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

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Abstract

Ladybird beetles (Coleoptera:Coccinellidae) are ubiquitous, being found on every continent with the exception of Antarctica, and inhabiting a huge range of habitats. Roughly 6000 sp. have been described, comprising at least 490 genera. When disturbed, ladybirds 'reflex bleed' (autohemorrhage) from the femoro-tibial joints, producing a viscous, yellow liquid which has a very distinctive odour, this has been shown to be an effective deterrent against predation from both birds and arthropods. Feeding trials have demonstrated that many ladybirds are considered unpalatable and unacceptable as food sources by many potential predators. The bright colouration displayed by many coccinellids is thought to function as an aposematic warning. This has been demonstrated experimentally using Japanese quails, where colour was shown to be a more effective feeding deterrent than either taste or smell. A number of novel compounds have been isolated from this fluid, including alkaloids. It has been hypothesised that alkaloids within the defensive fluid will serve a toxic function, and this is the basis of the aposematic colouration.

The aim of this work was to investigate the toxinological properties of alkaloid extracts of the harlequin ladybird (*Harmonia axyridis*) as well as a synthetic analogue of hippodamine, an alkaloid isolated from the convergent ladybird (*Hippodamia convergens*). We wished to establish whether these alkaloids display activity against nicotinic acetylcholine receptors (nAChR), and if so, whether they show any selectivity towards vertebrate or invertebrate nAChR. To do this we collected harlequin ladybirds and extracted the alkaloids using methanol, followed by acid-base extraction. HPLC revealed a number of compounds with elution times consistent with alkaloids. To test for nAChR activity we employed whole-cell patch clamp combined with perfusion of human muscle cells (TE671) and locust (*Schistocerca gregaria*) neurons. Both *H.* axyridis extract and

hippodamine were found to be potent antagonists to nAChR in both TE671 cells and locust neurons. Furthermore both were found to be considerably more potent to locust nAChR than TE671. *H. axyridis* extract had IC_{50} values of 2.12 µg/ml and 39.9 ng/ml in TE671 and locust neurons respectively with a holding potential of -50mV. Hippodamine had IC_{50} values of 22.3 µM and 18.5nM in TE671 and locust neurons respectively with a holding potential of -50mV. F-test comparisons revealed that both test compounds were significantly more potent to locust neurons than to TE671 cells (*H. axyridis* p=0.0015, hippodamine p=0.0001) at holding potentials of -50mV and -100mV. F-test comparisons between holding potentials revealed a voltage dependence in hippodamine when applied to locust neurons (p=0.0153), but no other voltage dependent relationships were identified.

The results of these experiments reveal that ladybird alkaloids do display toxicity, potently antagonising the nAChR. Further to this, both of those tested displayed selective toxicity towards locust nAChR of between 41 and 1205 fold potency for *H. axyridis* extract and hippodamine respectively. It remains to be seen how this is achieved, but this high selectivity warrants further investigation into the use of these alkaloids as lead structures for insecticides.

Acknowledgments

Many people were instrumental for the completion of this project. I wish to thank my supervisors at The University of Nottingham; Dr. Ian Mellor and Dr. Ian Duce for their assistance and enthusiasm for the topic, both in and out of the lab. I also wish to thank my supervisors at Rothamsted Research; Dr. Mike Birkett and Prof. John Pickett for their chemistry support, and for allowing me to use the lab facilities including the HPLC at Rothamsted. A significant portion of the experiments would not have been possible without hippodamine, synthesised by Dr. Rob Stockman from The School of Chemistry at Nottingham University. Tim Smith has been able to track down everything I have needed to complete lab work.

A large number of people helped by collecting ladybirds, despite my insistence that they were toxic. In no particular order; Tamsin Majerus, Kiara Majerus, Emily Dawson, Ian Mellor, my parents and all the work experience students that were encouraged to rapidly develop an interest in ladybirds by being forced outside with a collecting tube.

Despite the excitement of electrophysiology, the lab would have been a much less interesting place without the colleagues I shared it with. Mark Burton, Frankie Cash, Katharina Reusch, Dr. David McClymont, Andrew Crofts and Jack Morton. Also my fellow MRes students, with whom I shared the trials and tribulations of trying to develop, and carry out a project in a limited time frame.

My parents have always encouraged my interests, as well as supplying ladybirds they have provided much needed support throughout the year of this project.

