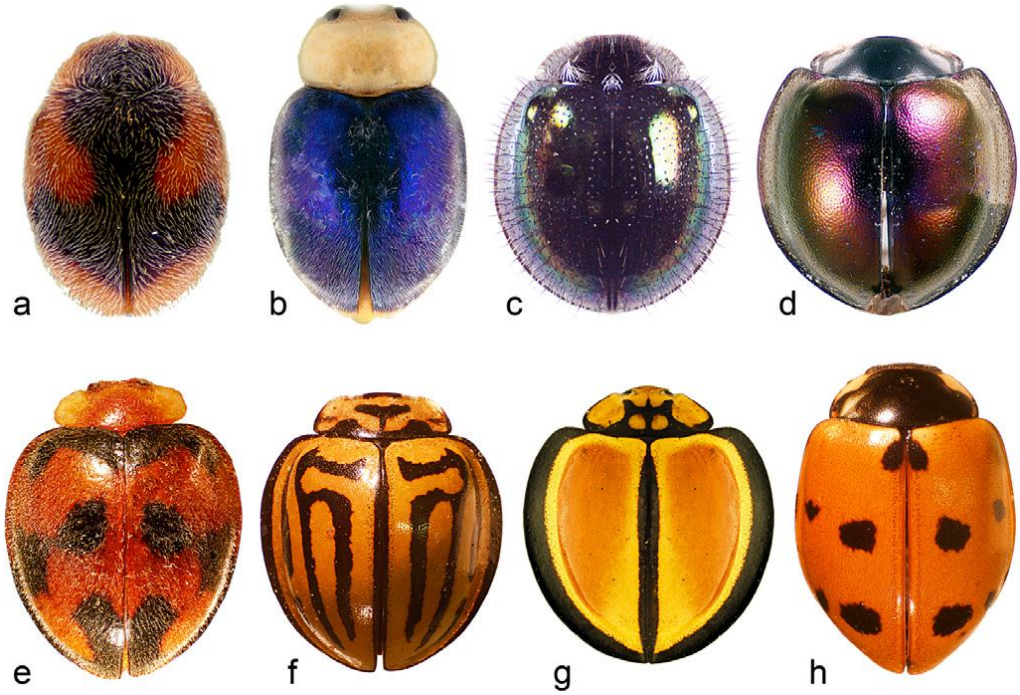
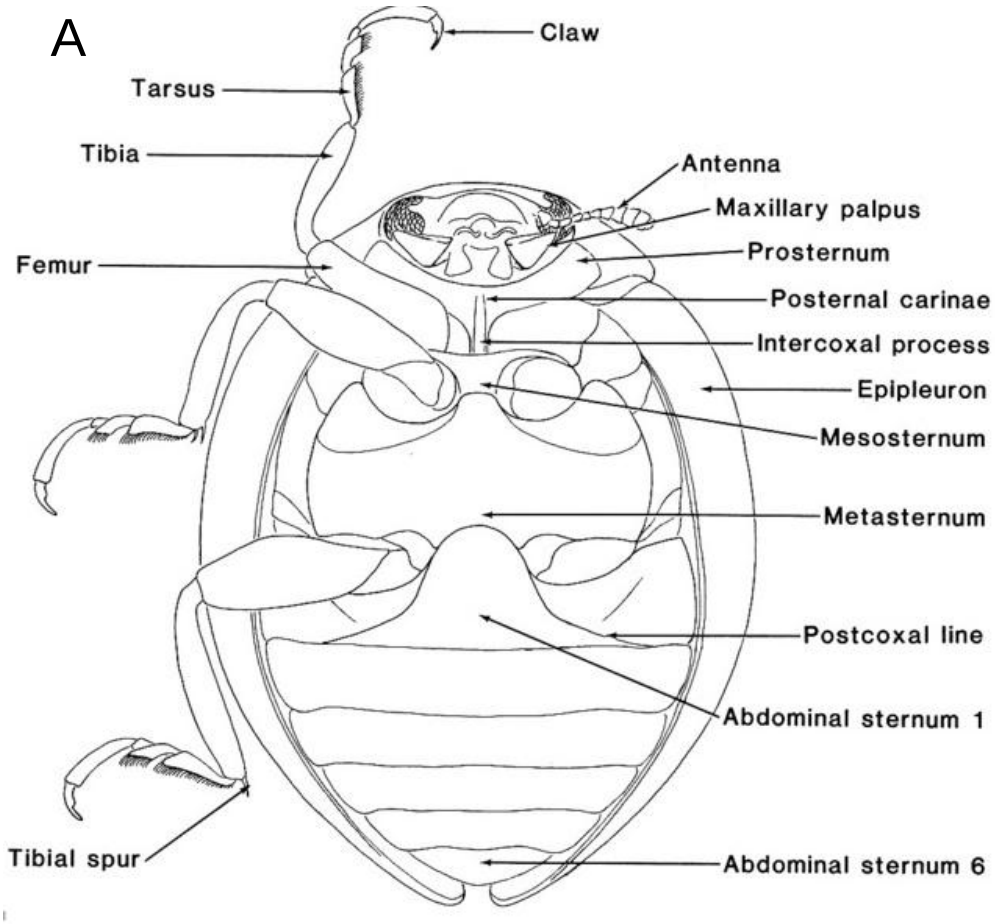


Fig 1.8.1 - Elytra (hard wing casing) colours, textures and patterns can vary greatly between different species of coccinellids. Dorsal views of (a) *Scymnus mitior*; (b) *Rhyncortalia australis*, (c) *Chaetolotis amy*; (d) *Halmus cupripennis*; (e) *Epilachna mjoebergi*; (f) *Archegleis delta*; (g) *Australoneda bourgeoisi*; (h) *Coccinella undecimpunctata* are shown. Taken from (Seago et al., 2011).

Usually, coccinellids have an oval/round form that is quite variable with a convex dorsal surface (Fig. 1.8.1) and flat ventral surface. They possess an exoskeleton consisting of cuticular plates joined by thinner, flexible areas. Eyes are compound, antennae consist of 7 or more segments and the mouthparts comprise of mandibles, usually with two inner facing teeth. A hard wing casing (elytron) covers the abdomen dorsally and flight wings fold away between the elytron and the dorsal abdomen surface (Fig. 1.8.2) (Gordon, 1985, Majerus, 1994). Eggs are elongate, oval and smooth with an outer shell called the chorion that contains pores (micropyles) that are thought to allow in spermatozoa and oxygen. They are laid in batches ranging from 2-100 and colours can range from off-white to dark orange (Majerus, 1994). The elytra of most coccinellids have a bright colouration and pattern that varies greatly between different coccinellid species (Fig. 1.8.1). Colours and patterns vary

between different coccinellid species and can vary between individuals of the same species (Bezzerides et al., 2007). There are four larval stages (instars) and many have elongate bodies with both dorsal and ventral surfaces covered in bumps giving a spiky appearance. Heads are square-like with rounded corners and antennae have up to 3 segments. Moulting occurs after the 4th larval instar followed by the transition from larva to pupa (Majerus, 1994). Adults then emerge from the pupal casing.



B

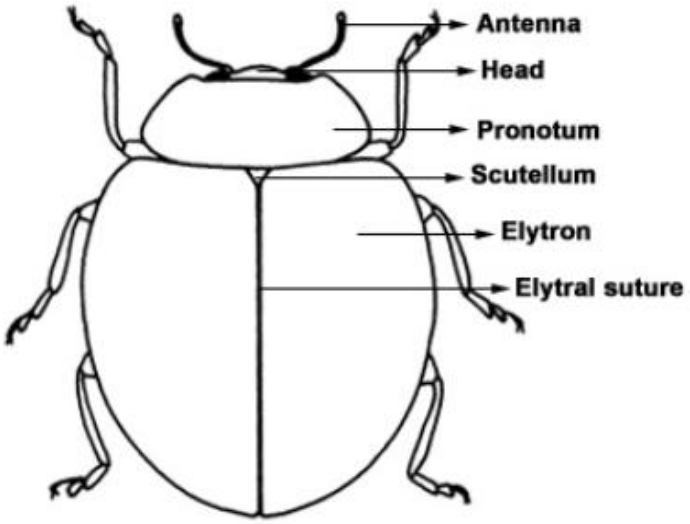


Fig. 1.8.2 – The key anatomical and morphological features of coccinellids from (A) a ventral and (B) a dorsal view. The ventral view is a schematic of *Coccinella novemnotata*. Ventral view taken from (Poorani, 2008) & dorsal view taken from (Gordon, 1985).

Female coccinellids contain a pair of ovaries, vagina, bursacopulatrix, spermatheca, accessory gland and 2 lateral oviducts that join to form a median oviduct. Sperm is deposited by the male into the bursacopulatrix and then passes on to the spermatheca. Eggs are formed in ovarioles, passed out the external orifice and laid (Majerus, 1994).

1.9 Predation involving ladybird Beetles

Coccinellids can vary from monophagy to polyphagy but most species are polyphagous (Hagen, 1962). Coccinellids can be aphidophagous (prey on aphids), coccidophagous (prey on other coccinellids) and phytophagous (consume plants). They are also known to feed on extra floral nectary secretions as well as pollen and fungi (Hagen, 1962). There is evidence that different aphidophagous coccinellids prefer different species of aphids (Finlayson et al., 2010) and so can be divided into groups depending on their

preferred prey. When populations of their preferred prey are low they will feed on egg and larval stages of other insects (Gordon, 1985). Egg cannibalism is common and the amount to which this occurs varies between species (Phoofolo and Obrycki, 1998). Many coccinellids co-occur with other coccinellid species and therefore are in direct competition for prey in the area in which they occupy. Species that predate on the same prey in the same area are classified into a guild of predators and intraguild predation (IGP) can occur between different species within a guild. This is defined as a combination of predation and competition as it involves the killing and eating of species that predate on the same prey in the same area (Polis et al., 1989). It is seen to increase when aphid populations decrease so IGP is thought to be adaptive (Sato and Dixon, 2004). Successful IGP would allow the elimination of competitors and the aphid population to regenerate so it is very beneficial for the species involved. Some species of coccinellids are much more aggressive and successful intraguild predators (Michaud, 2002, Nedved et al., 2010) and this is thought to be a reason for the decline in the populations of native coccinellids when an exotic species invades or is introduced (Ware and Majerus, 2008).

1.10 Chemical defence of ladybird beetles

It has long been thought that many coccinellids contain a form of chemical defence as they are rarely predated upon by other organisms (King and Meinwald, 1996). Toxic effects have been observed when blue tits (*Parus caeruleus*) were fed adult *Coccinella septempunctata* (*C. septempunctata*) beetles (Marples et al., 1989) and coccinellids have also been reported to cause allergic reactions (rhinoconjuctivitis) in humans (Yarbrough et al., 1999). The European fire ant, *Myrica rubra* (*M. rubra*), showed less aggressive behaviour towards *Harmonia axyridis* (*H. axyridis*) than *C.*

septempunctata (Finlayson et al., 2009). *Epilachna paenulata* adults are rejected by wolf spiders (*Lycosa* spp.) (Camarano et al., 2006) and crab spiders (*Misumenops trucuspidatus)* reject *H. axyridis* in favour of other coccinellid species (Koch, 2003). The survival of the common green lacewing, *Chrysoperla carnea,* was limited when feeding on the eggs of the 2-spot ladybird, *Adalia bipunctata*. No individuals survived when feeding on *H. axyridis* eggs (Santi and Maini, 2006). This evidence points to some form of chemical defence in coccinellid adults and eggs that is varied between different coccinellid species.

Defensive secretions are widespread in the arthropod (Eisner and Meinwald, 1966) and insect worlds (Kay et al., 1969). These secretions occur when the beetles are handled or molested and are strongly odorant (Eisner and Meinwald, 1966). Adult coccinellids are known to do this by a process called “reflex bleeding” which involves a defensive fluid being secreted from the tibio-femoral joints of the legs with each leg able to secrete independently (Fig. 1.10.1) (Happ and Eisner, 1961, Holloway et al., 1991). Secretions had no noticeable ill effects in *C. septempunctata* (Holloway et al., 1991). This defensive fluid or reflex blood is thought to be the haemolymph of the beetle as the emitted droplets have the same diagnostic features as haemolymph (Happ and Eisner, 1961). Haemocyte like immune cells were also found to be present in the reflex blood of *C. septempunctata* and *A. bipunctata* (Karystinou et al., 2004). This was the case for the reflex blood of *H. axyridis* and is thought to come at a great nutritive cost (Sato et al., 2009). 4th instar larvae of *H. axyridis* were able to secrete up to 20% of their body weight at a single time (de Jong et al., 1991). Such a large secretion should negatively affect the beetle and this was examined in *H. axyridis.* Beetles that had been caused to reflex bleed were compared to beetles that had not bled at all and

the pronotum was found to be significantly larger when no reflex bleeding had occurred. There was no significant effect on development time (Grill and Moore, 1998).

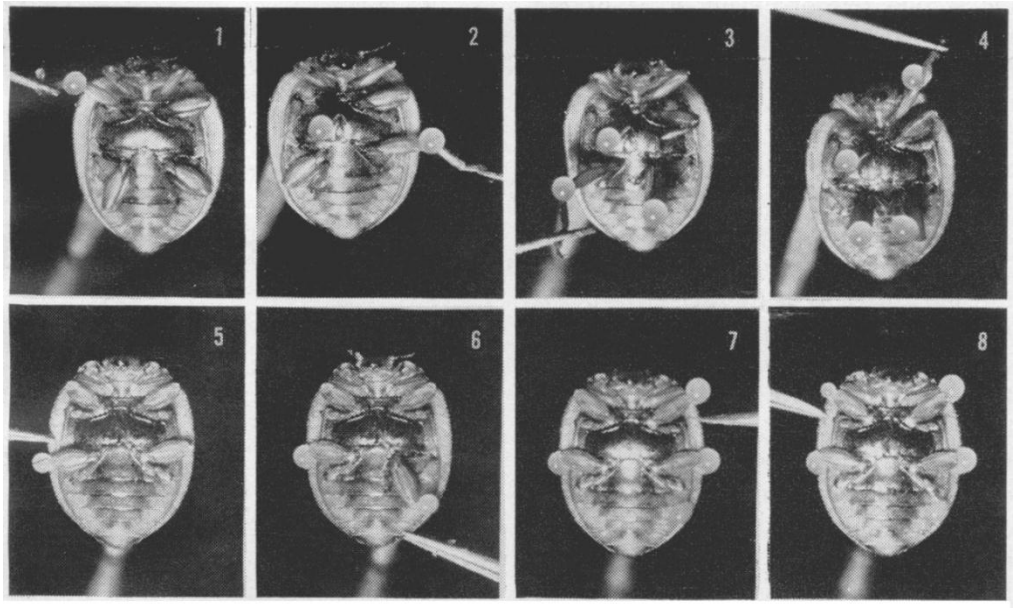
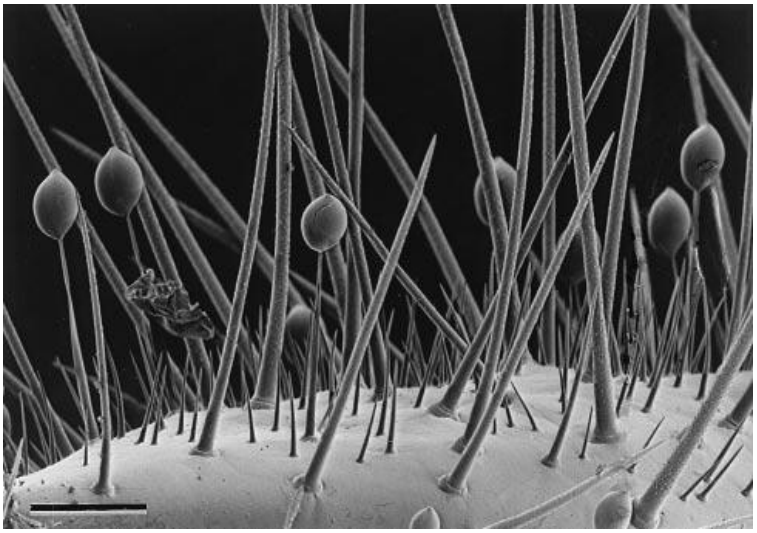


Fig. 1.10.1 - Photos 1-8 exhibit the ability of *E. varivestis* to reflex bleed upon pinching of a leg. Bleeding occurred from the legs closest to where the pinching occurred and legs were able to bleed independently of other legs. Taken from (Happ & Eisner 1961).

Coccinellids introduced into the nest of the ant *Formica exsectoides* secreted drops of reflex blood when attacked and contact with the fluid caused the ants to cease their attacks. Dragging of the mouthparts is a known response to defensive arthropod secretions and the ants exhibited this behaviour after contact was made (Happ and Eisner, 1961). Ants exposed to *Epilachna varivestis* (*E. varivestis*) pupae exhibited the same repellent effects and mouthpart dragging behaviour (Attygalle et al., 1993). This was due to the pupae having droplets of secretory fluid at the ends of glandular hairs on their surface (Fig. 1.10.2) (Attygalle et al., 1993, Schroeder et al., 1998). The odour of the secretory fluids plays a role in the repellent effects of the defensive secretions. Pyrazines are found in the secretions of some coccinellids and are

known to be responsible for a warning odour (Hautier et al., 2008). Odour can also be used as an attractant signal for other individuals of the same species (Al Abassi et al., 1998).

A



B

Fig. 1.10.2 - (A) Glandular hairs of *Subcoccinella vigintiquatorpunctata* with droplets of defensive fluid containing a mixture of macrocyclic polyamines (Bar = 100µm). (B) Glandular hairs of an *E. varivestis* pupa with droplets of defensive fluid at the tips. Taken from (Schroeder et al., 1998).

The bright elytra colouring can therefore be described as an aposematic warning signal for the chemical defence the coccinellid possesses. The aposematic warning itself can be a deterrant and is seen as the first line of defence (Majerus, 1994). This was shown by altering the colours of the elytra of *Cynegetis impunctata* and measuring the rate of attack

by avian predators (*Parus major)*. There was a greater number of attacks on beetles that had their elytra altered compared to unaltered beetles and even more attacks occurred when the elytra were removed. Removal of the spotted pattern also caused an increase in the number of attacks (Dolenska et al., 2009).

1.11 Alkaloids

Coccinellids are known to produce a group of compounds called alkaloids and secrete them as part of their reflex blood. These are varied between species and thought to contribute to the defensive properties of reflex blood (King and Meinwald, 1996, Pasteels et al., 1973) and their bitter taste (Tursch et al., 1975). *C. septempunctata* can secrete up to 12 µg of alkaloids in one reflex bleeding event (Holloway et al., 1991). Dissection of *C. septempunctata* followed by GC-MS analysis detected alkaloids present throughout the body (Holloway et al., 1991) lending evidence to the theory that reflex blood is haemolymph.

The first alkaloids to be identified were coccinelline and precoccinelline from *C. septempunctata* and coccinelline was found to repel ants (Pasteels et al., 1973). Further alkaloids were identified in *Hippodamia convergens* called hippodamine, convergine and myrrhine. These were found to share the same tricyclic tertiary amine structure as coccinelline and precoccinelline (Fig. 1.11.1) (Tursch et al., 1975).

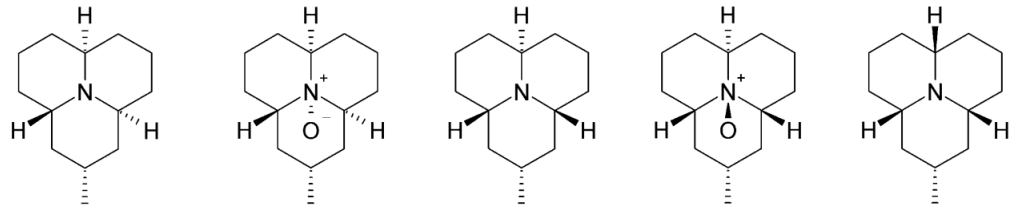


Fig. 1.11.1 – Members of the tricyclic tertiary amine family of alkaloids isolated from *H. convergens* (1&2), *C. septempunctata* (3&4) and *Myrrha octodecimguttata* (5). Taken from (Sloggett et al., 2009).

Defensive fluid from *E. varivestis* was found to contain 5 compounds, the major compound being an azamacrolide called epilachnene (Attygalle et al., 1993, Farmer et al., 1997). Pupal defensive secretions of *Epilachna borealis* were found to contain a combinatorial library of macrocyclic polyamines called polyazamacrolides with ring sizes containing up to 98 members. This library was based on the oligomerisation of a few heterologous building blocks (Schroder et al., 1998a) and these building blocks were found to be (a-1)-(2hydroxyethylamino)alkanoic acids (Schroder et al., 1998b). Polyazamacrolides were also detected in the 24-pointed ladybird *Subcoccinella vigintiquatorpunctata* (Schroeder et al., 1998). A different structure of alkaloids exist in the 22-pointed ladybird *Psyllobora vigintiduopunctata* called Psylloborine A, a dimeric alkaloid (Schroder and Tolasch, 1998). The alkaloids Harmonine and 3-hydroxypiperidin-2-one were isolated from *H. axyridis* and *Ailocaria hexaspilota* (Alam et al., 2002). The alkaloids of many other species are yet to be characterised but it is clear from the structures analysed that a great diversity exists between species (Fig. 1.11.2).

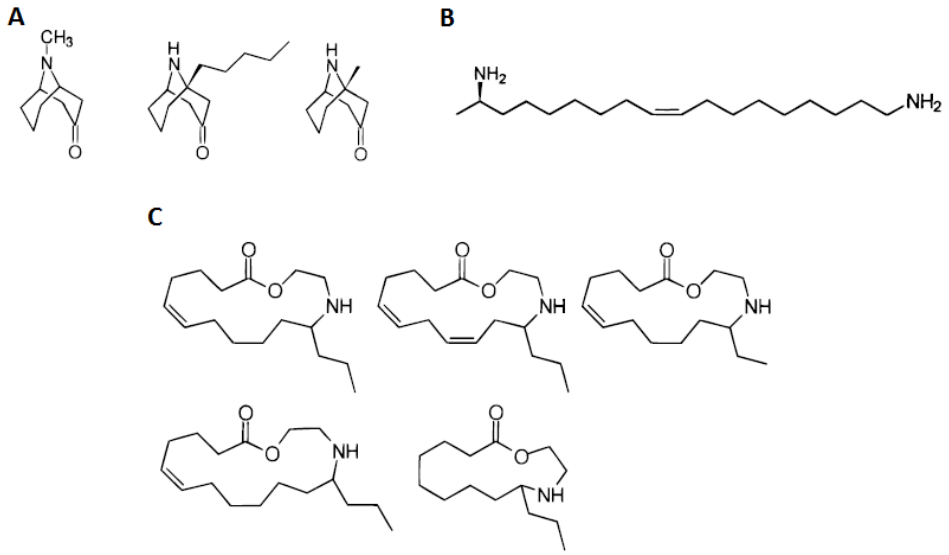


Fig. 1.11.2 - Alkaloid structures show a great diversity: (A) 9-azabicyclo[3.3.1]nonanes, (B) Harmonine & (C) Azamacrolides. Adapted from (King and Meinwald, 1996).