Contents

Abbreviations

A-BgTX	Alpha bungarotoxin
ACh	Acetylcholine
AChE	Acetylcholinesterase
ConA	Conconavalin A
DCM	Dichloromethane
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
FCS	Foetal calf serum
GABA	Gamma amino-butyric acid
Gln	Glutamine
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IC_{50}	Half maximal current
nAChR	Nicotinic acetylcholine receptor
NaOH	Sodium hydroxide
Pen/Strep	Penicillin/Streptomycin
PLRV	Potato Leafroll Virus
PSV	Pea Streak Virus
SEM	Scanning electron micrograph
THF	Tetrohydrofuran
VCSC	Voltage gated sodium channel
V_H	Holding potential

1. Introduction

1.1 Ladybird Beetles

Ladybird beetles (Coleoptera:Coccinellidae) are ubiquitous, found on every continent with the exception of Antarctica (Roy & Majerus, 2010), and inhabiting a huge range of habitats from sea level to at least 4260m (Mani, 1968). Coccinellids comprise almost certainly the most well known family within the largest order in existence, the beetles (Majerus, 1994). Although constrained in terms of body plan (Figure 1.1.1), ladybirds display great heterogeneity in terms of size, diet, colouration and habitat preferences. The majority of species are predatory carnivores (Samways *et al*, 1997) and they are often exploited as agents of biological control (Caltagirone & Doutt, 1989; Kindlman & Dixon, 1993; Dixon *et al*, 1997; Obrycki & Kring, 1998; Dixon, 2000; Al Abassi *et al*, 2000) owing to their predilection for homopterous agricultural pests such as aphids and scale insects (Twardowski *et al*, 2005). However, some phytophagous ladybirds are considered to be crop pests themselves, for example the Mexican bean beetle (*Epilachna varivestis*) (Singh & van Emden, 1979). Other species specialise upon pollen and fungus (Minelli & Pasqual, 1977). Coccinellids are holometabolous and go through four larval instars (Fig. 1.1.6) before pupating into adult beetles (Iperti, 1999). Unlike many holometabolous insects, such as lepidopterans and hymenopterans, ladybird larvae rely on the same food resources as adult beetles and both beetles and larvae have been used previously in biological control strategies (Gurney & Hussey, 1970).

Around 6000 species have been described to date (Seago *et al*, 2011) but the phylogenetic relationships within the Coccinellidae are not well resolved. Seven sub-families have been put forward, and these have been further divided into 42 tribes by some authors (Fürsch, 1990; Kovář, 1996; Ślipiński & Tomaszewska, 2010). Only one of these sub-families, the Coccinellinae is strongly supported as

monophyletic (Robertson *et al*, 2008; Giorgi *et al*, 2009; Magro *et al*, 2010). Seago *et al* (2011) used both maximum parsimony and bayesian inference to establish