These alkaloids are not detected in aphid prey and so are thought to be synthesised *de novo* (Tursch et al., 1975). Feeding of radiolabelled acetate to *E. paenulata* (Camarano et al., 2009) and *A. bipunctata* (Laurent et al., 2002) followed by GC-MS analysis showed high incorporation of acetate into the alkaloids. Feeding of radiolabelled oleic acid and serine showed high incorporation into the alkaloid epilachnene (Attygalle et al., 1994). Investigation of coccinelline synthesis in *C. septempunctata* by feeding of radiolabelled CH_3COONa showed high incorporation into coccinelline indicating coccinelline being formed from a β-polyketoacid and this was found to occur in the fat body (Laurent et al., 2002). This strongly points to the alkaloids being formed from fatty acid precursors across multiple species of coccinellids and that the fat body is responsible for alkaloid synthesis. The alkaloids of *H. axyridis* have also been implicated as having a similar method of creation (Haulotte et al., 2012).

1.12 The multi-coloured asian lady beetle, *Harmonia axyridis*

H. axyridis is an invasive coccinellid originating from Japan/ East Asia (Fig. 1.12.1). It is also known as the Harlequin beetle or Halloween beetle as aggregations on buildings are commonly observed around late October in North America (Mahr, 1996). Presumed native distribution of *H. axyridis* extends from the Altai Mountains in the west to the Pacific coast in the east, and from southern Siberia in the north to southern China in the south (Koch, 2003). The antennae and mouthparts are usually yellow-brown, head yellow-white and legs vary from yellow-brown to black. The elytron is normally red and contains 9 black spots arranged in a 2,3,3,1 pattern (Fig. 1.12.2) (Chapin and Brou, 1991, Bezzerides et al., 2007).

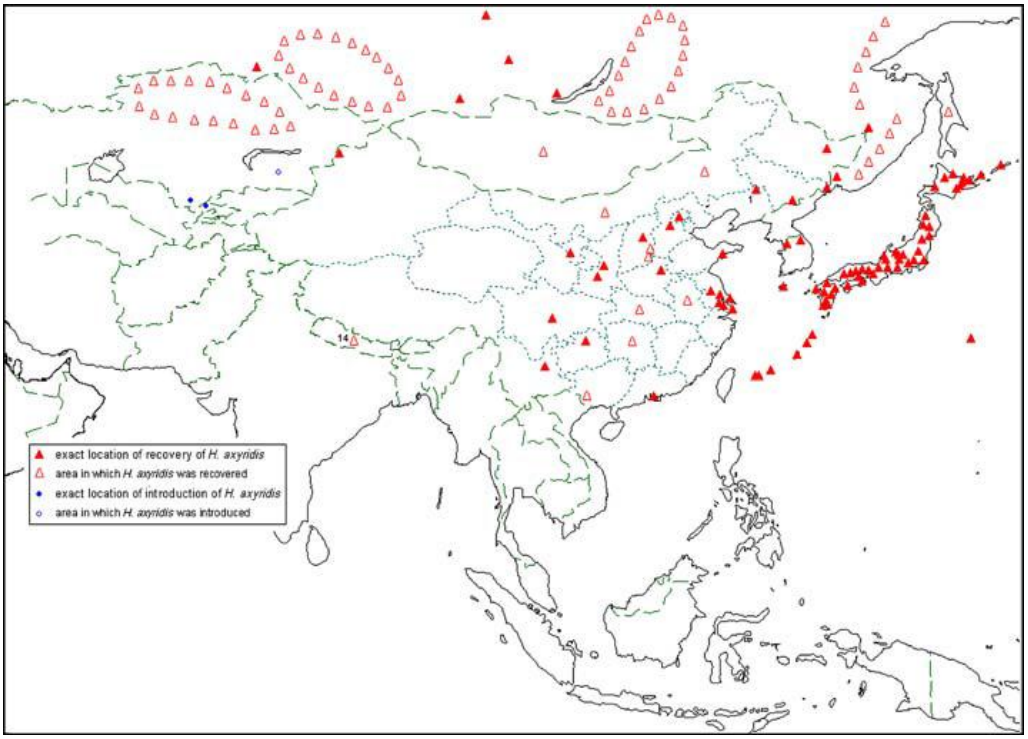


Fig. 1.12.1 - The native distribution of *H. axyridis* in East Asia. Taken from (Poutsma et al., 2008).

Fig. 1.12.2 - The colours and patterns of *H. axyridis* elytra can vary significantly between individuals. Taken from (Bezzerides et al., 2007).

H. axyridis exhibits a variety of traits that are thought to contribute to its success as an invasive species. The defensive alkaloids harmonine and 3-hydroxypiperidin-2-one are known to be produced (Alam et al., 2002) and pyrazines that cause a foul odour are known to be present (Sloggett et al., 2011). *H. axyridis* has a generalist diet and can tolerate a wide diversity of prey although newly encountered species are more difficult to predate on (Sloggett and Davis, 2010). This was demonstrated by the defensive alkaloid from the exotic *A. bipunctata,* adaline*,* being detectable in *H. axyridis* for longer than the defensive alkaloid of the native *Propylea japonica* so a specialised metabolism for *P. japonica* alkaloids is theorised to exist while newly encountered alkaloids are thought to be cleaved by non-specific processes (Sloggett et al., 2011, Sloggett and Davis, 2010). *H. axyridis* appears to be a top predator in the aphidophagous guild of predators and has a greater ability to use heterospecifics for food (Koch, 2003). The aggressiveness of predation on aphids by *H axyridis* has also shown to be greater when compared to 3 other coccinellids (Finlayson et al., 2010) and individuals are more repellent to *M. rubra* (Finlayson et al., 2009). These tools can make *H. axyridis* a successful intraguild predator and this is thought to be the reason for its success in invading new areas and as a biological control

agent (Koch, 2003). On the other hand, success may be due to defence against predation rather than aggressive IGP (Sloggett et al., 2009). It is clear that either way, the defensive chemistry (Fig. 1.12.3) plays a large role.

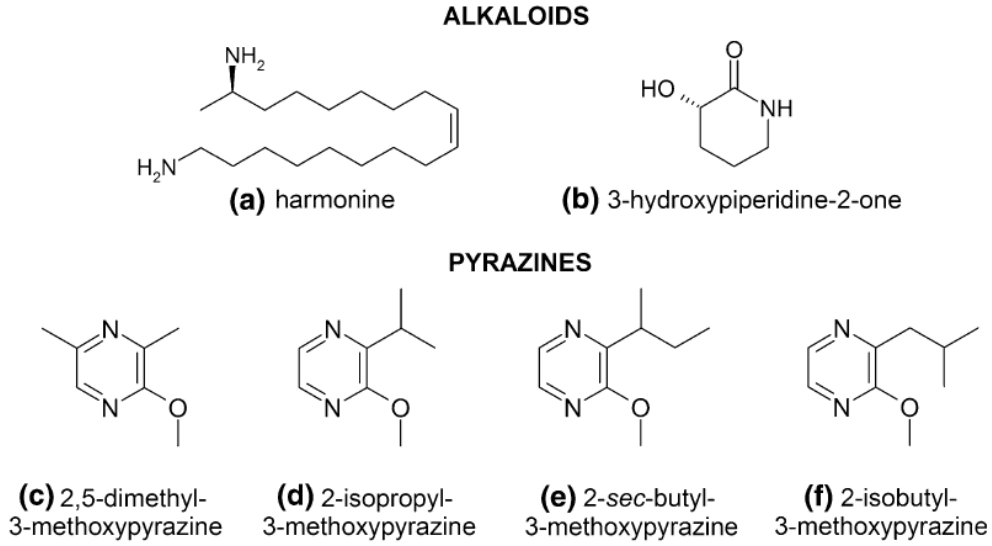


Fig. 1.12.3 - The alkaloids and pyrazines produced by *Harmonia axyridis*. Taken from (Sloggett et al., 2011).

1.13 Intraguild predation involving *Harmonia axyridis*

Many areas of the world are habitable for *H. axyridis* (Fig. 1.13.1) (Poutsma et al., 2008) and this has allowed *H. axyridis* to be extremely successful in invading areas outside its natural range. This has allowed *H. axyridis* to out-compete native coccinellids and supplant them as the dominant coccinellid species. This has occurred in the citrus systems of Florida where the native *Cycloneda sanguinea* has been replaced (Michaud, 2002), in Kentucky with *Coleomegilla maculata* (Cottrell and Yeargan, 1998) and in Western Oregon where *H. axyridis* has replaced *C. septempunctata* which itself was introduced as an exotic species (LaMana and Miller, 1996).

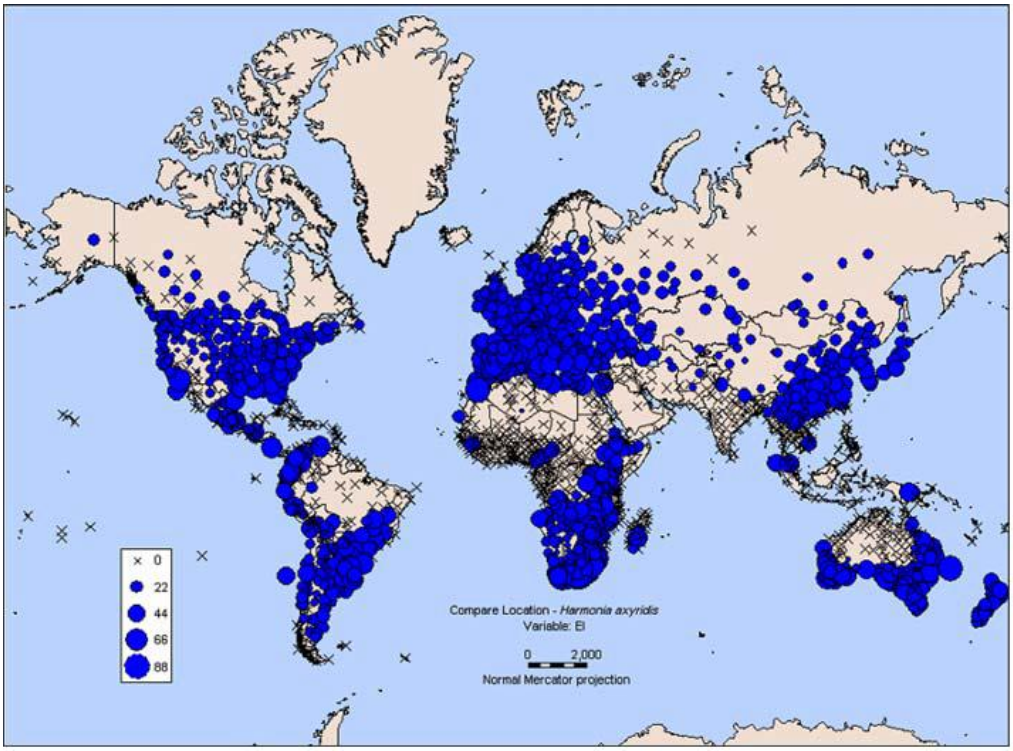


Fig. 1.13.1 – A CLIMEX world map showing econoclimatic indices (EI). The larger the EI, the more habitable the area is for *H. axyridis* and the larger the circle, the larger the EI. Crosses represent areas with an EI of 0. Taken from (Poutsma et al., 2008).

It is thought that IGP plays a role in the invasive, exotic species becoming dominant over the native species (Hautier et al., 2011). IGP occurrence was measured by GC-MS analysis of *H. axyridis* individuals and traces of exogenous alkaloids were found indicating that intraguild predation had occurred in the areas these individuals had occupied (Hautier et al., 2011). Interactions between *H. axyridis* and native British species in areas where *H. axyridis* was likely to come into contact with native species showed IGP to be asymmetrical in favour of *H. axyridis* (Ware and Majerus, 2008). Field studies over a 2 year period of *C. septempunctata* and *H. axyridis* showed that both species laid eggs during an increase or peak of aphid population. *H. axyridis* was observed to lay more eggs, have a lower pupal mortality and 4th instar larvae could develop when the aphid population was low (Hironori and Katsuhiro, 1997).

Laboratory studies have shed further light on why *H. axyridis* is such a good intraguild predator. Larvae of *H. axyridis* were more aggressive when paired with *C. impunctata, Scymnus rubromaculatus* (Nedved et al., 2010) and *C. sanguinea* larvae (Michaud, 2002). *H. axyridis* larvae were more aggressive and won more contests even when the opponent was 2-3 times bigger (Michaud, 2002). Studies have shown that *H. axyridis* eggs are better protected than other species of ladybird. *H. axyridis* larvae could complete development on *H. axyridis* and *C. septempunctata* eggs as well as aphids. Only 3 out of 16 *C. septempunctata* larvae developed when fed *H. axyridis* eggs (Yasuda and Ohnuma, 1999). Another study also showed *H. axyridis* eggs to be toxic to *C. septempunctata* (Kajita et al., 2010). This was also the case for *C. maculata* and *Olla v-nigrum* whereby consumption of *H. axyridis* eggs increased mortality for both species. *H. axyridis* was able to survive on eggs from both species (Cottrell, 2004). *C. carnea* survival was also found to be lowered when fed *H. axyridis* eggs compared to eggs of other coccinellids and aphids (Phoofolo and Obrycki, 1998). A reduction in the rate of consumption of eggs occurs when *H. axyridis, C Septempunctata* and *A. bipunctata* consume heterospecific eggs. However, this reduction is smaller in *H. axyridis* (Sato and Dixon, 2004). Aphids infected with the pathogenic fungus *Pandera neoaphidis* are predated upon less by *C. septempunctata* but *H. axyridis* was found to consume equal amounts of infected and uninfected prey suggesting the ability to survive on lower quality prey (Roy et al., 2008).

It is clear that *H. axyridis* is more aggressive when coming into contact with rival coccinellids and with the consumption of food. It is also clear that chemical defence plays an important role in the unpalatability and toxicity of its eggs.

1.14 Aims

This project aims to ascertain whether the *H. axyridis* alkaloids could potentially demonstrate any anthelmintic effects by using the free-living *C. elegans* as a model parasitic nematode. The alkaloids have been shown to be selective for insect neurons and they have been proposed to exhibit this action through the insect nAChR. *C. elegans* nAChRs are involved in the activation of pharyngeal pumping and body wall muscle movement. Due to the ease of genetic manipulation knockouts are readily available which will be used to identify possible targets in whole organism bioassays. The *Xenopus laevis* oocyte expression system and the TEVC technique will also be used to examine human α7, *C. elegans* ACR-16 and *Drosophila* alpha2/chick beta2 (Dalpha2/chickbeta2) nAChRs to characterise more precisely the alkaloid target and selectivity.

2. Materials and Methods

2.1 Extraction and purification of ladybird alkaloids

2.1.1 Collection of ladybird beetles

Adult *Coccinella septempunctata* and *Harmonia axyridis* beetles were collected from the University Park campus of the University of Nottingham (UK) and Rothamsted Research (Hertfordshire, UK) from September 2010 to December 2010 and stored in clear, universal tubes at -20 ˚C.

2.1.2 Solvent extraction

Collected beetles were counted, weighed and transferred from storage at -20 ˚C to different conical flasks dependent on the beetle species. HPLC grade methanol was added (approximately 50 ml per 100 beetles) and the flasks were left to stir overnight using a magnetic stirrer. The liquid was poured off into a separate flask and this was kept at 4 ˚C. The methanol was replaced and this was repeated for a further night.

2.1.3 Acid-Base extraction

The liquid remaining after the solvent extraction was placed in a rotary evaporator and left until the methanol had all evaporated. 1M hydrochloric acid (HCl) was used to wash the remaining solid out of the spherical flask and this was transferred to a separation funnel. HPLC grade ether was also used to wash the flask and this was also placed into the separation funnel. The liquids were mixed and left to separate. The aqueous layer was decanted back into the beaker and the organic layer was discarded. This process was repeated twice beginning with the addition of ether and the aqueous layers were combined.

The aqueous layer collected was made alkaline with sodium hydroxide (NaOH) to a pH of greater than 10 and this was added to the separation

funnel. The beaker was washed with HPLC grade dichloromethane and this was also added to the separation funnel. The liquids were mixed and left to separate and the aqueous layer was decanted. The organic layer was kept and the process was repeated using the decanted aqueous layer twice from the addition of dichloromethane. The organic layers were combined and the remaining dichloromethane was evaporated using a rotary evaporator and when a small volume remained, this was evaporated by directing a pressurised stream of nitrogen gas onto the surface of the liquid. The remaining solid was then dissolved in dimethyl sulfoxide (DMSO).

2.2 Maintenance of *C. elegans*

2.2.1 Strains

The following strains were used: N2 Bristol wild-type, DA465 *[eat-2(ad465)II]*, DA1110 *[eat-18(ad1110)I]* (McKay et al., 2004), ZZ37 *[unc-63(x37)I]* and RB918 *[acr-16(ok789)V]* (Touroutine et al., 2005). Strains ZZ37 and RB918 were kindly provided by Professor David Sattelle (University of Manchester, UK) and all strains originated from the *Caenorhabditis* Genetics Centre (University of Minnesota, US).

2.2.2 *Escherichia Coli* (*E. coli*) culture

Luria-Bertani broth (LB) agar (10 g tryptone, 10 g NaCl, 5 g yeast extract & 17 g agar made up to 1 litre in ddH_2O and autoclaved) was poured into 90mm petri dishes and was streaked with LB (10 g tryptone, 10 g NaCl & 5 g yeast extract made up to 1 litre in dH_2O and autoclaved) containing the uracil deficient *E. coli* strain OP50 once solidified. The plates were incubated at 37 ˚C overnight and a single colony of OP50 was used to inoculate approximately 5ml of LB. The inoculated LB was incubated at 37 ˚C on an orbital shaker at 250 rpm overnight. 100 µl of inoculated LB was added to 50

ml LB in universal tubes and incubated on an orbital shaker overnight again. The tubes were then stored at 4 ˚C for up to 4 weeks.

2.2.3 *C. elegans* culture

Nematode growth medium (NGM) (17 g agar, 3 g NaCl, 2.5 g bacteriological peptone, 1 ml 5 mg/ml cholesterol & 975 ml dH_2O autoclaved followed by the addition of 1 ml 1M $CaCl_2$, 1 ml 1M $MgSO_4$ & 25 ml 1M KPO_4 pH 6) was poured into sterile, 45 mm petri dishes. Once the agar had solidified, a drop of LB containing *E. coli* strain OP50 was added then spread to form a lawn on the agar surface and plates were incubated overnight at 37 ˚C. After incubation worms were transferred individually from other plates using a worm pick (platinum wire fused to a glass capillary tube) or by cutting chunks of agar from populated plates and transferring them to unpopulated plates ("chunking"). The plates were kept incubated at 15 ˚C. This was repeated once populated plates had become overpopulated and the *E. coli* food source had depleted (Brenner, 1974).

2.2.5 Synchronised culture

Plates abundant with laid eggs were selected and washed with bleaching solution (0.6 ml 5M NaOH, 1.5 ml NaClO solution (available chlorine 10-15%, Sigma) & 10 ml dH_2O mixed thoroughly). This was transferred to 15 ml tubes and topped up with sterile K-medium (53 mM NaCl, 32 mM KCl (Williams and Dusenberry, 1990)). The tubes were then centrifuged for 3mins at 2000rpm. The supernatant was removed and replaced with K-medium. The pellet was re-suspended and the tubes were centrifuged again. This wash was repeated a further 2 times and once the K-medium had been replaced a final time; the tubes were placed on a rotator overnight (Baugh and Sternberg, 2006). After this the tubes were placed on ice to allow the worms to settle at the bottom and the majority of the K-medium was removed. The remaining

suspension was pipetted onto seeded NGM plates and the plates were incubated at 15 ˚C once dry.

2.3 *C. elegans* assays

2.3.1 Pharyngeal pumping

2 plates containing synchronised cultures of *C. elegans* were washed with K-medium into 50 ml universal tubes and placed on ice for 10-15 mins to settle the worms at the bottom. K-medium was then removed until approximately 5 ml remained and the worms were suspended in the solution. 4 µl alkaloid extract was diluted with 196 µl *E. coli* + K-medium in small Eppendorf tubes and to this, 200 µl of the worm suspension was added giving a final volume of 400 µl. The tubes were mixed gently, placed on their side and incubated at 20 ˚C for the desired amount of time.

After incubation, 2 x 100 µl samples were transferred to 45 mm plates containing only NGM. A light microscope was then used to count the contractions of the terminal bulb manually using a stopwatch for 1 min. This method was adapted from (Avery and Horvitz, 1990).

2.3.2 Manual thrashing counts

Synchronised cultures of *C. elegans* were grown as described previously and 2 plates were washed with approximately 2-3 ml K-medium into 50 ml universal tubes. The suspension was diluted down with K-medium to ensure that 198 µl of the suspension contained approximately 10-20 worms. 2 µl of DMSO or drug/extract containing DMSO was added to wells of a flat-bottomed 96 well plate and serial diluted down to give the desired concentration range. To this 198 µl of the worms in K-medium was added (diluted down to ensure the volume contained 10-20 worms). The plates were then incubated at 20 ˚C for 2, 4 and 6 h. Thrashes were defined as the

complete bend of the body to one side and back (Glendinning et al., 2011) and were counted under a microscope.

2.3.3 Data analysis

Data of the effect of alkaloids was normalised by expressing the data as a percentage of the 1% DMSO control group for each experiment. GraphPad Prism 5 (GraphPad software, US) was used to plot graphs and fit curves using the "log(inhibitor) vs. normalised response – variable response" non-linear regression equation. Prism 5 was also used to analyse the differences between curves using an extra sum-of-squares F-test. IBM SPSS Statistics 20 (IBM, US) was used to analyse differences using independent samples t-tests.

2.4 Production nAChR subunits and associated protein cRNA

2.4.1 Transformation of *E. coli* with plasmid DNA

The plasmid vectors pcDNA3.1/Zeo (+) (Invitrogen) encoding human α7 and *C. elegans ric-3*, pGEM-T Easy (Promega) encoding *C. elegans acr-16*, pcDNA3.1 (Invitrogen) encoding chick β2 and pcDNA3 (Invitrogen) encoding *Drosophila* α2 (*D*α2) were kindly provided by Professor David Sattelle (University of Manchester, UK).

Chemically competent cells were created using *E. coli* strain XL1-Blue. An overnight culture of cells was prepared in 5 ml LB and incubated in an orbital shaker at 37 ˚C and 250 rpm. 50 ml LB was added and incubated for a further 2 h. The culture was placed on ice for 15 min and then centrifuged at 2000 rpm for 10 min at room temperature. The supernatant was removed and pellets re-suspended in 10 ml ice cold, filter sterilised 0.1M $CaCl_2$, left for 15 min and then centrifuged at 2000 rpm for 10 min at room temperature.

1 µl of plasmid DNA (pDNA) was added to 200 µl of chemically competent cells and left on ice for 20 min. The cells were then placed in a water bath at 42 ˚C for 1 min then immediately placed on ice for 2 min. 1 ml LB + 10 mM glucose was added and the cells were placed in a water bath at 37 ˚C for 30 min. 100 µl of cells were then spread over 95 mm Petri dishes containing LB agar containing 50 µg/ml Ampicillin and incubated overnight at 37 ˚C.

2.4.2 Isolation of plasmid DNA from transformed *E. coli*

A starter culture of *E. coli* was prepared by inoculating 5 ml LB using a sterile pipette tip to pick a single colony of the transformed *E. coli* from the LB agar plates. This was incubated in an orbital shaker overnight at 37 ˚C and 250 rpm.

A QIAGEN QIAprep Spin Miniprep Kit (50) was used to isolate the plasmids from the starter culture (all centrifugation steps were carried out at 13,000 rpm at room temperature): 1.5 ml starter culture was centrifuged at for 3 min. The supernatant was removed; a further 1.5 ml culture was added and centrifuged again for 3 min. The supernatant was removed; the pellet was re-suspended in 250 µl Buffer P1 and transferred to a fresh 1.5 ml Eppendorf tube. 250 µl Buffer P2 was added and mixed thoroughly by inverting 4-6 times until the solution became clear. 350 µl Buffer N3 was added, mixed thoroughly by inverting 4-6 times and centrifuged for 10 min. A QIAprep spin column was washed by placing the column in a 1.5 ml Eppendorf tube and adding 0.5 ml Buffer PB. The column was then centrifuged for 1 min and the flow-through was discarded. The supernatant was added to the column by pipetting and centrifuged for 1 min. The flow-through was discarded and 0.75 ml Buffer PE was added then centrifuged for 1 min. The column was centrifuged again for 3 min to ensure all the buffer had flowed through. The column was then placed

in a fresh 1.5 ml Eppendorf tube and 30 µl Buffer EB was added and left to stand for 1 min. The column was then centrifuged for 1 min to elute the DNA.

A Thermo-scientific NanoDrop 1000 spectrophotometer was used to measure the concentration of pDNA and the concentrations were as follows: 776 ng/µl for *C. elegans acr-16*; 529 ng/µl for human α7; 527 ng/µl for *D*α2; 475 ng/µl for chick β2 and 461 ng/µl for *C. elegans ric-3*.

2.4.3 Linearisation of plasmid DNA

Linearisation of pDNA (approximately 10 µg) was carried out using the following restriction enzymes: *Eco*RI for *D*α2, *Xba*I for chick β2/human α7, *Nde*I for *C. elegans acr-16* and *Not*I for *C. elegans ric-3* (all New England Biolabs). The mixtures used for the restriction digest were as follows: *C. elegans acr-16* (14 µl pDNA, 5 µl NEbuffer IV, 5 µl Bovine serum albumin (BSA), 5 µl *Nde*I & 21 µl ddH_2O), *C. elegans ric-3* (21 µl pDNA, 5 µl NEbuffer III, 5 µl BSA, 5 µl *Not*I & 14 µl dH_2O), human α7 (20 µl pDNA, 5 µl NEbuffer II, 5 µl BSA, 5 µl *Xba*I & 15 µl dH_2O), *D*α2 (20 µl pDNA, 5 µl NEbuffer IV, 5 µl BSA, 5 µl *Eco*RI & 15 µl dH_2O) & chick β2 (21 µl pDNA, 5 µl NEbuffer II, 5 µl BSA, 5 µl *Xba*I & 14 µl ddH_2O). The mixtures were incubated at 37 ˚C for 3 h.

Linearisation was checked after 2 h by running a 1 µl sample on a 1.5% TAE gel with a 1kb ladder. This was made using 1xTAE (4.84 g Tris base, 1.142 ml acetic acid, 2 ml 0.5M EDTA pH 8 made up to 1 litre with ddH_20 then autoclaved) and the gel was made by adding 0.75 g Agarose to 50 ml 1xTAE then melting in a microwave for approximately 2 min followed by the addition of 1 µl ethidium bromide. 1 µl of linearised pDNA/1kb ladder was mixed with 1 µl loading buffer on clean parafilm and loaded into the wells of the gel and run for 1 h at 70 V. One clear band was visible for each subunit which ensures that no other cuts were made by the restriction enzyme.

The restriction digest was terminated by adding 2.5 µl EDTA, 5 µl ammonium acetate and 100 µl 100% ethanol. This was left for 1 h at -20 ˚C and then centrifuged at 13,000 rpm/4 ˚C for 15 min. The supernatant was removed by pipetting and the tubes were left to dry in a sterile cabinet for 5 min. The pellet was then re-suspended in 4 µl ddH_2O. The following concentrations were determined by the spectrophotometer: 269 ng/µl for human α7, 792 ng/µl for *C. elegans acr-16*, 681 ng/µl for *C. elegans ric-3*, 805 ng/µl for *D*α2 and 446 ng/µl for chick β2.

2.4.4 cRNA production

Production of capped RNA (cRNA) from the linearised plasmids was carried out using the T7 mMESSAGE mMACHINE kit (Ambion) for human α7, *C. elegans ric-3*, chick β2 and *D*α2. The SP6 mMESSAGE mMACHINE kit (Ambion) was used for *C. elegans acr-16*. The mixtures used for the transcription reaction were as follows: *C. elegans acr-16*/*C. elegans ric-3*/*D*α2 (1 µl linearised pDNA (0.1-1 µg/µl), 15 µl 2x NTP/CAP, 3 µl 10x Reaction Buffer, 3 µl Enzyme Buffer, 6 µl nuclease free H_2O) and human α7/chick β2 (2 µl linearised pDNA, 15 µl 2x NTP/CAP, 3 µl 10x Reaction Buffer, 3 µl Enzyme Buffer, 5 µl nuclease free H_2O). The mixtures were incubated at 37 ˚C for 2 h 45 min followed by the addition of 30 µl lithium chloride precipitation solution and 30 µl nuclease free H_2O. The mixture was left to precipitate overnight at -20 ˚C. Following this the mixtures were centrifuged at 13,000 rpm/4 ˚C for 15 min. The supernatant was removed and the pellet washed with 1 ml 70% ethanol. This was centrifuged again at 13,000 rpm/4 ˚C for 15 min and the supernatant was removed. The remaining pellet was re-suspended in 15 µl nuclease free H_2O and the concentration of RNA was determined. The concentrations obtained were as follows: 293 ng/µl for chick β2, 1848 ng/µl for

human α7, 3805 ng/µl for *D*α2, 1012 ng/µl for *C. elegans acr-16* and 3992 ng/µl for *C. elegans ric-3*.

2.5 Electrophysiological recordings of *Xenopus laevis* oocytes

2.5.1 Preparation and injection of oocytes

Mature stage IV-V oocytes were obtained by ovariectomy of female *Xenopus laevis* from the European Xenopus Resource Centre (University of Portsmouth, UK) and incubated in 0.2mg/ml collagenase (type 1A, Sigma) in Ca^{2+} free Barth's GTP solution (96mM NaCl, 2mM KCl, 5mM HEPES, 2.5mM pyruvic acid & 0.5mM theophylline, pH 7.5, then autoclaved followed by the addition of 5ml 10mg/ml gentamicin per litre) for 1-2 h followed by thorough washing with Ca^{2+} free GTP. Oocytes were selected based on a clear divide between the light and dark poles and for their structural integrity then placed in fresh Ca^{2+} free GTP. Human α7/*C. elegans acr-16* was injected in a 5:1 ratio with *C. elegans ric-3* and *D*α2 was injected in a 1:1 ratio with chick β2. Injection concentrations did not exceed 1 µg/µl.

Injection needles were pulled from 3.5nl glass bores (World Precision Instruments, Inc.) using a David Kopf vertical pipette puller (model 700c, DK Instruments, California) and a small part of the tip was broken off. The needle was then "back filled" with filtered paraffin and placed into a Nanoliter injector (World Precision Instruments, Inc.). 1µl subunit cRNA was pipetted onto the surface of a sterile petri dish and the glass needle was manoeuvred into the solution. The cRNA was taken up by the needle ensuring that no air bubbles were present. Viable oocytes were then selected and placed into a dish containing GTP (96mM NaCl, 2mM KCl, 1.8mM $CaCl_2$, 5mM HEPES, 2.5mM pyruvic acid & 0.5mM theophylline, pH 7.5, then autoclaved followed by the addition of 5ml 10mg/ml gentamicin per litre) lined with grooved plastic to hold

the oocytes in place. 50 nl cRNA was injected into the cytoplasm of each oocyte and then oocytes were transferred to a separate dish with fresh GTP. Damaged or discoloured oocytes were removed and the rest were incubated at 18 ˚C for 2-4 days. GTP was changed daily and any damaged oocytes were removed.

2.5.2 Two electrode voltage clamp procedure

Microelectrodes were pulled from borosilicate glass capillaries (TW150F-4, World Precision Instruments Inc.) using a Sutter P-97 programmable puller and used to impale oocytes placed in a perfusion bath. The microelectrodes were filled with 3M KCl and resistance was 0.5MΩ or less. Oocytes displaying a resting membrane potential of at least -20 mV were clamped at -100 mV using an Axoclamp 2A or Geneclamp 500 amplifier (Axon Instruments Inc., USA). Output currents were transferred using a CED 1401 plus interface (Cambridge Electronic Design) to a PC and WinEDR software (Dr John Dempster, Institute of Pharmacy & Biomedical Sciences, University of Strathclyde, UK) was used for recording and analysis. Standard oocyte saline (SOS) (100mM NaCl, 2mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 5mM HEPES, adjusted to pH 7.6 with NaOH) + 0.5µM Atropine to prevent any muscarinic response was applied using a gravity fed perfusion system under the control of a ValveLink 8 valve controller (Automate Scientific Inc., USA). ACh and alkaloid extract were dissolved in SOS + 0.5µM atropine.

100µM (10^{-4}M) ACh was applied 2-3 times for a maximum of 5 s to test whether oocytes had a stable response then the effect of the alkaloid was tested by applying alkaloid extract solution at varying concentrations for 10 s prior to the application of ACh.

2.5.3 Data analysis

Data of the effect of alkaloids on the response to 10^{-4}M ACh was normalised by expressing the data as a percentage of the response to 10^{-4}M ACh alone. GraphPad Prism 5 (GraphPad software, US) was used to plot graphs and fit curves using the “log(inhibitor) vs. response – variable slope (four parameters)” non-linear regression equation. Prism 5 was also used to analyse the differences between curves using an extra sum-of-squares F-test.

3. Results I – Effects of Alkaloid on *C. elegans*

3.1 Pharyngeal pumping assays

The pharynx is a well characterised neuromuscular system in *C. elegans* and nAChRs play a large role in the generation of rapid pharyngeal pumping (McKay et al., 2004). The quantification of pharyngeal pumping provides a simple assay to determine the effects of neuroactive compounds and as ladybird alkaloids are thought to target nAChRs the effects of alkaloid extracts from 2 different ladybird species on wild-type (Bristol N2) *C. elegans* were determined (Fig. 3.1.1).

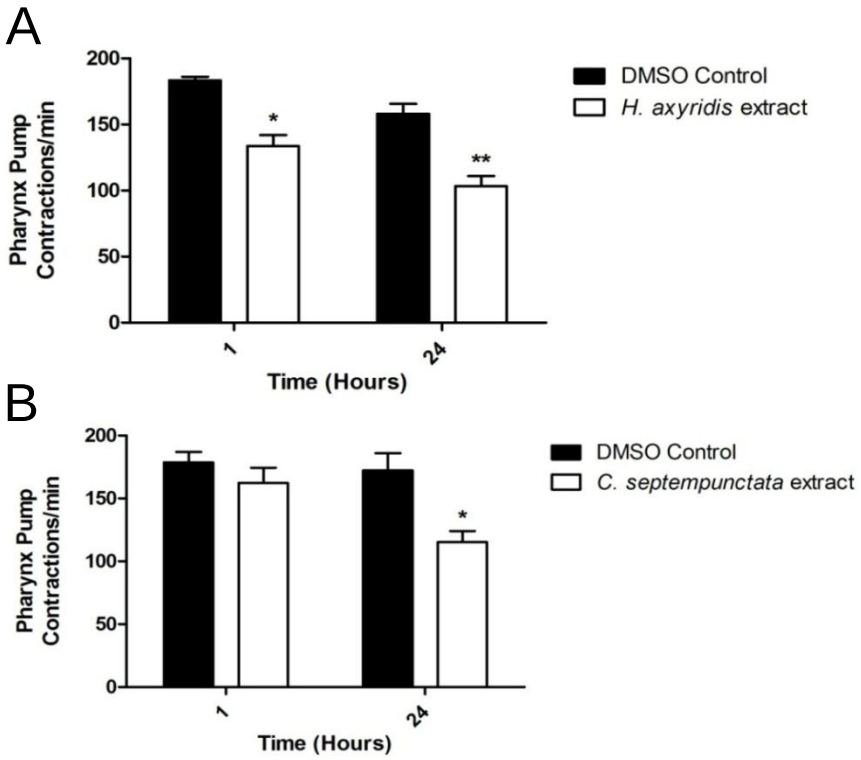


Fig. 3.1.1 – Adult N2 hermaphrodite *C. elegans* were exposed to alkaloid extract (0.3 mg/ml in DMSO) in K-medium or K-medium containing 1% DMSO as a control. The rate of pharyngeal pumping was measured after 1 h and 24 h. Each bar represents the mean ±SEM of 3 independent experiments containing 6 replicates each. Strains were compared using an independent-samples t-test (* P<0.05 & ** P<0.01). (A) Incubation with *H. axyridis* extract showed a significant reduction in pharyngeal pumping after 1 h (P=0.018) and 24 h (P=0.007) compared with1% DMSO control. (B) Incubation with *C. septempunctata* extract showed no significant difference after 1 h (P=0.338) but a significant reduction after 24 h (P=0.031).

The alkaloid extract of the Harlequin ladybird (*H. axyridis*) and the 7-spot ladybird (*C. septempunctata*) were dissolved in DMSO at a concentration of 30 mg/ml then diluted by 100 to give a final concentration of 0.3 mg/ml with 1% of the total solution containing DMSO. Adult N2 worms were incubated for 1 and 24 hours at 20 ˚C and the rate of pharyngeal pumping was determined (Fig 3.1.1). *H. axyridis* alkaloid extract reduced the mean rate of pharyngeal pumping compared to 1% DMSO control after 1 h (from 183.22 ±2.86 to 133.61 ±8.25 pump contractions/min) and 24 h (from 158 ±7.65 to 103.28 ±7.68 pump contractions/min) (Fig. 3.1.1A). An independent samples t-test showed this reduction to be statistically significant in both cases (P=0.018 & 0.007). *C. septempunctata* alkaloid extract also showed reductions in the mean rate of pharyngeal pumping after 1 h (178.56 ±8.33 to 162.33 ±12.06 pump contractions/min) and 24 h (172.33 ±13.56 to 115.28 ±8.81 pump contractions/min) (Fig. 3.1.1.B). However, statistical analysis showed that the reduction after 1 h was not significant (P=0.338) and the reduction after 24 h was significant (P=0.031). Therefore *H. axyridis* alkaloids were selected for further analysis.

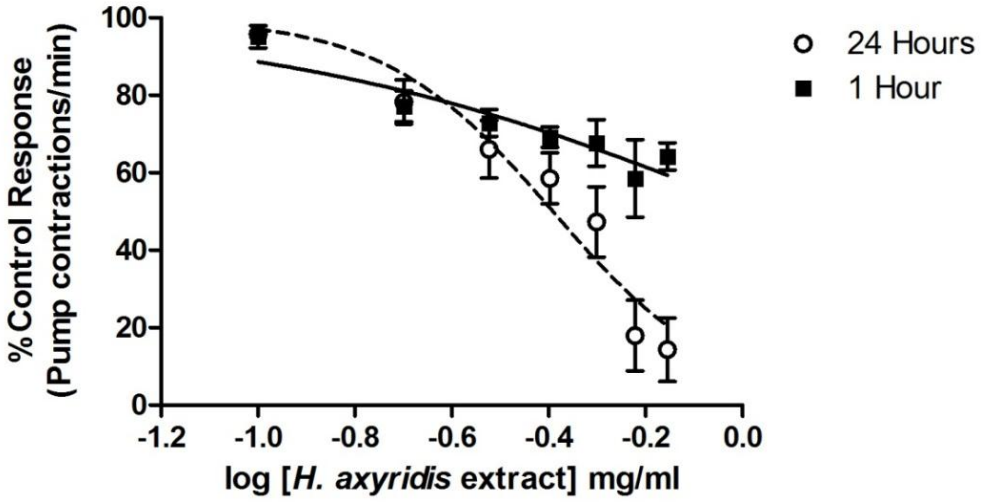


Fig. 3.1.2 - The rate of adult N2 hermaphrodite pharyngeal pumping was measured in response to concentrations of *H. axyridis* extract from 0.1-0.7mg/ml after 1 and 24 h. Each point represents the mean ±SEM of 3 independent experiments each containing 6 replicates expressed as a % of the control value (1% DMSO). Curves were fitted in GraphPad Prism 5 using the "log(inhibitor) vs. normalised response – variable slope" non-linear regression equation. IC_{50} values were determined as 1.087mg/ml for 1 h and 0.406mg/ml for 24 h.

Exposure to various concentrations of *H. axyridis* alkaloids revealed a concentration dependant inhibitory effect on the rate of pharyngeal pumping (Fig. 3.1.2).The IC_{50} after 1 h was determined as 1.087 mg/ml (95% CI = 0.652 to 1.81) based on the extrapolation of the non-linear regression curve to 0% and the IC_{50} after 24 h was determined as 0.406 mg/ml (95% CI = 0.357 to 0.46). The pumping rate was reduced to approximately 20% of the control rate at the maximum concentration of 0.7mg/ml *H. axyridis* alkaloid extract. An extra sum-of-squares F-test comparison of the IC_{50} values showed that the two values were siginificantly different ($P<0.0001$) therefore the effect observed after 24 h is significantly greater than the effect after 1 h.

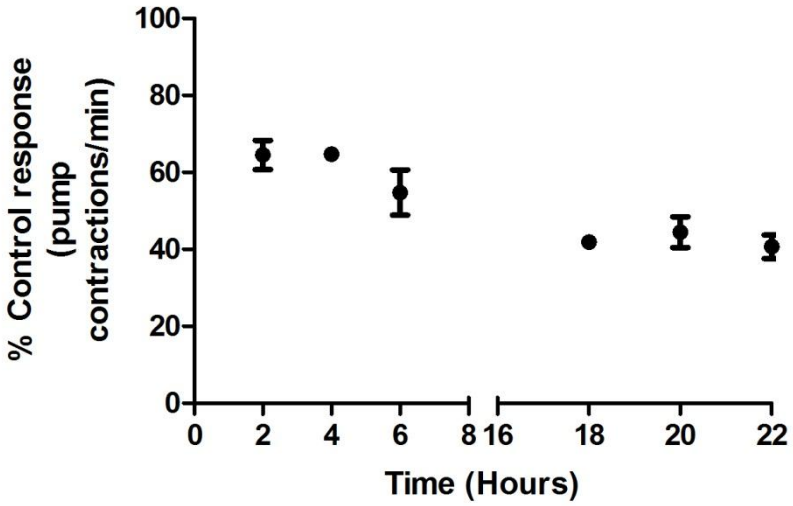


Fig. 3.1.3 – The effect of *H. axyridis* alkaloid extract at 0.7mg/ml was measured after 2, 4 and 6 h as well as overnight after 18, 20 and 22 h. Each point represents the mean ±SEM of 3 independent experiments containing 6 replicates each expressed as a % of a 1% DMSO control value.

The effect of 0.7 mg/ml *H. axyridis* alkaloids were measured after 2, 4, 6, 18, 20 and 22 h (Fig. 3.1.3). A reduction in the mean rate of pharyngeal pumping was observed after 2 h to 64.502 ±3.773% of the control rate and decreases were also observed to 64.697 ±1.346% and 54.745 ±5.882% for 4 and 6 h respectively. Overnight, the mean rate decreased to 41.835 ±1.131% for 18 h, 44.438 ±4.012 for 20 h and 40.689 ±3.039% for 22 h.

As the alkaloid extract of *H. axyridis* has been demonstrated to reduce the response of human and insect nAChRs; *C. elegans* strains containing mutations in nAChRs were examined to investigate the mode of action (Table 3.1.1). The strains DA465 contains a mutation in a nAChR non-α subunit and DA1110 contains a mutation in an associated transmembrane protein that are both expressed in the pharynx (Table 3.1.1). The pharyngeal pumping rate of both strains are reduced as a result of the mutations and so examination of *H. axyridis* alkaloid extract in these mutants could give insight into the mode of

action. The basal pumping rate of each mutant in 1% DMSO control solution of each mutant was compared to N2 (Fig. 3.1.4).

Table 3.1.1 – Transgenic *C. elegans* strains used for experiments with *H. axyridis* alkaloid extract.

Strain	Mutation	Info
DA465	***eat-2(ad465)II*** (C>T substitution)	Non-α nAChR subunit. Reduces rate of pharyngeal pumping (McKay et al., 2004).
DA1110	***eat-18(ad1110)I*** (G>T substitution)	Small transmembrane protein associated with *eat-2* nAChRs. Reduces rate of pharyngeal pumping (McKay et al., 2004).
ZZ73	***unc-63(x37)I*** (G>A substitution)	Levamisole-sensitive nAChR α subunit in body wall muscle. Causes locomotor defects (Touroutine et al., 2005).
RB918	***acr-16(ok789)V*** (Deletion)	Levamisole-insensitive nAChR α subunit in body wall muscle (Touroutine et al., 2005).