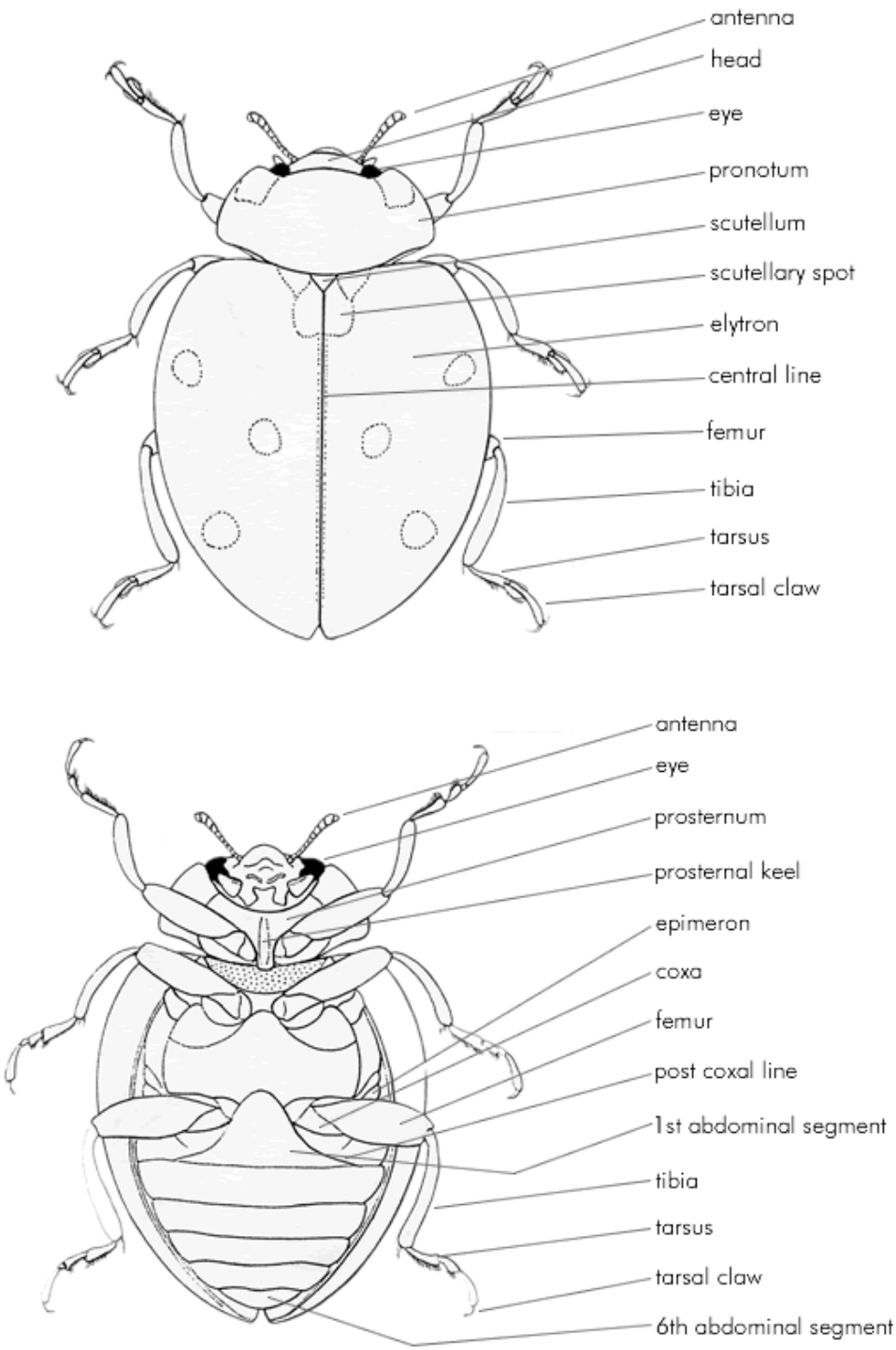


Figure 1.1.1 Diagrams showing the generalised anatomy of ladybird beetles as shown from above (top) and below (bottom). Diagrams courtesy of the Cambridge Ladybird Survey (www.ladybird-survey.org).

the evolutionary relationships within the Coccinellidae. Their results supported the division of the Coccinellidae into two subfamilies; the Coccinellinae and the Microweisinae which are further split into 20 tribes, and four tribes respectively.

1.2 Ladybird Defences:

It has long been assumed that ladybirds produce toxins, and that the bright colouration displayed by many species (Fig. 1.1.2) serves as an aposematic warning (e.g. Majerus, 1994; Daloze *et al*, 1995; Glisan King & Meinwald, 1996; Grill & Moore, 1998; Al Abassi *et al*, 2000; Sloggett, 2010). Aposematism can be defined as the display of warning colouration showing that the bearer is an unprofitable prey item. This is usually associated with a chemical defence in the form of toxins or other compounds. This is supported by the fact that there are few accounts of species which readily prey upon ladybirds, despite coccinellids being one of the most commonly encountered and easily identified invertebrates (Majerus, 1994). In addition to this, spiders which predate non-selectively have been shown to avoid feeding on ladybirds which have become entrapped and incapacitated by their webs (Nentwig, 1983; Nentwig, 1986; Young, 1989; Yasuda & Kimera, 2001 but see Sloggett, 2010). Ladybird colour and patterns were found to be more effective feeding deterrents to Japanese quails (*Coturnix coturnix japonicas*) than either taste or smell (Marples *et al*, 1994). All life stages are brightly coloured (Fig 1.1.6) but a predation bioassay has demonstrated that adult beetles are better defended than either the eggs or larvae of the Mexican ladybird *Epilachna paenulata* (Camarano *et al*, 2006).

Feeding trials have shown that many ladybirds are considered unpalatable and unacceptable as food sources by many predators including; birds, hedgehogs, beetles, lizards, toads (Frazer & Rothschild, 1960) and ants (Marples, 1993a) under laboratory conditions. This is in contrast to reports of wild birds which have been seen to feed upon ladybirds (e.g. Krištín, 1984; Krištín, 1986) and nest dropping

analysis which has shown that house martins (*Delichon urbica*) are able to successfully fledge offspring on a diet mainly composed of ladybirds (Muggleton, 1978). It is possible that this disparity is because wild animals are generally less well nourished than their laboratory housed counterparts, and can ill afford to reject prey, even if it is of low palatability, as long as it is not deleterious (Muggleton, 1978).

Figure 1.1.2 Photographs showing harlequin ladybirds (*Harmonia axyridis*) demonstrating the bright, possibly aposematic colouration displayed by many ladybird species. These show the most commonly encountered colour variants seen in the invasive UK population; succinea (orange) and spectabilis (melanic).

When disturbed, ladybirds 'reflex bleed' (autohemorrhage) (Fig. 1.1.3) from the femoro-tibial joints (Hodek, 1973; Majerus & Kearns, 1989; Holloway *et al*, 1991; Laurent *et al*, 2005; Roy *et al*, 2005), producing a viscous yellow liquid

Figure 1.1.3 Eyed ladybird (*Anatis ocellata*) reflex bleeding from the femoro-tibial joints. Photograph courtesy of The Royal Society.