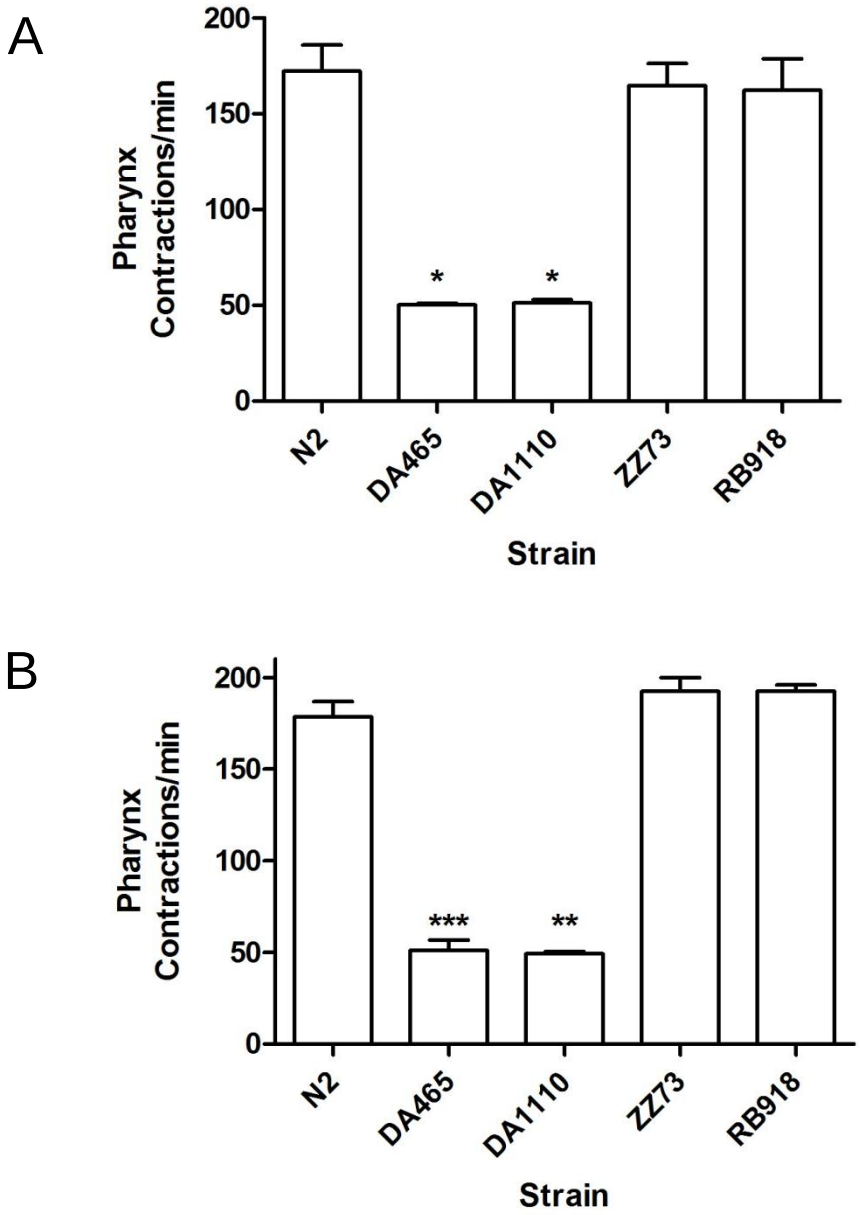
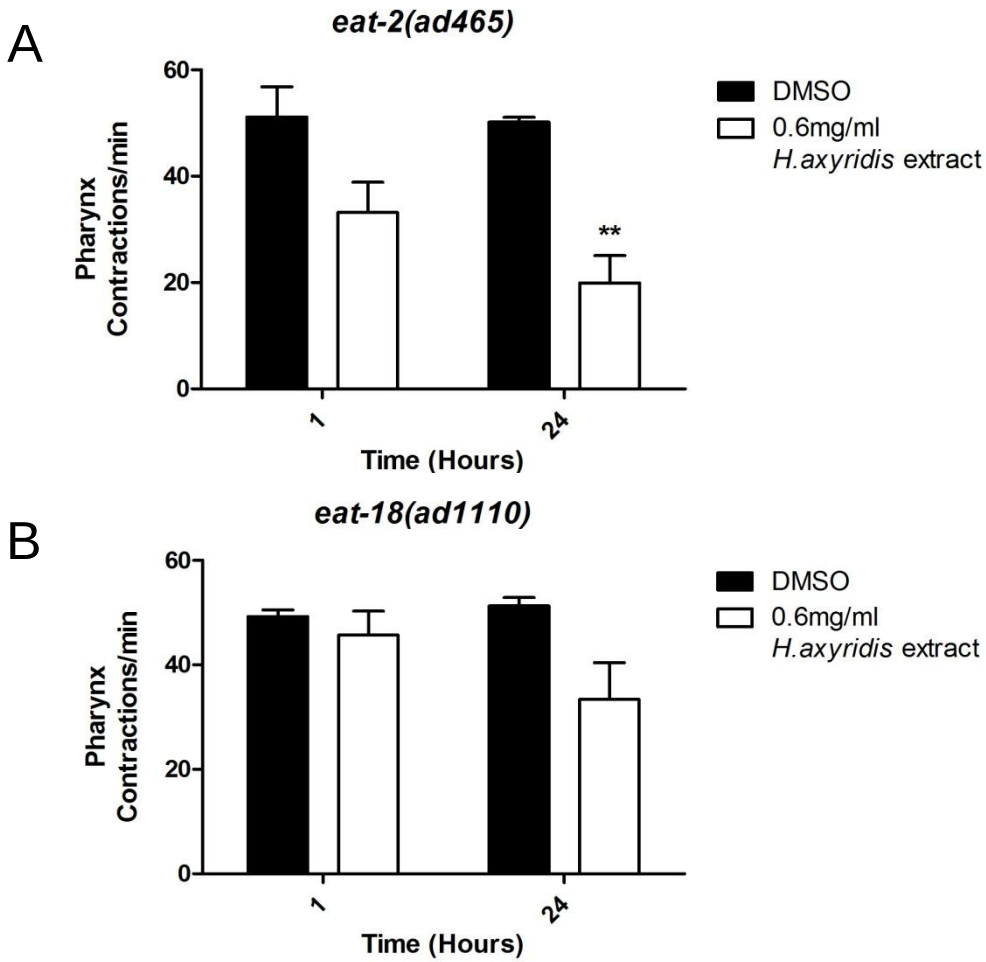


Fig. 3.1.4 – The basal pharyngeal pumping rates in 1% DMSO were measured and the different nAChR mutants were compared to the wild-type N2 strain when incubated for (A) 1 h and (B) 24 h. Each bar represents the mean ±SEM of 3 independent experiments containing 6 replicates each. Strains were compared using an independent-samples t-test (* $P<0.05$, ** $P<0.01$ & *** $P<0.001$).

An independent samples t-test showed significant reductions in the basal rate of pumping after 1 and 24 h in DA1110 (P=0.003 & 0.011) and DA465 (P=0.012 & <0.001). The mean basal rate after 1 and 24 h of DA1110 (51.11 ±5.724 & 50.17 ±0.928) and DA465 (49.22 ±1.309 & 51.28 ±1.587) dropped to approximately 25% of the wild-type N2 basal rate (178.6 ±8.334 &

175.3 ±13.56) but with no change from 1 to 24 h. The basal pumping rates of the remaining strains examined, ZZ73 (P=0.291 & 0.695) and RB918 (P=0.193 & 0.664) showed no significant difference from N2 (Fig. 3.1.4).

The effect of *H. axyridis* alkaloid extract was then examined on each strain to ascertain whether the mutated genes were involved in the effects of the extract. The genes involved were *eat-2*, *eat-18*, *unc-63* and *acr-16* (Table 3.1.1). A concentration of 0.6mg/ml was used and the pumping rate was examined after 1 and 24 h (Fig. 3.1.5).



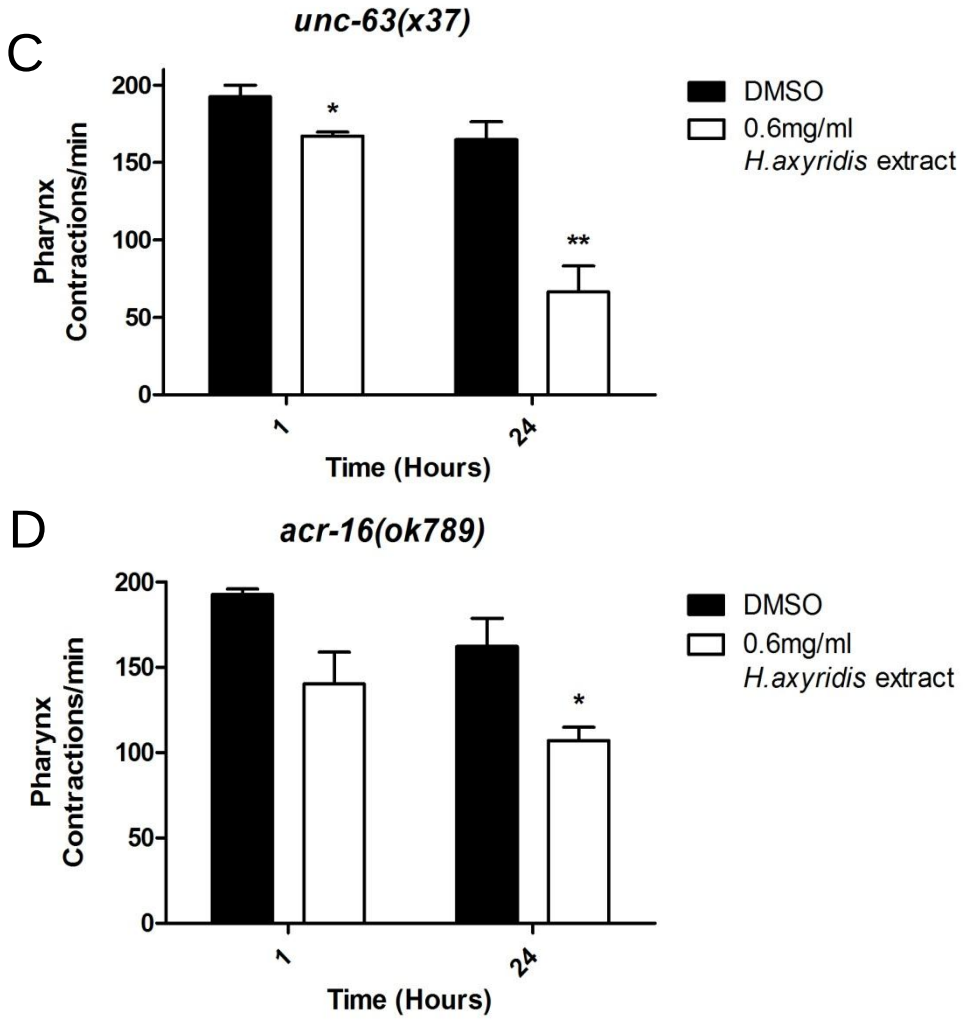


Fig. 3.1.5 – The effect of 0.6 mg/ml *H. axyridis* alkaloid extract on the rate of pharyngeal pumping on different nAChR mutant strains, (A) *eat-2(ad465)*, (B) *eat-18(ad1110)*, (C) *unc-63(x37)* and (D) *acr-16(ok789)*, after 1 and 24 h. Each bar represents the mean ±SEM of 3 independent experiments containing 6 replicates each. Strains were compared using an independent-samples t-test (* P<0.05, ** P<0.01 & *** P<0.001).

The results were examined using an independent samples t-test and *unc-63(x37)* showed significant reductions from the DMSO control after 1 (167.11 ±2.567, P=0.036) (Table 3.1.2) and 24 h (66.44 ±16.685, P=0.011) (Table 3.1.3). Significant reductions were observed only after 24 h in *eat-2(ad465)* (19.943 ±5.148, P=0.004) and *acr-16(ok789)* (107.167 ±7.688, P=0.038) while *eat-18(ad1110)* showed no significant differences (P=0.527 & 0.068) (Table 3.1.2).

Table 3.1.2 – Statistical analysis of mutant strains pumping rate after 1 h incubation with 0.6mg/ml *H. axyridis* alkaloid extract compared to 1% DMSO control.

Strain/Mutation	Mean ±SEM (Pump contractions/min)		Independent samples t-test results
	DMSO Control	Alkaloids (1 h)	
DA465 *[eat-2(ad465)]*	51.112 ±5.742	33.167 ±5.703	t=2.221 P=0.091
DA1110 *[eat-18(ad1110)]*	49.223 ±1.309	45.667 ±4.622	t=0.740 P=0.527
ZZ73 *[unc-63(x37)]*	192.333 ±7.669	167.110 ±2.567	t=3.119 P=0.036 *
RB918 *[acr-16(ok798)]*	192.556 ±3.283	147.167 ±18.745	t=2.753 P=0.104

NB. * indicates a significant difference: * P<0.05, ** P<0.01 & *** P<0.001.

Table 3.1.3 – Statistical analysis of mutant strains pumping rate after 24 h incubation with 0.6mg/ml *H. axyridis* alkaloid extract compared to 1% DMSO control.

NB. * indicates a significant difference: * P<0.05, ** P<0.01 & *** P<0.001.

Strain/Mutation	Mean ±SEM (Pump contractions/min)		Independent samples t-test results
	DMSO Control	Alkaloids (24 h)	
DA465 *[eat-2(ad465)]*	50.167 ±0.928	19.943 ±5.148	t=5.77 P=0.004 **
DA1110 *[eat-18(ad1110)]*	51.277 ±1.587	33.39 ±7.045	t=2.477 P=0.068
ZZ73 *[unc-63(x37)]*	164.833 ±11.498	66.444 ±16.685	t=4.856 P=0.011 *
RB918 *[acr-16(ok798)]*	162.333 ±16.387	107.167 ±7.688	t=3.048 P=0.038 *****

These effects were then compared to the effects of 0.6mg/ml *H. axyridis* alkaloid extract on N2. The rates of pumping were normalised as

different control rates were observed and compared after 1 and 24 h (Fig. 3.1.6).

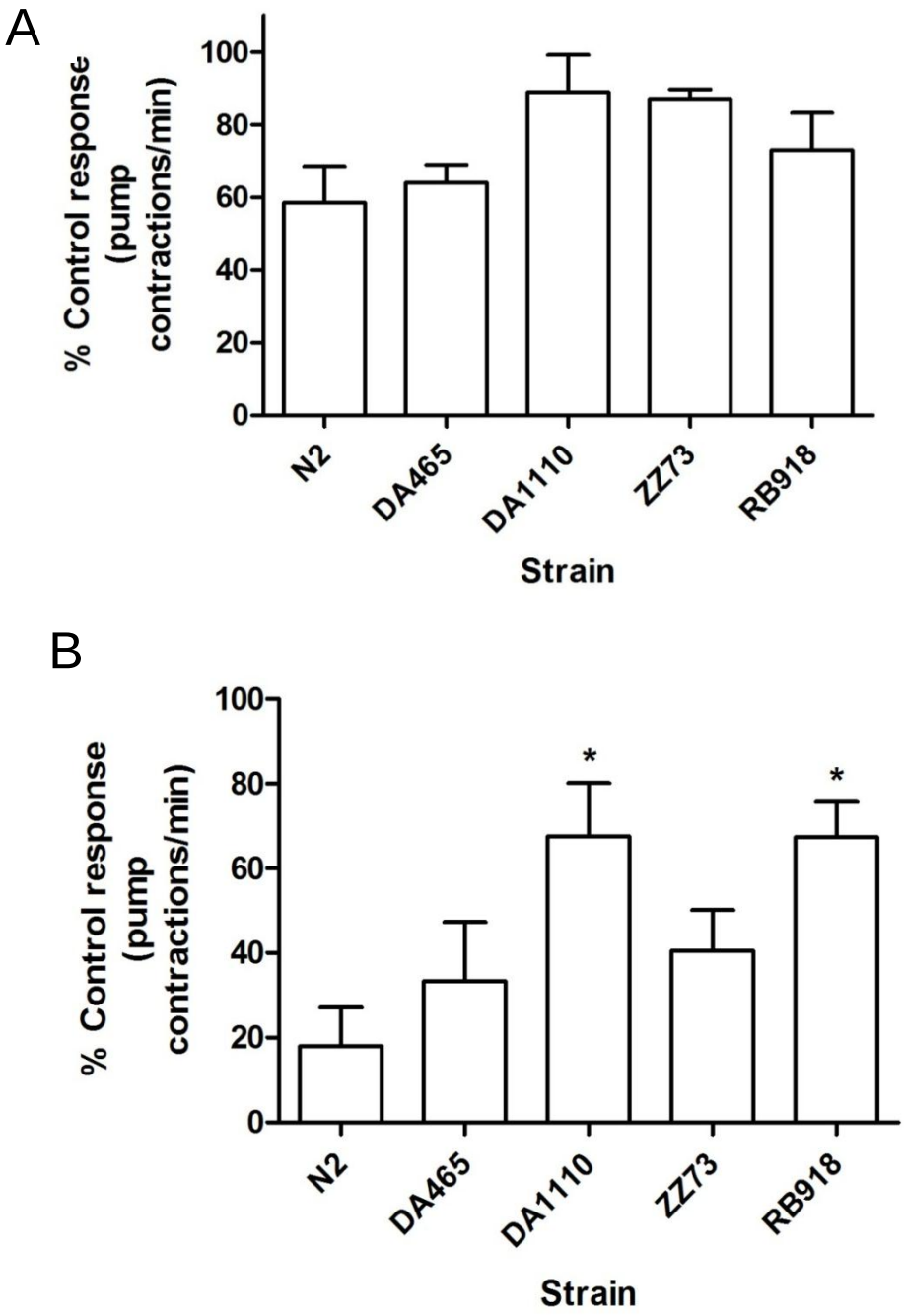


Fig. 3.1.6 – The effects of 0.6mg/ml alkaloids on the pumping rate of each mutant strain was compared to wild-type N2 after (A) 1 h and (B) 24 h. Each point represents the mean ±SEM of 3 independent experiments containing 6 replicates each expressed as a % of a 1% DMSO control value. Strains were compared using an independent-samples t-test (* $P<0.05$).

Comparison of the effects of 0.6mg/ml *H. axyridis* alkaloid extract showed that no significant differences from the N2 level of reduction was observed after 1 h (Table 3.1.4). This was also the case after 24 h for DA465 (P=0.412) and ZZ73 (P=0.166). However, after 24 h the mean pumping rates of DA1110 (51.28 ±1.587 ,P=0.034) and RB918 (162.3 ±16.39, P=0.016) were significantly larger than the N2 rate (Table 3.1.4).

Table 3.1.4 – Statistical analysis of mutant strains pumping rate in presence of 0.6mg/ml *H.axyridis* alkaloid extract compared to N2

Strain	**Mutation**	**Normalised mean ±SEM (Pump contractions/min)**		**Independent samples t-test results**	
		1 hour	**24 hours**	**1 hour**	**24 hours**
N2	**-**	58.56 ±9.993	18 ±9.161	-	-
DA465	***eat-2(ad465)***	64.05 ±4.947	18 ±9.161	t=-0.492 P=0.68	t=-0.915 P=0.412
DA1110	***eat-18(ad1110)***	88.97 ±17.65	67.43 ±12.73	t=-2.131 P=0.100	t=-3.512 P=0.034 *
ZZ73	***unc-63(x37)***	87.09 ±2.642	40.48 ±9.65	t=-2.76 P=0.051	t=-1.69 P=0.166
RB918	***acr-16(ok789)***	72.98 ±10.24	67.33 ±8.271	t=-1.008 P=0.37	t=-3.997 P=0.016 *

NB. * indicates a significant difference: * P<0.05, ** P<0.01 & *** P<0.001.

3.1.2 Swimming (thrashing) assays

Movement using body wall muscle in *C. elegans* is governed by a different neuromuscular system from that of the pharynx and is known to involve nAChRs. Many anthelmintics that target nAChRs affect body wall movement so the rate of swimming (thrashing) in solution can give a good idea as to the functioning of this system and potentially whether a compound acts on a body wall nAChR. The effect of *H. axyridis* alkaloid extract was therefore determined on the rate of swimming (thrashing) of *C. elegans* in solution (Fig. 3.2.1).

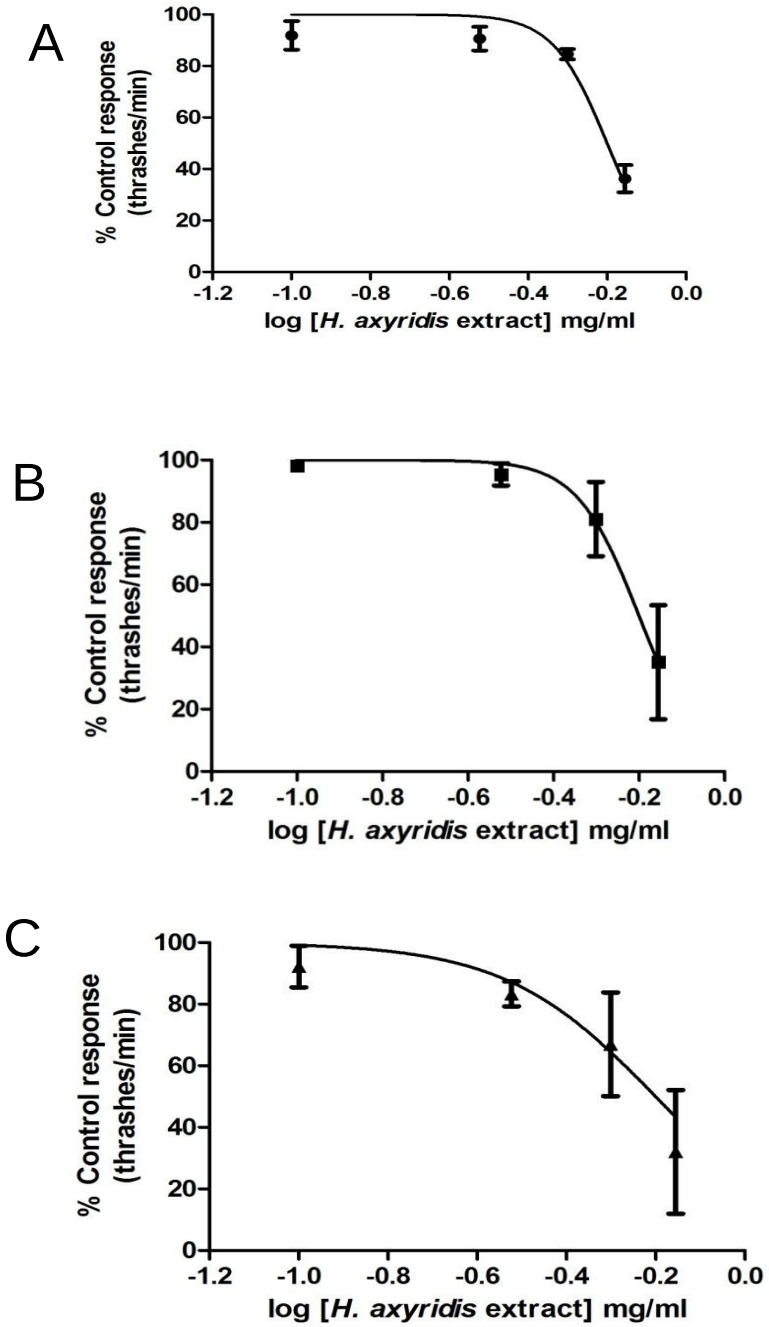


Fig. 3.2.1 - The concentration of *H. axyridis* extract was varied from 0.1-0.7mg/ml and the rates of adult N2 hermaphrodite swimming (thrashing) was measured after (A) 2, (B) 4 and (C) 6 h. Each point represents the mean ±SEM of 3 independent experiments containing 6 replicates each expressed as a % of a 1% DMSO control treatment. Curves were fitted in GraphPad Prism 5 using the "log(inhibitor) vs. normalised response – variable slope" non-linear regression equation. IC_{50} values were determined as 0.64 mg/ml after 2 h, 0.63 mg/ml after 4 h and 0.58 mg/ml after 6 h.

Adult N2 hermaphrodites were incubated in wells containing 0.1, 0.3, 0.5 and 0.7mg/ml *H. axyridis* alkaloid extract. The rate of swimming (thrashing) was counted after 2, 4 and 6 h (Fig. 3.2.1A, B & C). A concentration dependant inhibitory effect was observed with IC_{50} values of 0.64 mg/ml (95% CI = 0.60 to 0.69) after 2 h, 0.63 mg/ml (95% CI = 0.55 to 0.72) after 4 h and 0.58 mg/ml (95% CI = 0.44 to 0.76) after 6 h. An F-test comparison of the IC_{50} values showed no significant difference between 2 and 4 h (P=0.82) and 2 and 6 h (P=0.38) (Fig. 3.2.1).

4. Results II – Alkaloid effects on recombinant nAChRs

The oocytes of *Xenopus laevis* were used to express nAChR subunit cRNA in order to form functional recombinant nAChRs and allow the recording of currents through the nAChRs when ACh is applied. This allowed the direct testing of the effects of *H. axyridis* alkaloid extract on recombinant *C. elegans*, human and hybrid *Drosophila*/chicken nAChRs. However, the concentration was unknown. Alkaloid material was limited and a large amount is required for studies using *Xenopus* oocytes. To maximise the usage of the alkaloids an estimated molecular weight of 200 was used based on the chemical structures of alkaloids known to be present in *H. axyridis*.