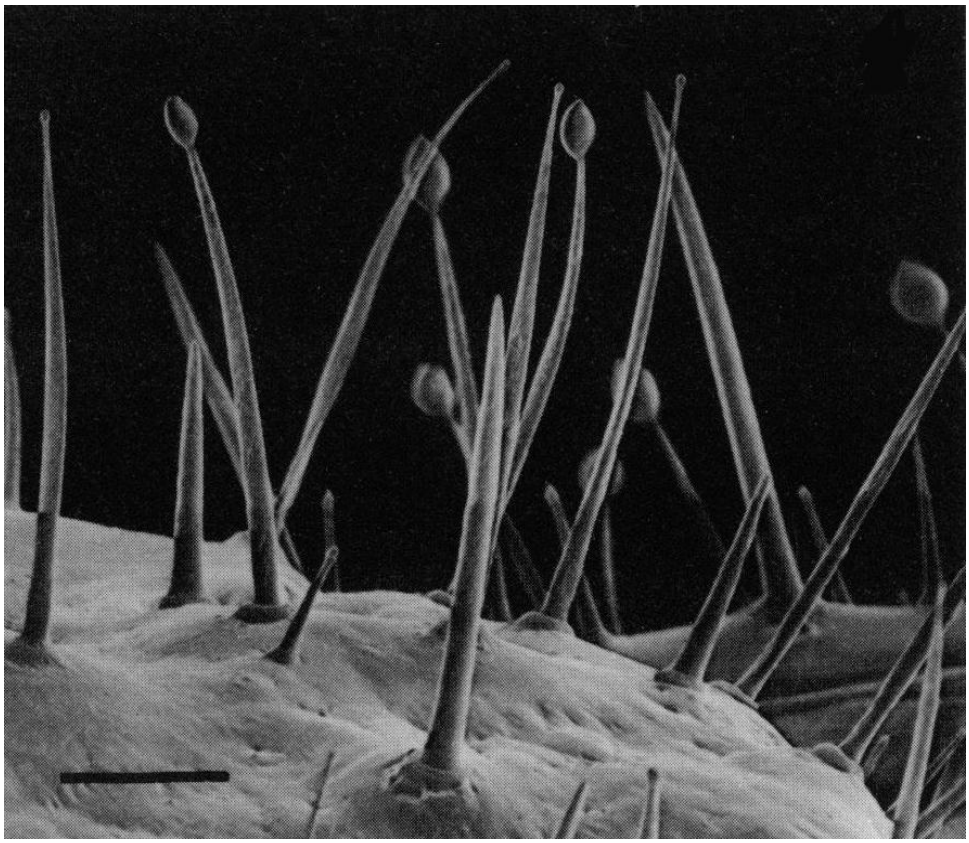


Figure 1.1.4 SEM of glandular hairs from *Epilachna varivestis* with droplets of alkaloid containing fluid. Scale bar shows 0.1mm. From Attygalle *et al* (1993).

which has a very distinctive odour and unpleasant flavour (Majerus, 1994; Pickering *et al*, 2007; Pickering *et al*, 2008; Sloggett, 2010). This behaviour has been shown to be an effective deterrent against predation from; Japanese quails (*C. c. japonicas*) (Marples *et al*, 1994), *Formica exsectoides* ants (Happ & Eisner, 1961) and *Myrmica rubra* ants (Tursch *et al*, 1971). When disturbed repeatedly ladybirds may lose up to 20% of their bodyweight through reflex bleeding (Holloway *et al*, 1991). As well as the ability to reflex bleed, pupae of the Mexican bean beetle (*E. varivestis*) are covered in glandular hairs. At the end of each hair is a bead of oil containing azomacrolide alkaloids (Fig. 1.1.4) (Attygalle *et al*, 1993).

1.3 Ladybird Toxicity:

A number of compounds have been isolated from the haemolymph, including; acetates, acids, alkanes, alcohols, aldehydes, benzaldehydes, benzopyroles, monoterpenes, sesquiterpenes, pyrazines and alkaloids (Durieux *et al*, 2010). Ladybirds are particularly rich sources of alkaloids and these compounds are thought to be responsible for the distinctive odour and bitter flavour that ladybirds are known to have (Tursch *et al*, 1971; Al Abassi *et al*, 2000; Laurent *et al*, 2005; Sloggett, 2010). It has been suggested that the alkaloids may serve a number of functions; as aggregation pheremones, to stimulate aggregations for mating and overwintering (Al Abassi *et al*, 1998) and as oviposition-dettering pheromones, to discourage females from laying eggs in areas where conspecific larvae are present (Klewer *et al*, 2007; Růžička & Zemek, 2008). It has also been shown that precoccinelline, an alkaloid produced by the 7-spot ladybird acts as a kairomone, attracting the parasitoid wasp *Dinocampus coccinellae* which then oviposit in the beetle (Fig. 1.1.5) (Majerus *et al*, 1997; Al Abassi *et al*, 2000) and dettering the aphid parasitoid *Aphidius ervi* (Nakashima *et al*, 2004).