4.1 Effect on *C. elegans* ACR-16

C. elegans acr-16 encodes a nAChR α subunit, ACR-16, that forms homopentameric nAChRs in the body wall muscle of *C. elegans* (Richmond and Jorgensen, 1999, Touroutine et al., 2005).

The *acr-16* subunit cRNA was injected into *Xenopus* oocytes and the peak amplitude was measured in response to 10^{-4}M ACh which is the EC_{50} of

the receptor to ACh (Raymond et al., 2000). Exposure to various concentrations of *H. axyridis* alkaloid extract revealed a concentration dependent inhibition of the ACh response (Fig. 4.1.1 & 4.1.2). Application of extract at a concentration of 10^{-4}M completely inhibited the response to ACh (Fig. 4.1.1B) and partial inhibition occurred at 10^{-5}M (Fig. 4.1.1C). No other concentration showed a noticeable decrease in ACh response (Fig. 4.1.1D-G). An IC_{50} value of $9.83x10^{-6}$M (95% CI = $5.2x10^{-6}$M to $1.86x10^{-5}$M) was determined (Fig. 4.1.2).

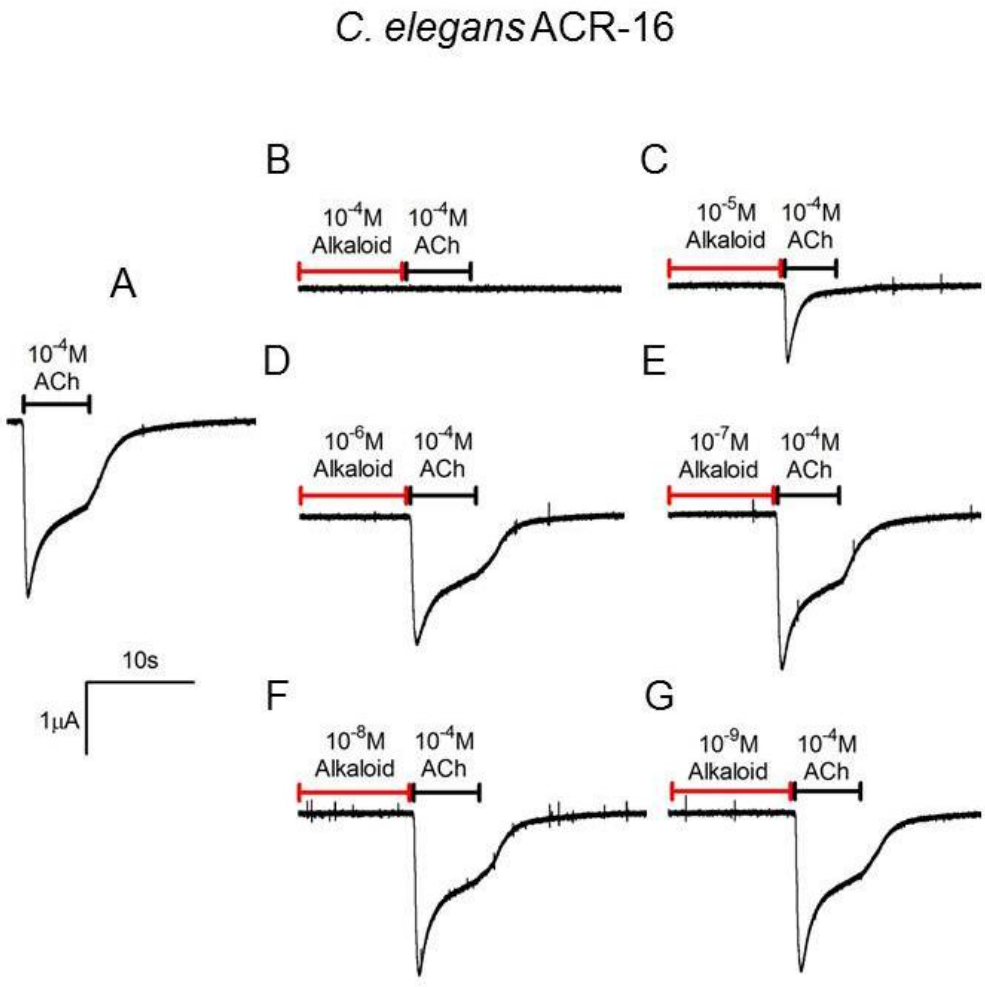


Fig 4.1.1 – The amplitude of the response to 10^{-4}M ACh (black bars) of *C. elegans* ACR-16 nAChRs expressed in *Xenopus* oocytes was measured after concentrations of *H. axyridis* extract from 10^{-9}-10^{-4}M were applied for 10 s prior to the application of Ach (red bars). Each trace represents the change in current once ACh was applied. (A) 10^{-4}M alone. (B) 10^{-4}M extract then 10^{-4}M ACh. (C) 10^{-5}M extract then 10^{-4}M ACh. (D) 10^{-6}M extract then 10^{-4}M ACh. (E) 10^{-7}M extract then 10^{-4}M ACh. (F) 10^{-8}M extract then 10^{-4}M ACh. (G) 10^{-9}M extract then 10^{-4}M ACh.

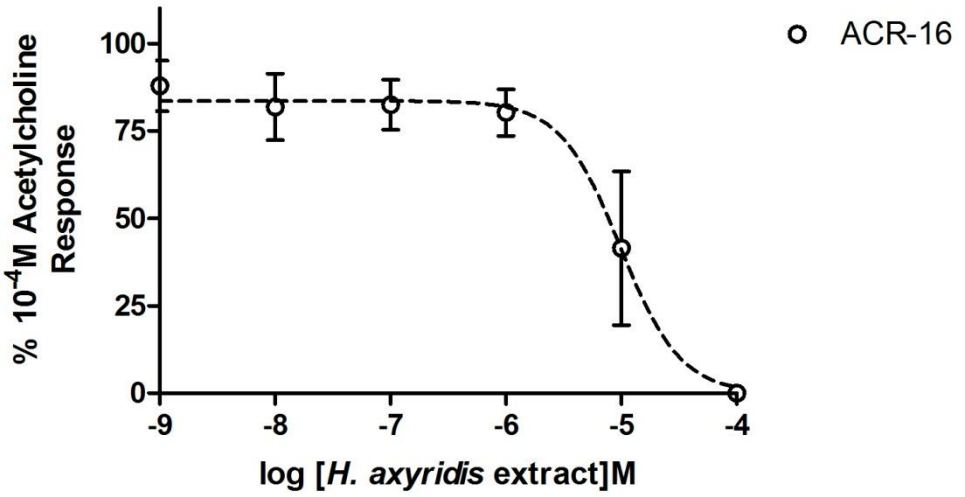


Fig. 4.1.2 - The amplitude of the response to 10^{-4}M ACh of *C. elegans* ACR-16 nAChRs expressed in *Xenopus* oocytes was measured after concentrations of *H. axyridis* extract from 10^{-9}-10^{-4}M were applied for 10 s prior to the application of ACh. Each point represents the mean ±SEM of 3 independent experiments each containing 2-3 replicates expressed as a % of the control value (10^{-4}M ACh). Curves were fitted in GraphPad Prism 5 using the "log(inhibitor) vs. response – variable slope (four parameters)" non-linear regression equation. The IC_{50} value was determined as $9.83x10^{-6}$M.

4.2 Effect on human α7

The human α7 nAChR subunit also forms homopentameric nAChRs and ACR-16 is the *C. elegans* homologue of human α7 (Ballivet et al., 1996). As *H. axyridis* alkaloid extract has been demonstrated to show selectivity for insect nAChRs (Richards, 2011), examining the human α7 nAChR will give indication as to whether selectivity for nematode over human nAChRs exists.

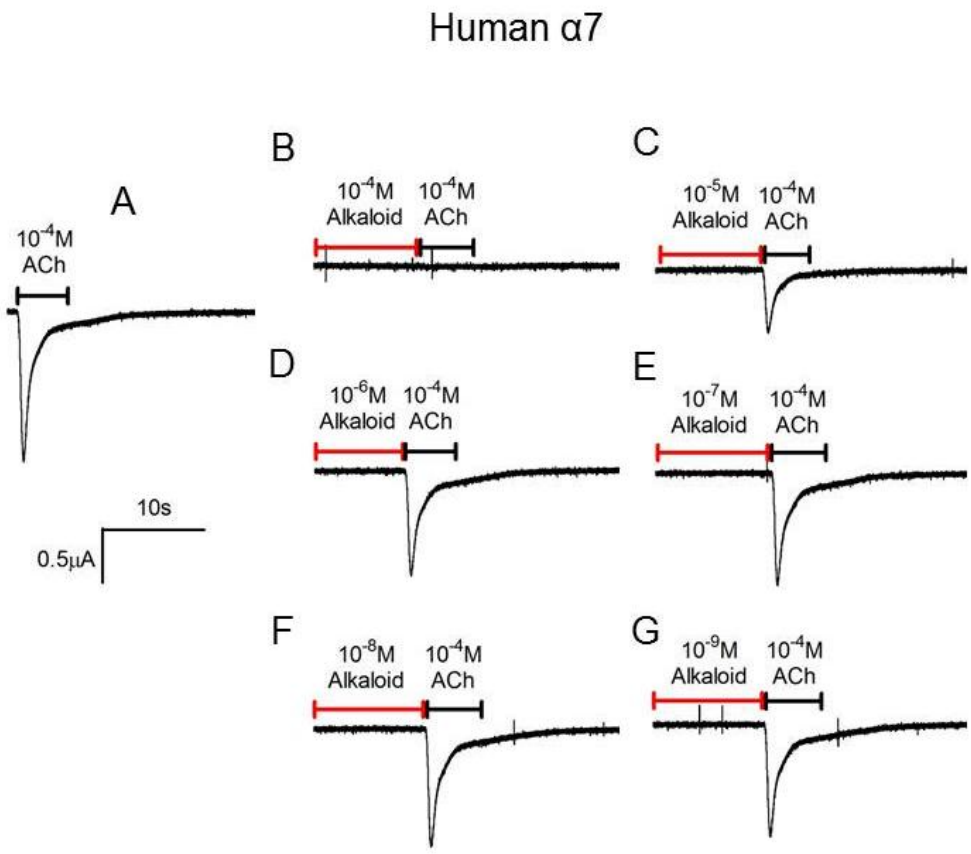


Fig. 4.2.1 - The amplitude of the response to 10^{-4}M ACh (black bars) of human α7 nAChRs expressed in *Xenopus* oocytes was measured after concentrations of *H. axyridis* extract from 10^{-9}-10^{-4}M were applied for 10 s prior to the application of Ach (red bars). Each trace represents the change in current once ACh was applied. (A) 10^{-4}M alone. (B) 10^{-4}M extract then 10^{-4}M ACh. (C) 10^{-5}M extract then 10^{-4}M ACh. (D) 10^{-6}M extract then 10^{-4}M ACh. (E) 10^{-7}M extract then 10^{-4}M ACh. (F) 10^{-8}M extract then 10^{-4}M ACh. (G) 10^{-9}M extract then 10^{-4}M ACh.

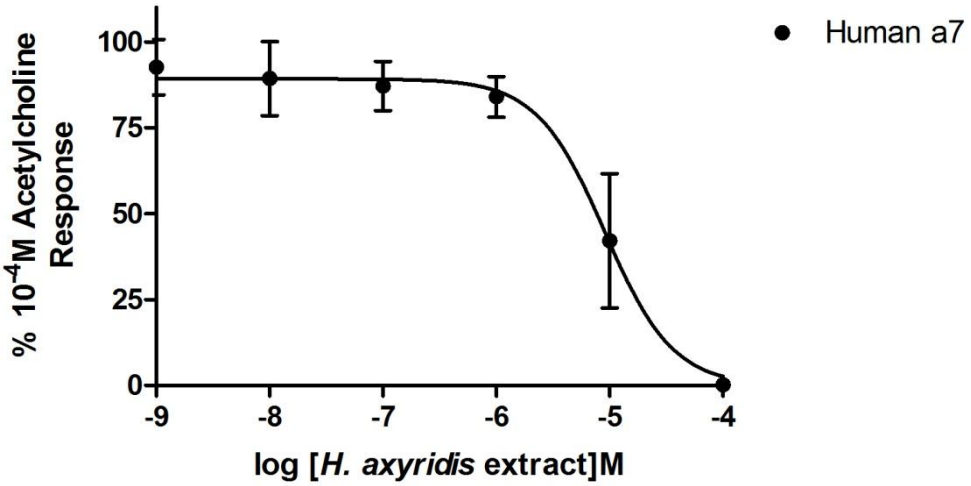


Fig. 4.2.2 - The amplitude of the response to 10^{-4}M ACh of human α7 nAChRs expressed in *Xenopus* oocytes was measured after concentrations of *H. axyridis* extract from 10^{-9}-10^{-4}M were applied for 10 s prior to the application of ACh. Each point represents the mean ±SEM of 3 independent experiments each containing 2-3 replicates expressed as a % of the control value (10^{-4}M ACh). Curves were fitted in GraphPad Prism 5 using the "log(inhibitor) vs. response – variable slope (four parameters)" non-linear regression equation. The IC_{50} value was determined as $9.09x10^{-6}$M.

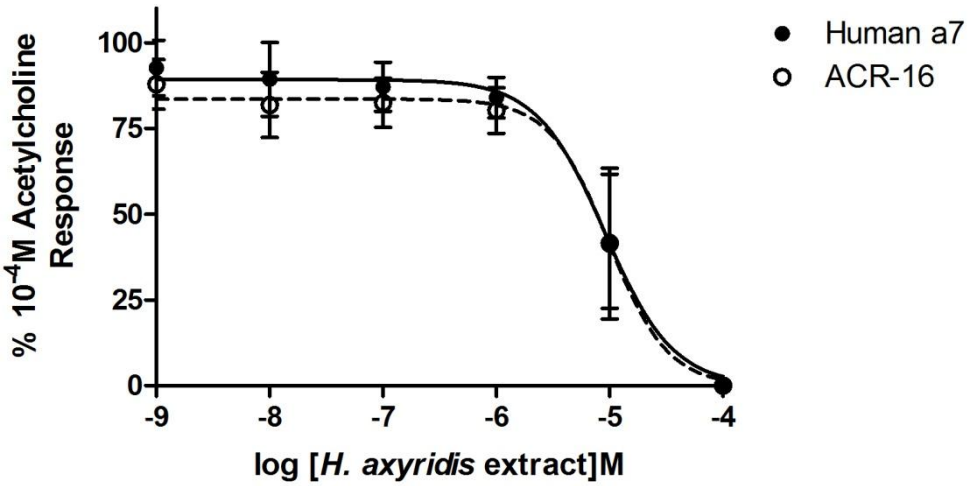


Fig. 4.2.3 – Comparison of the effect of *H. axyridis* alkaloids on the response to 10^{-4}M ACh of human α7 and *C. elegans* ACR-16 nAChRs expressed in *Xenopus* oocytes. Concentrations of *H. axyridis* extract from 10^{-9}-10^{-4}M were applied for 10 s prior to the application of ACh. Each point represents the mean ±SEM of 3 independent experiments each containing 2-3 replicates expressed as a % of the control value (10^{-4}M ACh). Curves were fitted in GraphPad Prism 5 using the "log(inhibitor) vs. response – variable slope (four parameters)" non-linear regression equation. The IC_{50} values were determined as $9.09x10^{-6}$M for human α7 and $9.83x10^{-6}$M for *C. elegans* ACR-16.

The human α7 subunit cRNA was injected into *Xenopus* oocytes and the peak amplitude was measured in response to 10^{-4}M ACh which is the EC_{50} of the receptor to ACh (Peng et al., 1994). Exposure to various concentrations of *H. axyridis* alkaloid extract revealed a concentration dependent inhibition of the ACh response (Fig. 4.2.1 & 4.2.2). Application of extract at a concentration of 10^{-4}M completely inhibited the response to ACh (Fig. 4.2.1B) and partial inhibition occurred at 10^{-5}M (Fig. 4.2.1C). No other concentration showed a noticeable decrease in ACh response (Fig. 4.2.1D-G). An IC_{50} value of $9.09x10^{-6}$M (95% CI = $4.66x10^{-6}$M to $1.78x10^{-5}$M) was determined (Fig. 4.2.2). An F-test comparison of the human α7 and *C. elegans* ACR-16 IC_{50} values revealed no significant difference (P=0.866).

4.3 Effect on *Drosophila* α2/chick β2

The difficulty of expressing pure insect nAChRs in *Xenopus* oocytes limits the ability to test potential compounds for modulating activity. Combination of insect α with mammalian β improves expression (Bertrand et al., 1994) and therefore the hybrid *Drosophila* α2/chicken β2 nAChR provides an ideal platform for the examination of the effects of *H. axyridis* alkaloids on an insect nAChR.

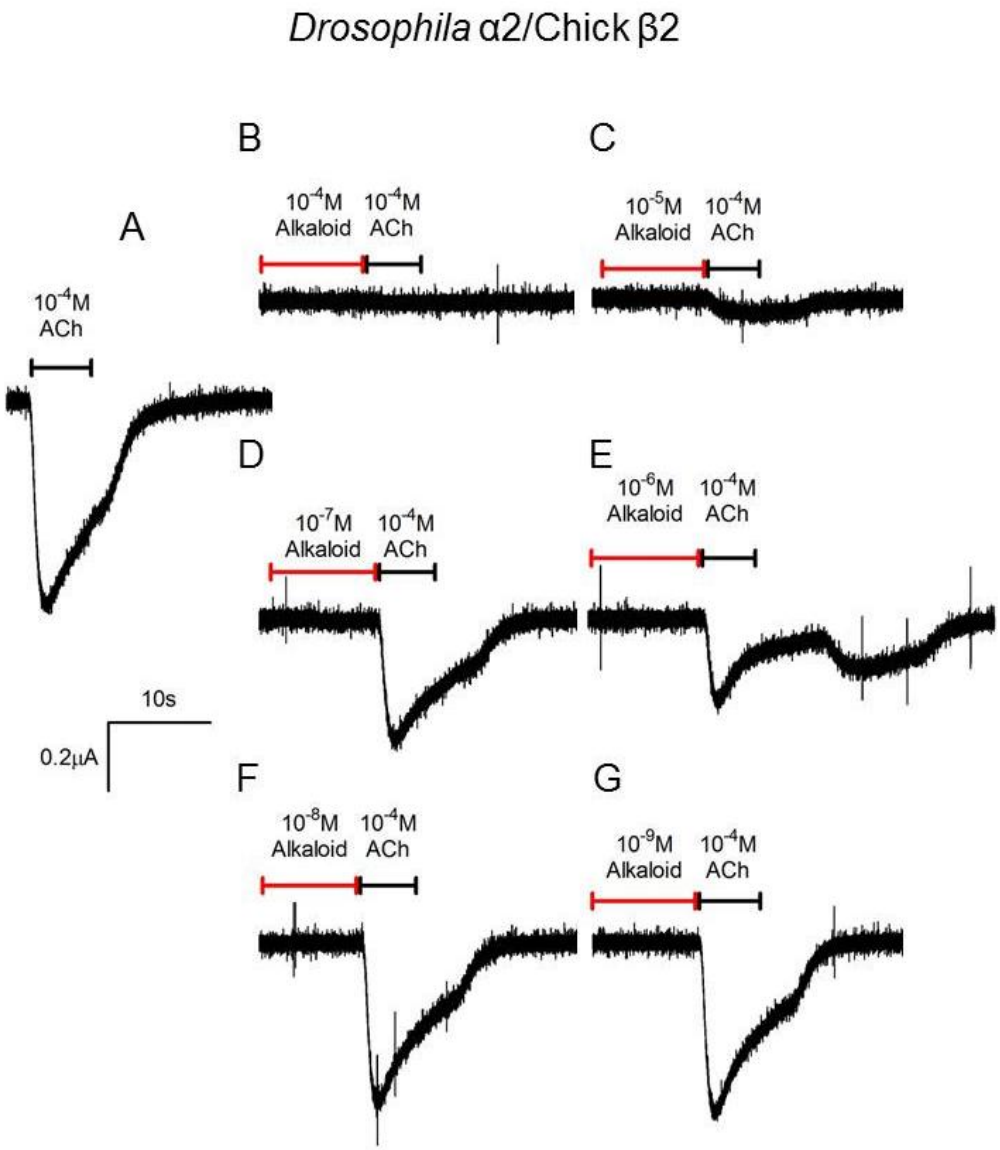


Fig. 4.3.1 - The amplitude of the response to 10^{-4}M ACh (black bars) of hybrid *Drosophila* α2/chick β2 nAChRs expressed in *Xenopus* oocytes was measured after concentrations of *H. axyridis* extract from 10^{-9}-10^{-4}M were applied for 10 s (red bars) prior to the application of ACh. Each trace represents the change in current once ACh was applied. (A) 10^{-4}M alone. (B) 10^{-4}M extract then 10^{-4}M ACh. (C) 10^{-5}M extract then 10^{-4}M ACh. (D) 10^{-7}M extract then 10^{-4}M ACh. (E) 10^{-6}M extract then 10^{-4}M ACh. (F) 10^{-8}M extract then 10^{-4}M ACh. (G) 10^{-9}M extract then 10^{-4}M ACh.

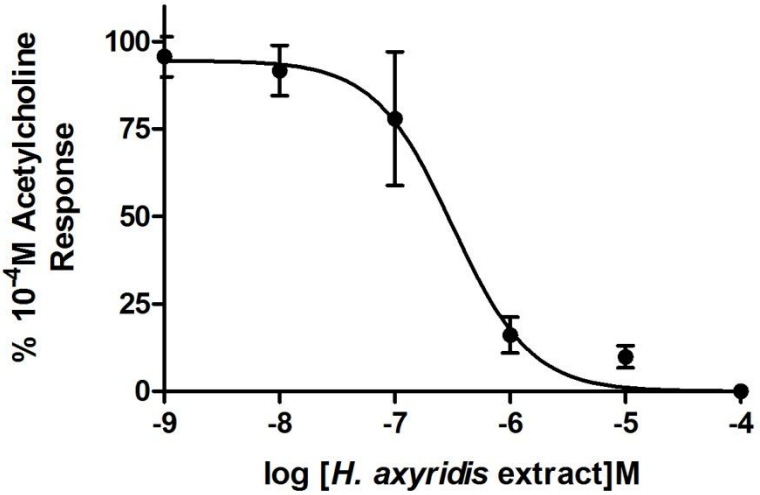


Fig. 4.3.2 - The amplitude of the response to 10^{-4}M ACh of hybrid *Drosophila* α2/chick β2 nAChRs expressed in *Xenopus* oocytes was measured after concentrations of *H. axyridis* extract from 10^{-9}-10^{-4}M were applied for 10 s prior to the application of ACh. Each point represents the mean of 2 independent experimens eacht containing 2-3 replicates expressed as a % of the control value (10^{-4}M ACh). Curves were fitted in GraphPad Prism 5 using the "log(inhibitor) vs. response – variable slope (four parameters)" non-linear regression equation. The IC_{50} value was determined as $3.16x10^{-7}$M.

Oocytes were injected with *Drosophila* α2 and chick β2 cRNA. The peak amplitude was measured in response to 10^{-4}M ACh. Exposure to various concentrations of *H. axyridis* alkaloid extract revealed a concentration dependent inhibition of the ACh response (Fig. 4.3.1 & 4.3.2). Application of extract at a concentration of 10^{-4}M completely inhibited the response to ACh (Fig. 4.3.1B) with partial inhibition occurring at 10^{-5}M (Fig. 4.3.1C), 10^{-6}M (Fig. 4.3.1D) and 10^{-7}M (Fig. 4.3.2). No other concentration showed a noticeable decrease in ACh response (Fig. 4.3.1F-G). An IC_{50} value of $3.16x10^{-7}$M for the *Drosophila* α2/chick β2 nAChR was determined (Fig. 4.3.2).

5. Discussion

5.1 Alkaloids of *H. axyridis* inhibit neuromuscular systems in *C. elegans*

The alkaloids of *H. axyridis* were found to inhibit the pharynx and body wall neuromuscular systems (Fig. 3.1.2 & 3.2.1). These systems are controlled by nAChRs (Richmond and Jorgensen, 1999, McKay et al., 2004) and as these alkaloids are known to target native insect and human nAChRs (Richards, 2011) the nAChR is the prime candidate for the mode of action. The non-α nAChR subunit EAT-2 is found in the pharynx (McKay et al., 2004) but the nAChR subunit composition remains unknown. The nAChRs involved in body wall movement are better characterised. A heteromeric levamisole-sensitive nAChR (Boulin et al., 2008) and a homomeric levamisole-insensitive nAChR (Touroutine et al., 2005) account for the ACh response of *C. elegans* body wall muscle (Richmond and Jorgensen, 1999). This presents a variety of different nAChRs in *C. elegans* that the alkaloids could target.

This was investigated by examining the effects of the alkaloids of the pharynx of different nAChR mutants in *C. elegans* (Table 3.1.1). The DA465 strain contains a mutation within the *eat-2* gene encoding a non-α subunit and the DA1110 strain contains a mutation in the *eat-18* gene encoding a small transmembrane protein that is associated with the EAT-2 protein. Mutations within these genes give a slow pumping phenotype (Raizen et al., 1995, McKay et al., 2004) and so the wild-type gene products are essential for fast rates of pumping. Mutant strains of the levamisole-sensitive (ZZ73) and levamisole-insensitive (RB918) nAChRs were also examined. The rate of pumping was reduced after 1 h compared to a 1% DMSO control in all mutants (Fig. 3.1.7A) but not statistically different from the N2 (wild type) strain (Table 3.1.4). After 24 h the strains DA1110 and RB918 showed a

statistically significant lesser reduction in the rate of pumping compared to N2 (Fig. 3.1.7B). As this would imply that the functioning of the alkaloids is being impaired, each mutated gene is implicated in playing a role in the action of the alkaloids.

The *acr-16* gene encodes a nAChR α subunit known to be expressed in the body wall muscle (Touroutine et al., 2005). Strain RB918 contains a null mutation where a large deletion removing a portion of *acr-16* abolishes the protein function. The lesser effects of the alkaloids on RB918 pharyngeal pumping (Fig. 3.1.7B) indicate that *acr-16* is involved in the action of the alkaloids and that *acr-16* is also expressed in the pharynx. However, there is no difference in the basal rate of pumping in RB918 (Fig. 3.1.4) therefore its expression is not as critical for pharyngeal pumping as *eat-2* and *eat-18*. It is likely that there is a background expression of *acr-16* in the *C. elegans* pharynx which either contributes to the pharyngeal muscle action potential or functions separately from the EAT-2 nAChR. Alkaloids still cause a reduction in the rate of pumping compared to controls in RB918 (Fig. 3.1.6D) so additional sites of action are likely. The small transmembrane protein encoded by *eat-18* is thought to be involved in the binding of ACh (McKay et al., 2004) and so the generation of the pharyngeal muscle action potential is hindered in DA1110. This is confirmed by the reduced basal rate of pumping observed in DA1110 (Fig. 3.1.4). The lesser effect of alkaloids in this strain indicates that EAT-18 is important for the functioning of the nAChR(s) in the pharynx targeted by the alkaloids.

DA465 contains a mutation in *eat*-2 expressed at the synapse of the MC neuron and pharyngeal muscle which is critical for fast pumping as a reduced basal pumping rate is observed in DA465 (Fig. 3.1.4). Alkaloids cause a reduction in the rate of pumping but there was no statistically

significant difference compared to N2 (Fig. 3.1.7). Although EAT-2 associates with EAT-18, EAT-18 is also thought to associate with other nAChRs in the pharynx that are expressed in areas other than the MC synapse (McKay et al., 2004). The lack of a difference in pumping rate compared to N2 means that the alkaloids are likely to affect other pharynx nAChRs that do not contain EAT-2. ZZ73 contains a mutation in *unc-63* that abolishes the levamisole-sensitive nAChR response in the body wall muscle (Richmond and Jorgensen, 1999). The mutation itself does not cause a difference from N2 (Fig. 3.1.4) and the alkaloid effect is also no different to N2 (Fig. 3.1.7). This would mean *unc-63* is either not involved in the action of the alkaloids and/or *unc-63* is not expressed in the pharynx. Further studies on the effect of the alkaloids on the swimming (thrashing) rate of *C. elegans* would allow further analysis of the potential role of *unc-63* and also allow the confirmation of the alkaloids effect through *acr-16*.

5.2 Alkaloids antagonise nAChRs expressed in *Xenopus* oocytes

The alkaloids were tested on recombinant nAChRs expressed in the membranes of *Xenopus* oocytes. Reduction in the current response of *C. elegans* ACR-16 (Fig. 4.1.1), human α7 (Fig. 4.2.1) and *Drosophila* α2/chick β2 (*D*α2/β2) (Fig. 4.3.1) nAChRs to ACh was observed when alkaloids were applied prior to the application of ACh. The ACh response after application of alklaloids did not completely return to the level of response observed without the prior application. This could be due to a slight desensitisation of the receptor or the decline in the quality of the oocyte after being impaled with glass microelectrodes. A 1% DMSO solution does not have an effect on the ACh response of nAChRs (Dr Ian Mellor, Personal Communication) and the reduction in response does not alter when then concentration of DMSO is diluted so this effect is unlikely to be due to the presence of DMSO. The

reduction in current indicates that the alkaloids inhibit the opening of the nAChRs in the oocyte membrane and cause less current to flow through the membrane. Therefore the effects observed in the *C. elegans* pharynx and body wall muscle can at least be partially attributed to the alkaloids actions on ACR-16. This putative pharynx ACR-16 nAChR could also have important associations with EAT-18.

ACR-16 is the *C. elegans* homologue of human α7 sharing 47% identity (Ballivet et al., 1996). Comparison allows selectivity of the alkaloids between mammals and nematodes to be examined and IC_{50} values of $9.09x10^{-6}$M for human α7 and $9.83x10^{-6}$M for ACR-16 were determined (Fig. 4.2.3). An F-test comparison of the IC_{50} values revealed no significant difference (P=0.866) so the alkaloids are equally potent to the human and *C. elegans* nAChRs. Preliminary analysis of the hybrid *D*α2/β2 nAChR gave an IC_{50} of $3.16x10^{-7}$M (Fig. 4.3.2) giving a difference an order of magnitude lower than human α7 and ACR-16. This confirms the mode of action of the alkaloids demonstrated by patch-clamp studies of locust neurons and TE671 muscle cells (Richards, 2011). The nAChRs in TE671 are muscle type (2α, β, γ and δ subunits) (Gerzanich et al., 1995) and therefore antagonistic activity of alkaloids has been demonstrated in invertebrate and vertebrate muscle and neuronal nAChRs. This implies that the alkaloids have activity against a broad spectrum of nAChRs but with selectivity for insect nAChRs.

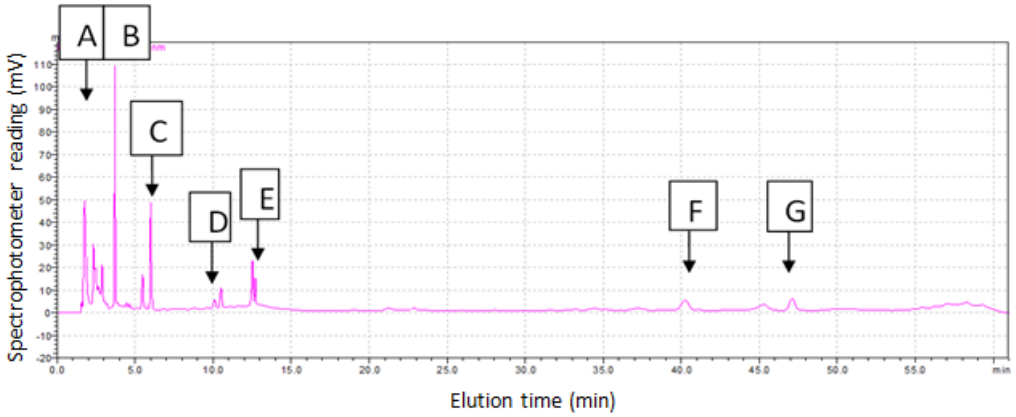


Fig. 5.2.1 – A high performance liquid chromatography profile of *H. axyridis* alkaloid extract after acid-base extraction from collected beetles. Taken from (Richards, 2011).

Interpretation of results should be made with caution as high performance liquid chromatography analysis of the alkaloid extract shows 7 different peaks and so 7 different compounds at varying concentrations exist within the mixture (Fig. 5.2.1) (Richards, 2011). The compound(s) responsible for the antagonistic effects on nAChRs are unknown and it is probable that different compounds have different pharmacological effects. The alkaloid extract is expected to contain a mixture of alkaloids and pyrazines (Sloggett et al., 2011) as well as plant alkaloids sequestered from aphid prey (Hautier et al., 2008). The pyrazines contribute to the foul odour of reflex blood (Sloggett et al., 2011) but also show antimicrobial effects (Kogel et al., 2012). These antimicrobial effects are shared with the *H. axyridis* alkaloid Harmonine (Röhrich et al., 2011) so there is a high probability that multiple components of the extract are active. Therefore the IC_{50} values obtained in the *C. elegans* assays (Fig. 3.1.2) and *Xenopus* oocyte studies (Fig. 4.2.3 & 4.3.2) are not entirely accurate. Separation of the alkaloid extract would allow the active ingredient(s) to be determined and more accurate IC_{50} values to be obtained.

The effect of the alkaloids is similar to that of nereistoxin whose synthetic analogue, Cartap, was used as a commercial insecticide (Sattelle et

al., 1985). Examination of the effects of neriestoxin on recombinant nAChRs in *Xenopus* oocytes gave IC_{50} values of $3.98x10^{-5}$M for chicken α4β2, $3.31x10^{-5}$M for chicken α7 and $1.29x10^{-5}$M for *D*α2/β2 (Raymond Delpech et al., 2003). These are higher values than those obtained from the alkaloid extract (Fig. 4.2.3 & 4.3.2) and the difference between mammalian and insect values show a decreased selectivity compared to the alkaloids. This means there is great potential for the alkaloids use as novel insecticides whose activity may be improved by the creation of synthetic analogues based on the alkaloid structure. This route would also be promising for improving the alkaloids selectivity for nematode nAChRs and so serving as leads for novel anthelmintics.

It is noted that there is voltage-dependency in the alkaloid inhibition of locust neuron and TE671 cell nAChRs which indicates that the effects may be due to open channel block (Dr Ian Mellor, Personal Observation). The philanthotoxin analogue, PhTX-343, shows voltage-dependant inhibition of nAChRs in TE671 cells and the data strongly suggests that this is due to blocking of the open channel (Brier et al., 2003). This inhibition is thought to be non-competitive and amino acid rings in the M2 region lining the channel pore are thought to interact with non-competitive inhibitors (Tikhonov et al., 2004). Modelling of PhTX-343 in the open pore of the nAChR showed binding to Threonine and Serine rings deep in the pore which are also bound by classical non-competitive inhibitors (Tikhonov et al., 2004). Comparison of the M1 and M2 regions of human α7 and other *C. elegans* nAChR subunits showed a conservation of the amino acid rings that form the non-competitive inhibitor binding sites (Fig. 5.2.2). These are conserved in human α7 and ACR-16 so the possibility that one of the alkaloid compounds may act by open channel block exists. Conservation of the amino acid rings in the *C. elegans*

UNC-63, UNC-38, LEV-8 and EAT-2 subunits gives the possibility that nAChRs containing these subunits could be affected in the same way. However, mutations in *eat-2* do not completely negate the effects of the alkaloids on pharyngeal pumping (Fig. 3.1.6A) so multiple nAChRs in the pharynx could be responsible for these effects. It should also be noted that the alkaloids were applied before the application of ACh so a closed channel blocking mechanism or competitive antagonism is also implied.

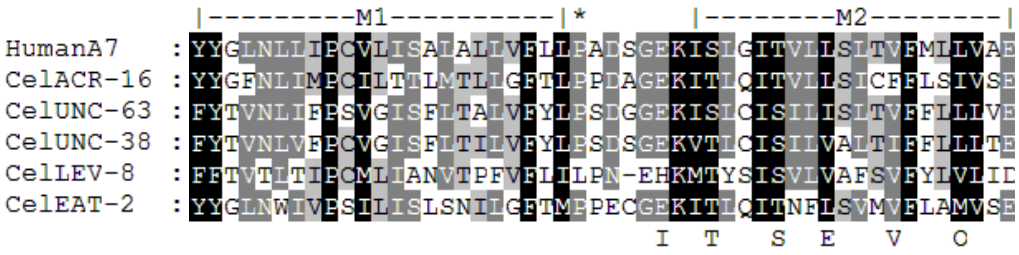


Fig. 5.2.2 – The M1-M2 sequences of human α7 and *C. elegans* ACR-16, UNC-63, UNC-38, LEV-8 and EAT-2 taken from and aligned using the UniProt database (http://www.ebi.ac.uk/uniprot/index.html) are shown. The Intermediate (I), Threonine (T), Serine (S), Equitorial (E), Valine (V) and Outer Leucine (O) rings involved in non-competitive inhibitor binding are labeled at the bottom. Adapted from (Doucette-Stamm et al., 1993; Tikhonov et al., 2004).

5.3 Alkaloids contribute to the chemical defence of ladybird beetles

The alkaloids are now known to act on the nAChR of insects (Fig. 4.3.2) and so can contribute to the chemical defence of *H. axyridis*. The alkaloids of *H. axyridis* are known to be present in the haemolymph (King and Meinwald, 1996) and possibly coating eggs (Hemptinne et al., 2000). Toxic effects are observed when eggs are consumed by other organisms (Phoofolo and Obrycki, 1998; Yasuda and Ohnuma, 1999; Cottrell, 2004; Santi and Maini, 2006; Kajita et al., 2010) and this can be explained by the neurotoxic effects of the alkaloids. As alkaloids are effective against mammalian nAChRs (Fig. 4.2.2) the alkaloids can possibly explain the toxicity observed when

beetles are consumed by bird predators (Marples et al., 1989). The bright elytra colouring of *H. axyridis* (Bezzerides et al., 2007) can therefore be confirmed as an aposematic warning of the chemical defence contained within the beetle.

Selectivity for the insect nAChR could be due to the natural predators and competitors of *H. axyridis*. Competition with other ladybird species of the same resources involves IGP (Polis et al., 1989). Eggs of competitor species are consumed in an effort to reduce the population of competitors and become the dominant species in an area. *H. axyridis* in particular has been extremely successful when invading areas in the US (Michaud, 2002; Cottrell and Yeargan, 1998; LaMana and Miller, 1996) and UK (Ware and Majerus, 2008). A combination of increased aggressiveness to other ladybird species (Michaud, 2002, Nedved et al., 2010) and a better chemical defence of eggs (Yasuda and Ohnuma, 1999, Kajita et al., 2010) help *H. axyridis* become an effective intraguild predator. Competition with insect predators over a long period of time puts a large selection pressure on the species involved. Development of a chemical defence effective against other species of insects would give *H. axyridis* a competitive advantage and explain why the alkaloids appear to be more selective for insect nAChRs. The action of the alkaloids on nAChRs shows similarities with toxins from other insect species such as PhTX (Brier et al., 2003) and toxins from wasps and ants (Zalat et al., 2005). The use of the same mode of action indicates that having a chemical defence targeting the nAChR is an effective form of protection.

Alkaloids can be toxic to other organisms but have no effects on the beetle itself. This could be due to nerve sheaths in insects that shield neurons (Hill and Usherwood, 1961). Differences in the amino acid sequences of nAChRs and other possible targets could also account for the resistance. This

is the case in puffer fish where tetrodotoxin is accumulated with no effect because of differences in the sodium ion channel target site (Venkatesh et al., 2005).

5.4 Future Work

The effect of the alkaloids on the nAChRs on the body wall muscle of *C. elegans* by examination of swimming (thrashing) behaviour in ZZ73 and RB918 will shed light on whether the alkaloids act on ACR-16 alone. This can also be examined and confirmed by the expression of the levamisole-sensitive nAChR in *Xenopus* oocytes. Analysing the effects of one particular concentration of alkaloid on different concentrations of ACh would show whether the antagonism is competitive or non-competitive.

The alkaloid extract contains multiple compounds (Fig. 5.2.1) and they need to be separated and assessed for activity. Once the active compounds are identified, different receptors from mammals, nematodes and insects need to be compared in order to find out whether any selectivity exists. Bioassays on pest insects and nematodes themselves would be needed to demonstrate the alkaloids effects on the target pests directly.

Thousands of other ladybird species exist containing a wealth of different alkaloids and their examination could lead to the identification of more potential leads for novel pesticides.

5.5 Conclusions

The alkaloid extract of *H. axyridis* was found to inhibit the functioning of the *C. elegans* pharynx and body wall neuromuscular systems. Examination of different strains containing mutations in nAChR subunits implicated the ACR-16 nAChR as a target for the alkaloids and that ACR-16 could be

expressed in the pharynx. The ACR-16 containing nAChR would fulfil a different role to the EAT-2 nAChR which controls rapid pharyngeal pumping. The action on ACR-16 was confirmed when alkaloids antagonised the response to ACh of recombinant ACR-16 nAChRs expressed in *Xenopus* oocytes. Human α7 and *D*α2/β2 nAChRs were also antagonised indicating a broad spectrum of activity. Comparison of IC_{50} values of the alkaloids on recombinant human α7, ACR-16 and *D*α2/β2 showed a greater selectivity for the insect nAChR and so there is great potential for the alkaloids to serve as leads for novel insecticides.

5. References

AL ABASSI, S., BIRKETT, M. A., PETTERSSON, J., PICKETT, J. A. & WOODCOCK, C. M. 1998. Ladybird beetle odour identified and found to be responsible for attraction between adults. *Cellular and Molecular Life Sciences,* 54**,** 876-879.

ALAM, N., CHOI, I. S., SONG, K. S., HONG, J. K., LEE, C. O. & JUNG, J. H. 2002. A new alkaloid from two coccinellid beetles Harmonia axyridis and Ailocaria hexaspilota. *Bulletin of the Korean Chemical Society,* 23**,** 497-499.

AMAR, M., THOMAS, P., WONNACOTT, S. & LUNT, G. G. 1995. A nicotinic acetylcholine receptor subunit from insect brain forms a non-desensitising homo-oligomeric nicotinic acetylcholine receptor when expressed in Xenopus oocytes. *Neurosci Lett,* 199**,** 107-10.

ARENA, J. P., LIU, K. K., PARESS, P. S., SCHAEFFER, J. M. & CULLY, D. F. 1992. Expression of a glutamate-activated chloride current in Xenopus oocytes injected with Caenorhabditis elegans RNA: evidence for modulation by avermectin. *Brain Res Mol Brain Res,* 15**,** 339-48.

ATTYGALLE, A. B., BLANKESPOOR, C. L., EISNER, T. & MEINWALD, J. 1994. Biosynthesis of a Defensive Insect Alkaloid - Epilachnene from Oleic-Acid and Serine. *Proceedings of the National Academy of Sciences of the United States of America,* 91**,** 12790-12793.

ATTYGALLE, A. B., MCCORMICK, K. D., BLANKESPOOR, C. L., EISNER, T. & MEINWALD, J. 1993. Azamacrolides: A family of alkaloids from the pupal defensive secretion of a ladybird beetle (Epilachna varivestis). *Proceedings of the National Academy of Sciences of the United States of America,* 90**,** 5204-5208.

AVERY, L. & HORVITZ, H. R. 1989. Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of C. elegans. *Neuron,* 3**,** 473-85.

AVERY, L. & HORVITZ, H. R. 1990. Effects of starvation and neuroactive drugs on feeding in Caenorhabditis elegans. *J Exp Zool,* 253**,** 263-70.

BAI, D., LUMMIS, S., LEICHT, W., BREER, H. & SATTELLE, D. 1991. Actions of imidacloprid and a related nitromethylene on cholinergic receptors of an identified insect motor neurone. *Pesticide Science,* 33**,** 197-204.

BALLIVET, M., ALLIOD, C., BERTRAND, S. & BERTRAND, D. 1996. Nicotinic acetylcholine receptors in the nematode Caenorhabditis elegans. *J Mol Biol,* 258**,** 261-9.

BARNARD, E. A., MILEDI, R. & SUMIKAWA, K. 1982. Translation of exogenous messenger RNA coding for nicotinic acetylcholine receptors produces functional receptors in Xenopus oocytes. *Proc R Soc Lond B Biol Sci,* 215**,** 241-6.

BARTLEY, D. J., JACKSON, F., JACKSON, E. & SARGISON, N. 2004. Characterisation of two triple resistant field isolates of Teladorsagia from Scottish lowland sheep farms. *Veterinary Parasitology,* 123**,** 189-99.

BAUGH, L. R. & STERNBERG, P. W. 2006. DAF-16/FOXO regulates transcription of cki-1/Cip/Kip and repression of lin-4 during C. elegans L1 arrest. *Current Biology,* 16**,** 780-785.

BAUMGARTNER, W., ISLAS, L. & SIGWORTH, F. J. 1999. Two-microelectrode voltage clamp of Xenopus oocytes: voltage errors and compensation for local current flow. *Biophys J,* 77**,** 1980-91.

BENNETT, H. M., WILLIAMSON, S. M., WALSH, T. K., WOODS, D. J. & WOLSTENHOLME, A. J. 2012. ACR-26: a novel nicotinic receptor subunit of parasitic nematodes. *Mol Biochem Parasitol,* 183**,** 151-7.

BERTRAND, D., BALLIVET, M., GOMEZ, M., BERTRAND, S., PHANNAVONG, B. & GUNDELFINGER, E. D. 1994. Physiological properties of neuronal nicotinic receptors reconstituted from the vertebrate beta 2 subunit and Drosophila alpha subunits. *Eur J Neurosci,* 6**,** 869-75.

BETHONY, J., BROOKER, S., ALBONICO, M., GEIGER, S. M., LOUKAS, A., DIEMERT, D. & HOTEZ, P. J. 2006. Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *Lancet,* 367**,** 1521-32.

BEZZERIDES, A. L., MCGRAW, K. J., PARKER, R. S. & HUSSEINI, J. 2007. Elytra color as a signal of chemical defense in the Asian ladybird beetle Harmonia axyridis. *Behavioral Ecology and Sociobiology,* 61**,** 1401-1408.