The strong evidence to suggest ladybirds are considered unpalatable by many species may be owed to the alkaloids found in the haemolymph (Fig. 1.1.7) serving a toxic function (Pasteels *et al*, 1973; Agarwala & Dixon, 1992; Daloze *et al*, 1995; Al Abassi *et al*, 1998; Al Abassi *et al*, 2000; Sato & Dixon, 2004; Dolenska *et al*, 2009; Sloggett, 2009a; Sloggett, 2009b; Sloggett, 2010) but this has never been established experimentally. A study conducted by Marples *et al* (1989) demonstrated that whole 7-spot ladybirds (*C. 7-punctata*) caused nestling blue tit (*Parus caeruleus*) death within two days when fed 5.3 beetles per day. Autopsy revealed that the birds had died of liver damage. An equivalent weight of 2-spot ladybirds (*Adalia bipunctata*) failed to cause death or retard growth. A follow up experiment confirmed that 2-spot ladybirds are neither toxic to, nor do they inhibit growth of nestling blue tits, and the same is true for 10-spot ladybirds (*Adalia 10-punctata*) and water ladybirds (*Anisosticta 19-punctata*). Pine and kidney spot ladybirds (*Exochomus 4-pustulatus* and *Chilocorus renipustulatus*) were both found to be toxic to very young nestlings and inhibit growth (Marples, 1993b).

Figure 1.1.5 (left) 7-spot ladybird (*C. 7-punctata*) that has been parasitised by the wasp *Dinocampus coccinellae* (right). The wasp larvae paralyse the beetle and spin a cocoon between its legs. The adult wasp emerges from the end of the cocoon.

The only invertebrate toxicity assessments using ladybirds have been conducted on other ladybirds, and are mainly concerned with the ecological impacts of toxicity on intraguild predation. Intraguild predation is the phenomenon where species which share a food resource also feed upon one another (Polis *et al*, 1989). Ladybirds are well known for this behaviour and often serve as an experimental model (Hemptinne *et al*, 2000; Sato & Dixon, 2004; Ware *et al*, 2008; Sloggett, 2009a; Sloggett, 2009b; Kajita *et al*, 2010). Sato and Dixon (2004) revealed a complex relationship between three species of ladybird often found in close association; the harlequin ladybird (*H. axyridis*), the 7-spot ladybird (*C. 7-punctata*) and the 2-spot ladybird (*Adalia bipunctata*). It was discovered that larvae of each species were able to safely consume conspecific eggs and *C. 7-punctata* could safely consume *A. bipunctata* eggs. However, larvae of *H. axyridis* suffered 65% and 15% mortality when fed eggs from *A. bipunctata* and *C. 7-punctata* respectively. Both *A. bipunctata* and *C. 7-punctata* suffered 100% mortality when fed eggs from *H. axyridis*. Finally, *A. bipunctata* suffered 46% mortality when fed eggs from *C. 7-punctata* (Sato & Dixon, 2004). These findings are largely concurrent with those of Yasuda & Ohnuma (1999), who found that *H. axyridis* appear to be fatally toxic to *C. 7-punctata* but not *vice versa*. A similar study which focussed just on *C. 7-punctata* and *A. bipunctata* found that not only are *C. 7-punctata* reluctant to feed on eggs from *A. bipunctata*, but they also fail to complete larval development when they do. *A. bipunctata* appeared to be less reluctant to feed on eggs from *C. 7-punctata* but 70% were unable to complete larval development when these eggs were included in the diet (Hemptinne *et al*, 2000). Another study, which quantified the alkaloids present in eggs of *H. axyridis* and *C. 7-punctata*, also found that *H. axyridis* is toxic to *C. 7-punctata*. It demonstrated that *C. 7-punctata* eggs with a high concentration of alkaloid significantly slowed development in *H. axyridis* (Kajita *et al*, 2010). These studies are limited by the fact that they only consider three species, and from these data,

the only species we can confidently say possesses a toxic defence against coccinellid predation is the harlequin ladybird. Another feeding trial experiment conducted by Sloggett (2009b) found that *H. axyridis* larvae fed on the eggs of *Coleomegilla maculata*, which are known to produce the alkaloids myrhhine and harmonine (Fig. 1.1.7) become incapacitated within 24-36 hours and do not recover. Although suggestive, these studies show that eating whole ladybirds, larvae or eggs can be deleterious in some species; the exact cause of death is not known, but the alkaloids are often implicated (Pasteels *et al*, 1973; Agarwala & Dixon, 1992; Daloze *et al*, 1995; Al Abassi *et al*, 1998; Al Abassi *et al*, 2000; Sato & Dixon, 2004; Dolenska *et al*, 2009; Sloggett, 2009a; Sloggett, 2009b; Sloggett, 2010).