BIANCHI, L. & DRISCOLL, M. 2006. Heterologous expression of C. elegans ion channels in Xenopus oocytes. *WormBook***,** 1-16.

BOULIN, T., FAUVIN, A., CHARVET, C. L., CORTET, J., CABARET, J., BESSEREAU, J. L. & NEVEU, C. 2011. Functional reconstitution of Haemonchus contortus acetylcholine receptors in Xenopus oocytes provides mechanistic insights into levamisole resistance. *Br J Pharmacol,* 164**,** 1421-32.

BOULIN, T., GIELEN, M., RICHMOND, J. E., WILLIAMS, D. C., PAOLETTI, P. & BESSEREAU, J. L. 2008. Eight genes are required for functional reconstitution of the Caenorhabditis elegans levamisole-sensitive acetylcholine receptor. *Proc Natl Acad Sci U S A,* 105**,** 18590-5.

BOULTER, J., CONNOLLY, J., DENERIS, E., GOLDMAN, D., HEINEMANN, S. & PATRICK, J. 1987. Functional expression of two neuronal nicotinic acetylcholine receptors from cDNA clones identifies a gene family. *Proc Natl Acad Sci U S A,* 84**,** 7763-7.

BOULTER, J., EVANS, K., MARTIN, G., MASON, P., STENGELIN, S., GOLDMAN, D., HEINEMANN, S. & PATRICK, J. 1986. Isolation and sequence of cDNA clones coding for the precursor to the gamma subunit of mouse muscle nicotinic acetylcholine receptor. *J Neurosci Res,* 16**,** 37-49.

BREER, H. & SATTELLE, D. B. 1987. Molecular properties and functions of insect acetylcholine receptors - a review. *J Insect Physiol,* 33**,** 771-790.

BREJC, K., VAN DIJK, W. J., KLAASSEN, R. V., SCHUURMANS, M., VAN DER OOST, J., SMIT, A. B. & SIXMA, T. K. 2001. Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature,* 411**,** 269-76.

BRENGUES, C., HAWKES, N. J., CHANDRE, F., MCCARROLL, L., DUCHON, S., GUILLET, P., MANGUIN, S., MORGAN, J. C. & HEMINGWAY, J. 2003. Pyrethroid and DDT cross-resistance in Aedes aegypti is correlated with novel mutations in the voltage-gated sodium channel gene. *Med Vet Entomol,* 17**,** 87-94.

BRENNER, S. 1974. The genetics of Caenorhabditis elegans. *Genetics,* 77**,** 71-94.

BRIER, T. J., MELLOR, I. R., TIKHONOV, D. B., NEAGOE, I., SHAO, Z., BRIERLEY, M. J., STROMGAARD, K., JAROSZEWSKI, J. W., KROGSGAARD-LARSEN, P. & USHERWOOD, P. N. 2003. Contrasting actions of philanthotoxin-343 and philanthotoxin-(12) on human muscle nicotinic acetylcholine receptors. *Molecular Pharmacology,* 64**,** 954-64.

BROWN, L. A., IHARA, M., BUCKINGHAM, S. D., MATSUDA, K. & SATTELLE, D. B. 2006. Neonicotinoid insecticides display partial and super agonist actions on native insect nicotinic acetylcholine receptors. *J Neurochem,* 99**,** 608-15.

BURSZTAJN, S. & GERSHON, M. D. 1977. Discrimination between nicotinic receptors in vertebrate ganglia and skeletal muscle by alpha-bungarotoxin and cobra venoms. *J Physiol,* 269**,** 17-31.

CAHILL, M., BYRNE, F., GORMAN, K., DENHOLM, I. & DEVONSHIRE, A. 1995. Pyrethroid and organophosphate resistance in the tobacco whitefly Bemisia tabaci (Homoptera: Aleyrodidae). *Bulletin of Entomological Research,* 85**,** 181-187.

CAMARANO, S., GONZALEZ, A. & ROSSINI, C. 2006. Chemical defense of the ladybird beetle Epilachna paenulata. *Chemoecology,* 16**,** 179-184.

CAMARANO, S., GONZALEZ, A. & ROSSINI, C. 2009. Biparental Endowment of Endogenous Defensive Alkaloids in Epilachna paenulata. *Journal of Chemical Ecology,* 35**,** 1-7.

CAMPBELL, W. C. 1985. Ivermectin: an update. *Parasitol Today,* 1**,** 10-6.

CASIDA, J. E. & QUISTAD, G. B. 1998. Golden age of insecticide research: past, present, or future? *Annual Review of Entomology,* 43**,** 1-16.

CELIE, P. H., VAN ROSSUM-FIKKERT, S. E., VAN DIJK, W. J., BREJC, K., SMIT, A. B. & SIXMA, T. K. 2004. Nicotine and carbamylcholine binding to nicotinic acetylcholine receptors as studied in AChBP crystal structures. *Neuron,* 41**,** 907-14.

CHAN, M. S. 1997. The global burden of intestinal nematode infections--fifty years on. *Parasitol Today,* 13**,** 438-43.

CHANGEUX, J. P., DEVILLERS-THIERY, A. & CHEMOUILLI, P. 1984. Acetylcholine receptor: an allosteric protein. *Science,* 225**,** 1335-45.

CHANGEUX, J. P. & EDELSTEIN, S. J. 1998. Allosteric receptors after 30 years. *Neuron,* 21**,** 959-80.

CHANGEUX, J. P., KASAI, M. & LEE, C. Y. 1970. Use of a snake venom toxin to characterize the cholinergic receptor protein. *Proc Natl Acad Sci U S A,* 67**,** 1241-7.

CHAPIN, J. B. & BROU, V. A. 1991. Harmonia-Axyridis (Pallas), the 3rd Species of the Genus to Be Found in the United-States (Coleoptera, Coccinellidae). *Proceedings of the Entomological Society of Washington,* 93**,** 630-635.

CLARKSON, C., MELLOR, I. R., LAMBERT, M. & JAROSZEWSKI, J. W. 2006. 5alpha-cardenolides from Kanahia laniflora inhibit ionotropic acetylcholine receptors. *Planta Med,* 72**,** 1418-20.

CLAUDIO, T., BALLIVET, M., PATRICK, J. & HEINEMANN, S. 1983. Nucleotide and deduced amino acid sequences of Torpedo californica acetylcholine receptor gamma subunit. *Proc Natl Acad Sci U S A,* 80**,** 1111-5.

CONSORTIUM, C. E. S. 1998. Genome sequence of the nematode C. elegans: a platform for investigating biology. *Science,* 282**,** 2012-8.

COOPER, E., COUTURIER, S. & BALLIVET, M. 1991. Pentameric structure and subunit stoichiometry of a neuronal nicotinic acetylcholine receptor. *Nature,* 350**,** 235-8.

COTTRELL, T. E. 2004. Suitability of exotic and native lady beetle eggs (Coleoptera : Coccinellidae) for development of lady beetle larvae. *Biological Control,* 31**,** 362-371.

COTTRELL, T. E. & YEARGAN, K. V. 1998. Intraguild predation between an introduced lady beetle, Harmonia axyridis (Coleoptera : Coccinellidae), and a native lady beetle, Coleomegilla maculata (Coleoptera : Coccinellidae). *Journal of the Kansas Entomological Society,* 71**,** 159-163.

CULETTO, E., BAYLIS, H. A., RICHMOND, J. E., JONES, A. K., FLEMING, J. T., SQUIRE, M. D., LEWIS, J. A. & SATTELLE, D. B. 2004. The Caenorhabditis elegans unc-63 gene encodes a levamisole-sensitive nicotinic acetylcholine receptor alpha subunit. *J Biol Chem,* 279**,** 42476-83.

DALE, R. P., JONES, A. K., TAMBORINDEGUY, C., DAVIES, T. G., AMEY, J. S., WILLIAMSON, S., WOLSTENHOLME, A., FIELD, L. M., WILLIAMSON, M. S., WALSH, T. K. & SATTELLE, D. B. 2010. Identification of ion channel genes in the Acyrthosiphon pisum genome. *Insect Mol Biol,* 19 Suppl 2**,** 141-53.

DE JONG, P. W., HOLLOWAY, G. J., BRAKEFIELD, P. M. & DE VOS, H. 1991. Chemical defence in ladybird beetles (Coccinellidae). II. Amount of reflex fluid, the alkaloid adaline and individual variation in 2-spot ladybirds (Adalia bipunctata). *Chemoecology,* 2**,** 15-19.

DEVILLERS-THIERY, A., GIRAUDAT, J., BENTABOULET, M. & CHANGEUX, J. P. 1983. Complete mRNA coding sequence of the acetylcholine binding alpha-subunit of Torpedo marmorata acetylcholine receptor: a model for the transmembrane organization of the polypeptide chain. *Proc Natl Acad Sci U S A,* 80**,** 2067-71.

DOLENSKA, M., NEDVED, O., VESELY, P., TESAROVA, M. & FUCHS, R. 2009. What constitutes optical warning signals of ladybirds (Coleoptera: Coccinellidae) towards bird predators: colour, pattern or general look? *Biological Journal of the Linnean Society,* 98**,** 234-242.

DOUCETTE-STAMM, L., MONTEGGIA, L. M., DONNELLY-ROBERTS, D., WANG, M. T., LEE, J., TIAN, J. & GIORDANO, T. 1993. Cloning and sequence of the human α7 nicotinic acetylcholine receptor. *Drug Development Research,* 30**,** 252-256.

EISNER, T. & MEINWALD, J. 1966. Defensive Secretions of Arthropods. *Science,* 153**,** 1341-&.

ELLIOTT, M. 1976. Properties and applications of pyrethroids. *Environ Health Perspect,* 14**,** 1-13.

EVANS, T. & CHAPPLE, N. 2002. The animal health market. *Nat Rev Drug Discov,* 1**,** 937-8.

FARMER, J. J., ATTYGALLE, A. B., SMEDLEY, S. R., EISNER, T. & MEINWALD, J. 1997. Defensive mechanisms of arthropods .144.

Absolute configuration of insect-produced epilachnene. *Tetrahedron Letters,* 38**,** 2787-2790.

FINLAYSON, C., ALYOKHIN, A., GROSS, S. & PORTER, E. 2010. Differential consumption of four aphid species by four lady beetle species. *Journal of Insect Science,* 10.

FINLAYSON, C. J., ALYOKHIN, A. V. & PORTER, E. W. 2009. Interactions of Native and Non-Native Lady Beetle Species (Coleoptera: Coccinellidae) With Aphid-Tending Ants in Laboratory Arenas. *Environmental Entomology,* 38**,** 846-855.

FLEMING, J. T., SQUIRE, M. D., BARNES, T. M., TORNOE, C., MATSUDA, K., AHNN, J., FIRE, A., SULSTON, J. E., BARNARD, E. A., SATTELLE, D. B. & LEWIS, J. A. 1997. Caenorhabditis elegans levamisole resistance genes lev-1, unc-29, and unc-38 encode functional nicotinic acetylcholine receptor subunits. *J Neurosci,* 17**,** 5843-57.

FRANKS, C. J., PEMBERTON, D., VINOGRADOVA, I., COOK, A., WALKER, R. J. & HOLDEN-DYE, L. 2002. Ionic basis of the resting membrane potential and action potential in the pharyngeal muscle of Caenorhabditis elegans. *Journal of Neurophysiology,* 87**,** 954-961.

GERZANICH, V., PENG, X., WANG, F., WELLS, G., ANAND, R., FLETCHER, S. & LINDSTROM, J. 1995. Comparative pharmacology of epibatidine: a potent agonist for neuronal nicotinic acetylcholine receptors. *Molecular Pharmacology,* 48**,** 774-82.

GIROLAMI, V., MAZZON, L., SQUARTINI, A., MORI, N., MARZARO, M., DI BERNARDO, A., GREATTI, M., GIORIO, C. & TAPPARO, A. 2009. Translocation of neonicotinoid insecticides from coated seeds to seedling guttation drops: a novel way of intoxication for bees. *Journal of Economic Entomology,* 102**,** 1808-15.

GLENDINNING, S. K., BUCKINGHAM, S. D., SATTELLE, D. B., WONNACOTT, S. & WOLSTENHOLME, A. J. 2011. Glutamate-gated chloride channels of Haemonchus contortus restore drug sensitivity to ivermectin resistant Caenorhabditis elegans. *PLoS One,* 6**,** e22390.

GORDON, R. D. 1985. The Coccinellidae (Coleoptera) of America north of Mexico. *Journal of the New York Entomological Society,* 93**,** 1-912.

GRILL, C. P. & MOORE, A. J. 1998. Effects of a larval antipredator response and larval diet on adult phenotype in an aposematic ladybird beetle. *Oecologia,* 114**,** 274-282.

GUEDES, R., LIMA, J., SANTOS, J. & CRUZ, C. 1995. Resistance to DDT and pyrethroids in Brazilian populations of Sitophilus zeamais Motsch. (Coleoptera: Curculionidae). *Journal of Stored Products Research,* 31**,** 145-150.

GUNNING, R., EASTON, C., GREENUP, L. & VE., E. 1984. Pyrethroid resistance in Heliothis armiger (Hubner) (Lepidoptera: Noctuidae) in Australia. *Journal of Economic Entomology,* 77**,** 1283-1287(5).

GURDON, J. B., LANE, C. D., WOODLAND, H. R. & MARBAIX, G. 1971. Use of frog eggs and oocytes for the study of messenger RNA and its translation in living cells. *Nature,* 233**,** 177-82.

HAGEN, K. S. 1962. Biology and Ecology of Predaceous Coccinellidae. *Annual Review of Entomology,* 7**,** 289-&.

HALLIWELL, J. V., PLANT, T. D. & STANDEN, N. B. 1987. Voltage clamp technique. *Microelectrode technique.* 2nd ed. Cambridge: The Company of Biologists Limited.

HAPP, G. M. & EISNER, T. 1961. Hemorrhage in a Coccinellid Beetle and Its Repellent Effect on Ants. *Science,* 134**,** 329-&.

HARROW, I. D. & GRATION, K. A. F. 1985. Mode of Action of the Anthelmintics Morantel, Pyrantel and Levamisole on Muscle-Cell Membrane of the Nematode Ascaris-Suum. *Pesticide Science,* 16**,** 662-672.

HAULOTTE, E., LAURENT, P. & BRAEKMAN, J. C. 2012. Biosynthesis of defensive coccinellidae alkaloids: incorporation of fatty acids in adaline, coccinelline and harmonine. *European Journal of Organic Chemistry,* 2012**,** 1907-1912.

HAUTIER, L., GREGOIRE, J.-C., DE SCHAUWERS, J., MARTIN, G. S., CALLIER, P., JANSEN, J.-P. & DE BISEAU, J.-C. 2008. Intraguild predation by Harmonia axyridis on coccinellids revealed by exogenous alkaloid sequestration. *Chemoecology,* 18**,** 191-196.

HAUTIER, L., SAN MARTIN Y GOMEZ, G., CALLIER, P., DE BISEAU, J.-C. & GREGOIRE, J.-C. 2011. Alkaloids provide evidence of intraguild predation on native coccinellids by Harmonia axyridis in the field. *Biological Invasions,* 13**,** 1805-1814.

HEMPTINNE, J. L., LOGNAY, G., GAUTHIER, C. & DIXON, A. F. G. 2000. Role of surface chemical signals in egg cannibalism and intraguild predation in ladybirds (Coleoptera : Coccinellidae). *Chemoecology,* 10**,** 123-128.

HENRY, M., BEGUIN, M., REQUIER, F., ROLLIN, O., ODOUX, J. F., AUPINEL, P., APTEL, J., TCHAMITCHIAN, S. & DECOURTYE, A. 2012. A common pesticide decreases foraging success and survival in honey bees. *Science,* 336**,** 348-50.

HILL, R. B. & USHERWOOD, P. N. 1961. The action of 5-hydroxytryptamine and related compounds on neuromuscular transmission in the locust Schistocerca gregaria. *J Physiol,* 157**,** 393-401.

HIRONORI, Y. & KATSUHIRO, S. 1997. Cannibalism and interspecific predation in two predatory ladybirds in relation to prey abundance in the field. *Entomophaga,* 42**,** 153-163.

HODGKIN, A. L. & HUXLEY, A. F. 1952. Propagation of electrical signals along giant nerve fibers. *Proc R Soc Lond B Biol Sci,* 140**,** 177-83.

HOLDEN-DYE, L. & WALKER, R. J. 2007. Anthelmintic drugs. *WormBook*, 1-13.

HOLLOWAY, G. J., DE JONG, P. W. & BRAKEFIELD, P. M. D. V., H. 1991. Chemical defence in ladybird beetles (Coccinellidae) I. Distribution of coccinelline and individual variation in defence in 7-spot ladybirds (Coccinella septempunctata). *Chemoecology,* 2, 7-14.

HOTEZ, P. J., BUNDY, D. A. P., BEEGLE, K., BROOKER, S., DRAKE, L., DE SILVA, N., MONTRESOR, A., ENGELS, D., JUKES, M., CHITSULO, L., CHOW, J., LAXMINARAYAN, R., MICHAUD, C., BETHONY, J., CORREA-OLIVEIRA, R., SHUHUA, X., FENWICK, A. & SAVIOLI, L. 2006. Helminth Infections: Soil-transmitted Helminth Infections and Schistosomiasis. *In:* JAMISON, D. T., BREMAN, J. G., MEASHAM, A. R., ALLEYNE, G., CLAESON, M., EVANS, D. B., JHA, P., MILLS, A. & MUSGROVE, P. (eds.) *Disease Control Priorities in Developing Countries.* 2nd ed. Washington (DC).

HU, Y., XIAO, S. H. & AROIAN, R. V. 2009. The New Anthelmintic Tribendimidine is an L-type (Levamisole and Pyrantel) Nicotinic Acetylcholine Receptor Agonist. *Plos Neglected Tropical Diseases,* 3.

IHARA, M., MATSUDA, K., OTAKE, M., KUWAMURA, M., SHIMOMURA, M., KOMAI, K., AKAMATSU, M., RAYMOND, V. & SATTELLE, D. B. 2003. Diverse actions of neonicotinoids on chicken alpha 7, alpha 4 beta 2 and Drosophila-chicken SAD beta 2 and ALS beta 2 hybrid nicotinic acetylcholine receptors expressed in Xenopus laevis oocytes. *Neuropharmacology,* 45, 133-144.

JESCHKE, P. & NAUEN, R. 2008. Neonicotinoids-from zero to hero in insecticide chemistry. *Pest Management Science,* 64, 1084-98.

JONES, A. K., BROWN, L. A. & SATTELLE, D. B. 2007. Insect nicotinic acetylcholine receptor gene families: from genetic model organism to vector, pest and beneficial species. *Invert Neurosci,* 7, 67-73.

JONES, A. K., GRAUSO, M. & SATTELLE, D. B. 2005. The nicotinic acetylcholine receptor gene family of the malaria mosquito, Anopheles gambiae. *Genomics,* 85, 176-87.

JONES, A. K., RAYMOND-DELPECH, V., THANY, S. H., GAUTHIER, M. & SATTELLE, D. B. 2006. The nicotinic acetylcholine receptor gene family of the honey bee, Apis mellifera. *Genome Res,* 16, 1422-30.

JONES, A. K. & SATTELLE, D. B. 2004. Functional genomics of the nicotinic acetylcholine receptor gene family of the nematode, Caenorhabditis elegans. *Bioessays,* 26, 39-49.

KAJITA, Y., OBRYCKI, J. J., SLOGGETT, J. J. & HAYNES, K. F. 2010. Intraspecific alkaloid variation in ladybird eggs and its effects on con- and hetero-specific intraguild predators. *Oecologia (Berlin),* 163, 313-322.

KAMINSKY, R., DUCRAY, P., JUNG, M., CLOVER, R., RUFENER, L., BOUVIER, J., WEBER, S. S., WENGER, A., WIELAND-

BERGHAUSEN, S., GOEBEL, T., GAUVRY, N., PAUTRAT, F., SKRIPSKY, T., FROELICH, O., KOMOIN-OKA, C., WESTLUND, B., SLUDER, A. & MASER, P. 2008. A new class of anthelmintics effective against drug-resistant nematodes. *Nature,* 452**,** 176-U19.

KANDEL, E. R., SCHWARTZ, J. H. & JESSELL, T. M. 2000. *Principles of neural science,* New York, McGraw-Hill.

KAO, P. N. & KARLIN, A. 1986. Acetylcholine receptor binding site contains a disulfide cross-link between adjacent half-cystinyl residues. *J Biol Chem,* 261**,** 8085-8.

KAPLAN, R. M. 2004. Drug resistance in nematodes of veterinary importance: a status report. *Trends in Parasitology,* 20**,** 477-481.

KARLIN, A. 1993. Structure of nicotinic acetylcholine receptors. *Curr Opin Neurobiol,* 3**,** 299-309.

KARLIN, A., HOLTZMAN, E., YODH, N., LOBEL, P., WALL, J. & HAINFELD, J. 1983. The arrangement of the subunits of the acetylcholine receptor of Torpedo californica. *J Biol Chem,* 258**,** 6678-81.

KARYSTINOU, A., THOMAS, A. P. M. & ROY, H. E. 2004. Presence of haemocyte-like cells in coccinellid reflex blood. *Physiological Entomology,* 29**,** 94-96.

KAY, D., ROTHSCHILD, M. & APLIN, R. 1969. PARTICLES PRESENT IN THE HEMOLYMPH AND DEFENSIVE SECRETIONS OF INSECTS ARCTIA-CAJA PANAXIA-DOMINULA ZYGAENA-FILIPENDULAE DANAUS-PLEXIPPUS COCCINELLA-SEPTEMPUNCTATA VESPULA-GERMANICA NECROPHORUS-INVESTIGATOR NOSOPHYLLUS-FASCIATUS LYMANTRIA-DISPAR LASIOCAMPA-QUERCUS. *Journal of Cell Science,* 4**,** 369-379.

KEYSER, K. T., BRITTO, L. R., SCHOEPFER, R., WHITING, P., COOPER, J., CONROY, W., BROZOZOWSKA-PRECHTL, A., KARTEN, H. J. & LINDSTROM, J. 1993. Three subtypes of alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors are expressed in chick retina. *J Neurosci,* 13**,** 442-54.

KING, A. G. & MEINWALD, J. 1996. Review of the defensive chemistry of coccinellids. *Chemical Reviews,* 96**,** 1105-1122.

KOCH, R. L. 2003. The multicolored Asian lady beetle, Harmonia axyridis: a review of its biology, uses in biological control, and non-target impacts. *J Insect Sci,* 3**,** 32.

KOGEL, S., EBEN, A., HOFFMANN, C. & GROSS, J. 2012. Influence of Diet on Fecundity, Immune Defense and Content of 2-Isopropyl-3-Methoxypyrazine in Harmonia axyridis Pallas. *J Chem Ecol,* 38**,** 854-64.

LAMANA, M. L. & MILLER, J. C. 1996. Field observations on Harmonia axyridis Pallas (Coleoptera: Coccinellidae) in Oregon. *Biological Control,* 6**,** 232-237.

LANSDELL, S. J., COLLINS, T., GOODCHILD, J. & MILLAR, N. S. 2012. The Drosophila nicotinic acetylcholine receptor subunits Dalpha5 and Dalpha7 form functional homomeric and heteromeric ion channels. *BMC Neurosci,* 13**,** 73.

LAURENT, P., BRAEKMAN, J.-C., DALOZE, D. & PASTEELS, J. M. 2002. In vitro production of adaline and coccinelline, two defensive alkaloids from ladybird beetles (Coleoptera: Coccinellidae). *Insect Biochemistry and Molecular Biology,* 32**,** 1017-1023.

LAVIOLETTE, S. R. & VAN DER KOOY, D. 2004. The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour. *Nat Rev Neurosci,* 5**,** 55-65.

LEVANDOSKI, M. M., PIKET, B. & CHANG, J. 2003. The anthelmintic levamisole is an allosteric modulator of human neuronal nicotinic acetylcholine receptors. *Eur J Pharmacol,* 471**,** 9-20.

LEWIS, J. A., WU, C. H., BERG, H. & LEVINE, J. H. 1980. The Genetics of Levamisole Resistance in the Nematode Caenorhabditis-Elegans. *Genetics,* 95**,** 905-928.

LIU, L., ALAM, M. S., HIRATA, K., MATSUDA, K. & OZOE, Y. 2008. Actions of quinolizidine alkaloids on Periplaneta americana nicotinic acetylcholine receptors. *Pest Management Science,* 64**,** 1222-8.

MAHR, S. 1996. *Know your friends: multicolored Asian lady beetle* [Online]. University of Wisconsin. Available: http://www.entomology.wisc.edu/mbcn/kyf210.html [Accessed 30/01/2012 2012].

MAJERUS, M. E. N. 1994. *Ladybirds*, Harper Collins.

MARPLES, N. M., BRAKEFIELD, P. M. & COWIE, R. J. 1989. DIFFERENCES BETWEEN THE 7-SPOT AND 2-SPOT LADYBIRD BEETLES COCCINELLIDAE IN THEIR TOXIC EFFECTS ON A BIRD PREDATOR. *Ecological Entomology,* 14**,** 79-84.

MARTINEZ-TORRES, D., CHANDRE, F., WILLIAMSON, M. S., DARRIET, F., BERGE, J. B., DEVONSHIRE, A. L., GUILLET, P., PASTEUR, N. & PAURON, D. 1998. Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector Anopheles gambiae s.s. *Insect Mol Biol,* 7**,** 179-84.

MATSUDA, K., BUCKINGHAM, S. D., FREEMAN, J. C., SQUIRE, M. D., BAYLIS, H. A. & SATTELLE, D. B. 1998. Effects of the alpha subunit on imidacloprid sensitivity of recombinant nicotinic acetylcholine receptors. *Br J Pharmacol,* 123**,** 518-24.

MATSUDA, K., KANAOKA, S., AKAMATSU, M. & SATTELLE, D. B. 2009. Diverse actions and target-site selectivity of neonicotinoids: structural insights. *Molecular Pharmacology,* 76**,** 1-10.

MCKAY, J. P., RAIZEN, D. M., GOTTSCHALK, A., SCHAFER, W. R. & AVERY, L. 2004. eat-2 and eat-18 are required for nicotinic

neurotransmission in the Caenorhabditis elegans pharynx. *Genetics,* 166**,** 161-9.

MELLO, C. C., KRAMER, J. M., STINCHCOMB, D. & AMBROS, V. 1991. Efficient gene transfer in C.elegans: extrachromosomal maintenance and integration of transforming sequences. *EMBO J,* 10**,** 3959-70.

MELLOR, I. R., BRIER, T. J., PLUTEANU, F., STROMGAARD, K., SAGHYAN, A., ELDURSI, N., BRIERLEY, M. J., ANDERSEN, K., JAROSZEWSKI, J. W., KROGSGAARD-LARSEN, P. & USHERWOOD, P. N. 2003. Modification of the philanthotoxin-343 polyamine moiety results in different structure-activity profiles at muscle nicotinic ACh, NMDA and AMPA receptors. *Neuropharmacology,* 44**,** 70-80.

MICHAUD, J. P. 2002. Invasion of the Florida citrus ecosystem by Harmonia axyridis (Coleoptera : Coccinellidae) and asymmetric competition with a native species, Cycloneda sanguinea. *Environmental Entomology,* 31**,** 827-835.

MISHINA, M., TOBIMATSU, T., IMOTO, K., TANAKA, K., FUJITA, Y., FUKUDA, K., KURASAKI, M., TAKAHASHI, H., MORIMOTO, Y., HIROSE, T. & ET AL. 1985. Location of functional regions of acetylcholine receptor alpha-subunit by site-directed mutagenesis. *Nature,* 313**,** 364-9.

NAUEN, R. & DENHOLM, I. 2005. Resistance of insect pests to neonicotinoid insecticides: current status and future prospects. *Arch Insect Biochem Physiol,* 58**,** 200-15.

NEDVED, O., KALUSHKOV, P., FOIS, X., UNGEROVA, D. & ROZSYPALOVA, A. 2010. Harmonia axyridis: six-legged alligator or lethal fugu? *IOBC Bulletin,* 58**,** 65-68.

NISHIWAKI, H., NAKAGAWA, Y., KUWAMURA, M., SATO, K., AKAMATSU, M., MATSUDA, K., KOMAI, K. & MIYAGAWA, H. 2003. Correlations of the electrophysiological activity of neonicotinoids with their binding and insecticidal activities. *Pest Management Science,* 59**,** 1023-1030.

NOAH. 2011. *Facts and figures about the UK animal medicines industry* [Online]. Available: http://www.noah.co.uk/focus/facts_figures.htm [Accessed 03/2012 2012].

NODA, M., TAKAHASHI, H., TANABE, T., TOYOSATO, M., KIKYOTANI, S., FURUTANI, Y., HIROSE, T., TAKASHIMA, H., INAYAMA, S., MIYATA, T. & NUMA, S. 1983a. Structural homology of Torpedo californica acetylcholine receptor subunits. *Nature,* 302**,** 528-32.

NODA, M., TAKAHASHI, H., TANABE, T., TOYOSATO, M., KIKYOTANI, S., HIROSE, T., ASAI, M., TAKASHIMA, H., INAYAMA, S., MIYATA, T. & NUMA, S. 1983b. Primary structures of beta- and delta-subunit precursors of Torpedo californica acetylcholine receptor deduced from cDNA sequences. *Nature,* 301**,** 251-5.

PASTEELS, J. M., DEROE, C., TURSCH, B., BRAEKMAN, J. C., DALOZE, D. & HOOTELE, C. 1973. DISTRIBUTION AND ACTIVITIES OF DEFENSIVE ALKALOIDS OF COCCINELLIDAE. *Journal of Insect Physiology,* 19**,** 1771-1784.

PENG, X., KATZ, M., GERZANICH, V., ANAND, R. & LINDSTROM, J. 1994. Human alpha 7 acetylcholine receptor: cloning of the alpha 7 subunit from the SH-SY5Y cell line and determination of pharmacological properties of native receptors and functional alpha 7 homomers expressed in Xenopus oocytes. *Mol Pharmacol,* 45**,** 546-54.

PHOOFOLO, M. W. & OBRYCKI, J. J. 1998. Potential for intraguild predation and competition among predatory Coccinellidae and Chrysopidae. *Entomologia Experimentalis Et Applicata,* 89**,** 47-55.

POLIS, G. A., MYERS, C. A. & HOLT, R. D. 1989. The Ecology and Evolution of Intraguild Predation - Potential Competitors That Eat Each Other. *Annual Review of Ecology and Systematics,* 20**,** 297-330.

POORANI, J. 2008. *Adult Morphology* [Online]. Available: http://www.angelfire.com/bug2/j_poorani/morphology.htm.

POUTSMA, J., LOOMANS, A. J. M., AUKEMA, B. & HEIJERMAN, T. 2008. Predicting the potential geographical distribution of the harlequin ladybird, Harmonia axyridis, using the CLIMEX model. *Biocontrol,* 53**,** 103-125.

QIAN, H., MARTIN, R. J. & ROBERTSON, A. P. 2006. Pharmacology of N-, L-, and B-subtypes of nematode nAChR resolved at the single-channel level in Ascaris suum. *FASEB J,* 20**,** 2606-8.

RAIZEN, D. M., LEE, R. Y. & AVERY, L. 1995. Interacting genes required for pharyngeal excitation by motor neuron MC in Caenorhabditis elegans. *Genetics,* 141**,** 1365-82.

RAYMOND DELPECH, V., IHARA, M., CODDOU, C., MATSUDA, K. & SATTELLE, D. B. 2003. Action of nereistoxin on recombinant neuronal nicotinic acetylcholine receptors expressed in Xenopus laevis oocytes. *Invert Neurosci,* 5**,** 29-35.

RAYMOND, V., MONGAN, N. P. & SATTELLE, D. B. 2000. Anthelmintic actions on homomer-forming nicotinic acetylcholine receptor subunits: chicken alpha7 and ACR-16 from the nematode Caenorhabditis elegans. *Neuroscience,* 101**,** 785-91.

REJZEK, M., STOCKMAN, R. A. & HUGHES, D. L. 2005. Combining two-directional synthesis and tandem reactions: an efficient strategy for the total syntheses of (+/-)-hippodamine and (+/-)-epi-hippodamine. *Org Biomol Chem,* 3**,** 73-83.

REYNOLDS, J. A. & KARLIN, A. 1978. Molecular weight in detergent solution of acetylcholine receptor from Torpedo californica. *Biochemistry,* 17**,** 2035-8.

RICHARDS, D. P. 2011. *Investigating ladybird (Coleoptera: Coccinellidae) alkaloids as novel sources for insecticides: Differential inhibition of the vertebrate and invertebrate nicotinic acetylcholine receptor using Harlequin ladybird (Harmonia axyridis) extract and synthetic hippodamine.* MRes, The University of Nottingham.

RICHMOND, J. E. & JORGENSEN, E. M. 1999. One GABA and two acetylcholine receptors function at the C. elegans neuromuscular junction. *Nat Neurosci,* 2**,** 791-7.

ROBERTSON, A. P., CLARK, C. L., BURNS, T. A., THOMPSON, D. P., GEARY, T. G., TRAILOVIC, S. M. & MARTIN, R. J. 2002. Paraherquamide and 2-deoxy-paraherquamide distinguish cholinergic receptor subtypes in Ascaris muscle. *J Pharmacol Exp Ther,* 302**,** 853-60.

ROY, H. E., BAVERSTOCK, J., WARE, R. L., CLARK, S. J., MAJERUS, M. E. N., BAVERSTOCK, K. E. & PELL, J. K. 2008. Intraguild predation of the aphid pathogenic fungus Pandora neoaphidis by the invasive coccinellid Harmonia axyridis. *Ecological Entomology,* 33**,** 175-182.

ROZENTAL, R., SCOBLE, G. T., ALBUQUERQUE, E. X., IDRISS, M., SHERBY, S., SATTELLE, D. B., NAKANISHI, K., KONNO, K., ELDEFRAWI, A. T. & ELDEFRAWI, M. E. 1989. Allosteric inhibition of nicotinic acetylcholine receptors of vertebrates and insects by philanthotoxin. *J Pharmacol Exp Ther,* 249**,** 123-30.

RÖHRICH, C. R., NGWA, C. J., WIESNER, J., SCHMIDTBERG, H., DEGENKOLB, T., KOLLEWE, C., FISCHER, R., PRADEL, G. & VILCINSKAS, A. 2011. Harmonine, a defence compound from the harlequin ladybird, inhibits mycobacterial growth and demonstrates multi-stage antimalarial activity. *Biol Lett*.

SACHS, F. 1991. Mechanical transduction by membrane ion channels: a mini review. *Mol Cell Biochem,* 104**,** 57-60.

SANTI, F. & MAINI, S. 2006. Predation upon Adalia bipunctata and Harmonia axyridis eggs by Chrysoperla carnea larvae and Orius laevigatus adults. *Bulletin of Insectology,* 59**,** 53-58.

SATO, S. & DIXON, A. F. G. 2004. Effect of intraguild predation on the survival and development of three species of aphidophagous ladybirds: consequences for invasive species. *Agricultural and Forest Entomology,* 6**,** 21-24.

SATO, S., KUSHIBUCHI, K. & YASUDA, H. 2009. Effect of reflex bleeding of a predatory ladybird beetle, Harmonia axyridis (Pallas) (Coleoptera: Coccinellidae), as a means of avoiding intraguild predation and its cost. *Applied Entomology and Zoology,* 44**,** 203-206.

SATTELLE, D. B., HARROW, I. D., DAVID, J. A., PELHATE, M., CALLEC, J. J., GEPNER, J. I. & HALL, L. M. 1985. Nereistoxin: actions on a CNS acetylcholine receptor/ion channel in the cockroach Periplaneta americana. *J Exp Biol,* 118**,** 37-52.

SATTELLE, D. B., JONES, A. K., SATTELLE, B. M., MATSUDA, K., REENAN, R. & BIGGIN, P. C. 2005. Edit, cut and paste in the nicotinic acetylcholine receptor gene family of Drosophila melanogaster. *Bioessays,* 27**,** 366-76.

SCHRODER, F. C., FARMER, J. J., ATTYGALLE, A. B., SMEDLEY, S. R., EISNER, T. & MEINWALD, J. 1998a. Combinatorial chemistry in insects: A library of defensive macrocyclic polyamines. *Science,* 281**,** 428-431.

SCHRODER, F. C., FARMER, J. J., SMEDLEY, S. R., EISNER, T. & MEINWALD, J. 1998b. Absolute configuration of the polyazamacrolides, macrocyclic polyamines produced by a ladybird beetle. *Tetrahedron Letters,* 39**,** 6625-6628.

SCHRODER, F. C. & TOLASCH, T. 1998. Psylloborine A, a new dimeric alkaloid from a ladybird beetle. *Tetrahedron,* 54**,** 12243-12248.

SCHROEDER, F. C., SMEDLEY, S. R., GIBBONS, L. K., FARMER, J. J., ATTYGALLE, A. B., EISNER, T. & MEINWALD, J. 1998. Polyazamacrolides from ladybird beetles: Ring-size selective oligomerization. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 13387-13391.

SEAGO, A. E., GIORGI, J. A., LI, J. & SLIPINSKI, A. 2011. Phylogeny, classification and evolution of ladybird beetles (Coleoptera: Coccinellidae) based on simultaneous analysis of molecular and morphological data. *Mol Phylogenet Evol,* 60**,** 137-51.

SEO, S., HENRY, J. T., LEWIS, A. H., WANG, N. & LEVANDOSKI, M. M. 2009. The positive allosteric modulator morantel binds at noncanonical subunit interfaces of neuronal nicotinic acetylcholine receptors. *J Neurosci,* 29**,** 8734-42.

SHAO, Y. M., DONG, K. & ZHANG, C. X. 2007. The nicotinic acetylcholine receptor gene family of the silkworm, Bombyx mori. *BMC Genomics,* 8**,** 324.

SHIMOMURA, M., YOKOTA, M., IHARA, M., AKAMATSU, M., SATTELLE, D. B. & MATSUDA, K. 2006. Role in the selectivity of neonicotinoids of insect-specific basic residues in loop D of the nicotinic acetylcholine receptor agonist binding site. *Molecular Pharmacology,* 70**,** 1255-63.

SLOGGETT, J. J. & DAVIS, A. J. 2010. Eating chemically defended prey: alkaloid metabolism in an invasive ladybird predator of other ladybirds (Coleoptera: Coccinellidae). *Journal of Experimental Biology,* 213**,** 237-241.

SLOGGETT, J. J., HAYNES, K. F. & OBRYCKI, J. J. 2009. Hidden costs to an invasive intraguild predator from chemically defended native prey. *Oikos,* 118**,** 1396-1404.

SLOGGETT, J. J., MAGRO, A., VERHEGGEN, F. J., HEMPTINNE, J.-L., HUTCHISON, W. D. & RIDDICK, E. W. 2011. The chemical ecology of Harmonia axyridis. *BioControl (Dordrecht),* 56**,** 643-661.

SMIT, A. B., SYED, N. I., SCHAAP, D., VAN MINNEN, J., KLUMPERMAN, J., KITS, K. S., LODDER, H., VAN DER SCHORS, R. C., VAN ELK, R., SORGEDRAGER, B., BREJC, K., SIXMA, T. K. & GERAERTS, W. P. 2001. A glia-derived acetylcholine-binding protein that modulates synaptic transmission. *Nature,* 411**,** 261-8.

SPARKS, T. C., CROUSE, G. D. & DURST, G. 2001. Natural products as insecticides: the biology, biochemistry and quantitative structure-activity relationships of spinosyns and spinosoids. *Pest Management Science,* 57**,** 896-905.

SULSTON, J. E. & HORVITZ, H. R. 1977. Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. *Dev Biol,* 56**,** 110-56.

SULSTON, J. E., SCHIERENBERG, E., WHITE, J. G. & THOMSON, J. N. 1983. The embryonic cell lineage of the nematode Caenorhabditis elegans. *Dev Biol,* 100**,** 64-119.

SUMIKAWA, K., HOUGHTON, M., EMTAGE, J. S., RICHARDS, B. M. & BARNARD, E. A. 1981. Active multi-subunit ACh receptor assembled by translation of heterologous mRNA in Xenopus oocytes. *Nature,* 292**,** 862-4.

SUTHERLAND, I. A., DAMSTEEGT, A., MILLER, C. M. & LEATHWICK, D. M. 2008. Multiple species of nematodes resistant to ivermectin and a benzimidazole-levamisole combination on a sheep farm in New Zealand. *N Z Vet J,* 56**,** 67-70.

TAKAHASHI, T., KUNO, M., MISHINA, M. & NUMA, S. 1985. A physiological study on acetylcholine receptor expressed in Xenopus oocytes from cloned cDNAs. *J Physiol (Paris),* 80**,** 229-32.

THANY, S. H. & TRICOIRE-LEIGNEL, H. 2011. Emerging Pharmacological Properties of Cholinergic Synaptic Transmission: Comparison between Mammalian and Insect Synaptic and Extrasynaptic Nicotinic Receptors. *Curr Neuropharmacol,* 9**,** 706-14.

TIKHONOV, D. B., MELLOR, I. R. & USHERWOOD, P. N. 2004. Modeling noncompetitive antagonism of a nicotinic acetylcholine receptor. *Biophys J,* 87**,** 159-70.

TOUROUTINE, D., FOX, R. M., VON STETINA, S. E., BURDINA, A., MILLER, D. M., 3RD & RICHMOND, J. E. 2005. acr-16 encodes an essential subunit of the levamisole-resistant nicotinic receptor at the Caenorhabditis elegans neuromuscular junction. *J Biol Chem,* 280**,** 27013-21.

TOWERS, P. R., EDWARDS, B., RICHMOND, J. E. & SATTELLE, D. B. 2005. The Caenorhabditis elegans lev-8 gene encodes a novel type of nicotinic acetylcholine receptor alpha subunit. *J Neurochem,* 93**,** 1-9.

TURSCH, B., DALOZE, D., BRAEKMAN, J. C., HOOTELE, C. & PASTEELS, J. M. 1975. CHEMICAL ECOLOGY OF ARTHROPODS .10. STRUCTURE OF MYRRHINE AND BIOSYNTHESIS OF COCCINELLINE. *Tetrahedron,* 31**,** 1541-1543.

VENKATESH, B., LU, S. Q., DANDONA, N., SEE, S. L., BRENNER, S. & SOONG, T. W. 2005. Genetic basis of tetrodotoxin resistance in pufferfishes. *Current Biology,* 15**,** 2069-72.

WARE, R. L. & MAJERUS, M. E. N. 2008. Intraguild predation of immature stages of British and Japanese coccinellids by the invasive ladybird Harmonia axyridis. *Biocontrol,* 53**,** 169-188.

WATSON, G. B., CHOUINARD, S. W., COOK, K. R., GENG, C., GIFFORD, J. M., GUSTAFSON, G. D., HASLER, J. M., LARRINUA, I. M., LETHERER, T. J., MITCHELL, J. C., PAK, W. L., SALGADO, V. L., SPARKS, T. C. & STILWELL, G. E. 2010. A spinosyn-sensitive Drosophila melanogaster nicotinic acetylcholine receptor identified through chemically induced target site resistance, resistance gene identification, and heterologous expression. *Insect Biochem Mol Biol,* 40**,** 376-84.

WEBSTER, M. 1999. Product warning: FRONTLINE. *Aust Vet J,* 77**,** 202.

WHITE, J. G., SOUTHGATE, E., THOMSON, J. N. & BRENNER, S. 1986. The structure of the nervous system of the nematode C. elegans. *Philosophical Transactions of the Royal Society of London,* 314**,** 1-340.

WHITEHORN, P. R., O'CONNOR, S., WACKERS, F. L. & GOULSON, D. 2012. Neonicotinoid pesticide reduces bumble bee colony growth and queen production. *Science,* 336**,** 351-2.

WILLIAMS, P. L. & DUSENBERRY, D. B. 1990. Aquatic toxicity testing using the nematode, *Caenorhabditis elegans*. *Environmental Toxicology & Chemistry,* 9**,** 1285-1290.

WILLIAMSON, S. M., ROBERTSON, A. P., BROWN, L., WILLIAMS, T., WOODS, D. J., MARTIN, R. J., SATTELLE, D. B. & WOLSTENHOLME, A. J. 2009. The nicotinic acetylcholine receptors of the parasitic nematode Ascaris suum: formation of two distinct drug targets by varying the relative expression levels of two subunits. *PLoS Pathog,* 5**,** e1000517.

WRIGLEY, J., MCARTHUR, M., MCKENNA, P. B. & MARIADASS, B. 2006. Resistance to a triple combination of broad-spectrum anthelmintics in naturally-acquired Ostertagia circumcincta infections in sheep. *N Z Vet J,* 54**,** 47-9.

YARBROUGH, J. A., ARMSTRONG, J. L., BLUMBERG, M. Z., PHILLIPS, A. E., MCGAHEE, E. & DOLEN, W. K. 1999. Allergic rhinoconjunctivitis caused by Harmonia axyridis (Asian lady beetle, Japanese lady beetle, or lady bug). *J Allergy Clin Immunol,* 104**,** 704-5.

YASUDA, H. & OHNUMA, N. 1999. Effect of cannibalism and predation on the larval performance of two ladybird beetles. *Entomologia Experimentalis Et Applicata,* 93**,** 63-67.

ZAIM, M. & GUILLET, P. 2002. Alternative insecticides: an urgent need. *Trends in Parasitology,* 18**,** 161-3.

ZALAT, S., ELBANA, S., RIZZOLI, S., SCHMIDT, J. O. & MELLOR, I. R. 2005. Modulation of nicotinic acetylcholine and N-methyl-d-aspartate receptors by some Hymenopteran venoms. *Toxicon,* 46**,** 282-90.

ZHANG, W., JIANG, F. & OU, J. 2011. Global pesticide consumption and pollution: with China as a focus. *Proceedings of the International Academy of Ecology and Environmental Sciences,* 1**,** 125-144.

ZINSER, E. W., WOLF, M. L., ALEXANDER-BOWMAN, S. J., THOMAS, E. M., DAVIS, J. P., GROPPI, V. E., LEE, B. H., THOMPSON, D. P. & GEARY, T. G. 2002. Anthelmintic paraherquamides are cholinergic antagonists in gastrointestinal nematodes and mammals. *Journal of Veterinary Pharmacology and Therapeutics,* 25**,** 241-250.