1.4 Alkaloids Isolated From Ladybirds:

The first ladybird alkaloid to be isolated was coccinelline (Fig. 1.1.7), a tri-cyclic azaphenalene found in the 7-spot ladybird (Tursch *et al*, 1971). Experiments with *Myrmica rubra* ants demonstrated that coccinelline is a very effective deterrent at a concentration of 0.5% (Tursch *et al*, 1975). Further experiments have shown that an isomer of coccinelline, convergine, from the convergent ladybird (*Hippodamia convergens*) has similar ant repelling properties to coccinelline (Pasteels *et al*, 1973) and the homotropane alkaloid adaline from the 2-spot ladybird is an effective feeding deterrent to fire ants (*Solenopsis invicta*) (Hill & Renbaum, 1982). Eisner *et al* (1986) identified a correlation between the concentration of the azabicyclononane alkaloid euphococcinine in the Mexican bean beetle (*Epilachna varivestis*) and the propensity with which the jumping spider *Phidippus regius* preyed upon beetles. The concentration of alkaloid was found to increase over the first seven days from beetle eclosion and predatory behaviour of *P. regius* was significantly lower for older, more alkaloid rich beetles (Eisner *et al*, 1986). The azamacralide alkaloid epilachnene was shown to act as a detterent to

H. axyridis and a topical irritant to American cockroaches (*Periplaneta americana*) (Rossini *et al*, 2000). A number of ladybird alkaloids from various structural families have been shown to deter predators or make previously palatable food items unpalatable (Camarano *et al*, 2006; Smedley *et al*, 2002).

Figure 1.1.6 Harlequin ladybird eggs (left) and larvae (right). Both eggs and larvae are brightly coloured and contain alkaloids. Many species of ladybird are intraguild predators, particularly during larval instars.

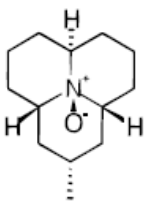
In viticulture, ladybirds are responsible for a phenomenon known as "ladybird taint" whereby beetles are harvested along with grapes. These then undergo the pressing process resulting in contamination of wine with compounds found in the haemolymph. Some of these, notably pyrazines produced by *H. axyridis* and *Hippodamia convergens* (such as 2-alkyl-3-methoxypyrazines) strongly influence the scent and flavour of wine at low concentrations (Cudjoe *et al*, 2004; Pickering *et al*, 2007; Pickering *et al*, 2008). In pinot noir the sensory threshold was found to be 3ng/L and 11ng/L for Reisling when using 3-isopropyl-2-methoxypyrazine (Kögel *et al*, 2011).

Other than these crude feeding and deterrence experiments and observations, there is very little known about the activity of ladybird alkaloids. Historically a number of diseases have been treated with ladybirds. Newell (1845) hints that ladybird beetles were used to treat measles and colic, but most

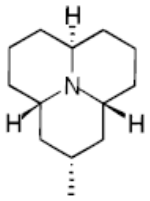
interestingly Jeager (1859) suggests that ladybirds could cure toothache when mashed and pushed into the pulp of a hollow tooth (cited in Majerus, 1994). These records must be taken for what they are, but the implication that ladybirds possess pain killing properties is possibly suggestive of ion channel blocking activity.

As previously stated, the first alkaloids isolated from ladybirds were coccinelline and its free base precoccinelline (Fig. 1.1.7) (Tursch *et al*, 1971) from the 7-spot ladybird. This initial discovery aroused the interests of chemists, and subsequently at least 36 novel ladybird alkaloids have been discovered from 41 species (Durieux *et al*, 2010) with the vast majority of species remaining unstudied. The alkaloids are structurally diverse and fall into a number of families including the azaphenalenes, homotropanes, dimeric alkaloids, pyrollidines, piperidines and azamacrolides (Glisan King & Meinwald, 1996). Most appear to be endogenously produced (Laurent *et al*, 2002; Laurent *et al*, 2003) because they have not been isolated from the aphids which ladybirds feed upon and they are found in similar concentrations in eggs, larvae (Fig. 1.1.6) and adult beetles (Tursch *et al*, 1975). 7-spot ladybirds were shown to incorporate ^{14}C-labelled acetate into the coccinelline they produced, suggesting that the azaphenalene alkaloids, produced by many species of ladybird, are synthesised via a polyacetate pathway (Tursch *et al*, 1975). A Kuhn-Roth oxidation of the radio-labelled coccinelline revealed that all of the carbon atoms were derived from the labelled acetate molecules. It was hypothesised that the homotropane ladybird alkaloids such as adaline are formed via the same biosynthetic pathway as the azaphenalenes (Tursch *et al*, 1973; Tursch *et al*, 1975; Ayer & Browne, 1977). This was confirmed by the isolation of *cis*-1-(*6*-methyl-2-piperidyl)-propan-2-one (Fig. 1.1.7) from the mealworm ladybird (*Cryptolaemus montrouzieri*) which is an intermediate between the two structural groups (Brown & Moore, 1982).

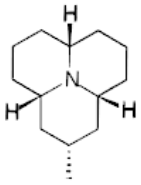
Azaphenalenes:



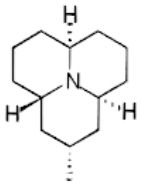
Coccinelline



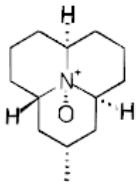
Precoccinelline



Myrrhine

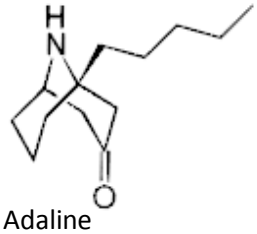


Hippodamine

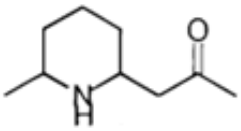


Convergine

Homotropane:

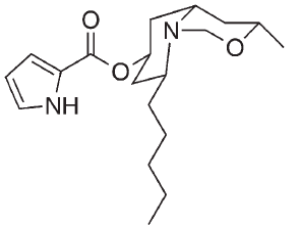


Adaline



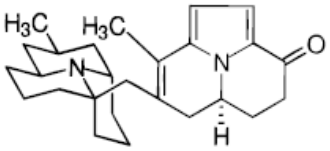
cis-1-(*6*-methyl-2-piperidyl)-propan-2-one

3-oxaquinolizidine:



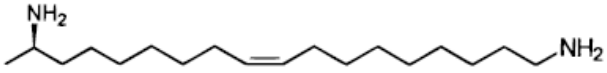
Hyperaspine (Laurent et al, 2005)

Dimeric:



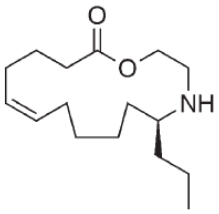
Exochomine

Acyclic amine:



Harmonine

Azomacrolide:



Epilachnene

Figure 7. Structures of alkaloids produced by ladybirds showing the diversity that exists. Structures from Glisan King & Meinwald (1996) unless otherwise stated.

A further labelling experiment has shown that dietarily aquired acetate is incorporated into the piperidine alkaloid 2-(2'-oxopropyl)-6-methylpiperidine by

Epilachna paenulata and that males transfer alkaloids to females during mating, meaning both parents contribute to the alkaloid content of eggs (Camarano *et al*, 2009). Four pyrrolizidine alkaloids sequestered by the ant attended aphid *Aphis jacobaea* from its host plant *Senecio jacobaea* have also been found at high concentrations in the 7-spot ladybird. These are the result of organic toxin recruitment events rather than ladybird biosynthesis and no other alkaloids found in ladybirds appear to be the result of sequestration (Hartmann & Toppel, 1987; Hartmann *et al*, 1989).

Only one other species has been found to contain ladybird alkaloids, the cantharid beetle *Chauliognathus pulchellus* from Australia. Moore and Brown (1978) isolated precoccinelline, hippodamine and propyleine from this species. It is not known whether this species produces these alkaloids *de novo* or sequesters them from ladybirds.

Synthetic routes to a number of ladybird alkaloids have been discovered (e.g. Ayer & Browne, 1977; Glisan King & Meinwald, 1994; Yamazaki *et al*, 1999). These include all of the azaphenalenes shown in figure 1.1.7. The structural diversity means that different scaffolds and techniques must be adopted to synthesise these alkaloids. Figure 1.1.8 shows the synthetic route to a number of azaphenalenes developed by Ayer *et al* (1977) and described in detail by Glisan King & Meinwald (1994). The process begins with the monolithium derivative of 2,4,6-collidine which is mixed with 3-brompropionaldehyde dimethyl acetal. The addition of phenyllithium produces an anion which can be treated with acetonitrile to produce a ketone. When reduced with sodium and isoamyl alcohol, a solution of saturated stereoisomeric amines is produced. This is then chromatographed and a racemic compound is formed, this can then be heated with *P*-toluenesulfonic acid to form a ketone with the stereogenic center required for the ladybird alkaloid myrrhine. Conversion of this ketone to thioketal followed by desulfurisation with

Raney nickel yields myrrhine. This can then undergo a number of processes to become any of the azaphenalene ladybird alkaloids.

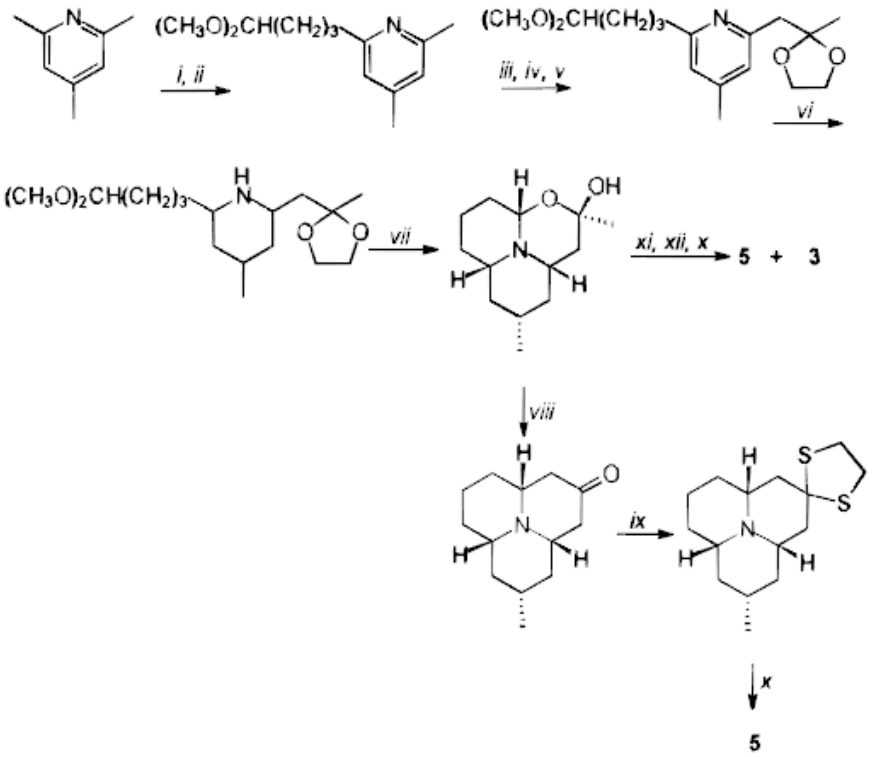


Figure 1.1.8 The synthetic route to azaphenalene ladybird alkaloids, developed by Ayer & Browne (1977). Figure from Glisan King & Meinwald (1994).

Alkaloids are a structurally and functionally diverse group of natural products (Harborne, 1993). They can be broadly defined as organic molecules which contain basic nitrogen in some form of ring structure. Differentiating alkaloids from other secondary, nitrogen containing compounds is difficult. Bringing together the descriptions put forward by Winterstein and Tier (1910) and Waller and Nowacki (1978) a compound can be considered an alkaloid if it; is basic in pH, contains nitrogen connected to two or more carbon atoms and contains at least one ring structure which is usually heterocyclical. Waller and Nowacki (1978) stated that the structural units of macromolecular cellular substances, hormones or vitamins should not be considered as alkaloids. In addition to this Eagleson *et al*

(1994) also exclude amino acids, peptides, nucleosides, amino sugars and antibiotics. Alkaloids are poorly soluble in water, but readily dissolve in organic solvents such as methanol, ether and chloroform and often display bioactivity, notably toxicity affecting the central nervous system (CNS). This forms the basis for believing that ladybird alkaloids may also possess toxicity.

Alkaloids are produced by a diverse array of organisms including; bacteria, fungi, plants and animals. Of these, plants produce the greatest number of alkaloid compounds, many of which are utilised by man as medical drugs (e.g. atropine from *Atropa belladonna*, morphine from *Papaver somniferum*), stimulants (e.g. caffeine from members of the genus *Coffea*, nicotine from members of the genus *Nicotiana*), recreational drugs (e.g. cocaine from members of the genus *Erythroxylum*, heroin also from *Papaver*) and research probes (e.g. tubocurarine from *Chondrodendron*). The structural heterogeneity of these compounds allows for a diverse range of bioactivities. In their native state they may act as toxins to predators or parasites, or they may serve to deter predators by tasting and smelling unpleasant.

Although most ladybirds only produce one or two structurally analogous alkaloids, some species of ladybird are known to produce a multitude. The largest number isolated from a single species is 12, from the Mexican bean beetle (*E. varivestis*) (Laurent *et al*, 2005).

1.5 Uses for Alkaloids:

The bioactive properties of many alkaloids make them very useful for a range of applications. Historically alkaloids of plant origin have been used by the medical and pharmaceutical industries to treat a diverse range of conditions. The huge structural diversity is matched by a huge range of physiological targets and activities. Examples include quinine, isolated from the bark of *Cinchona spp.* which is an effective anti-malarial, anti-pyretic, analgesic and anti-inflammatory. Atropine

from *Atropa belladonna* is a competitive antagonist of the muscarinic acetylcholine receptor and as such, has a number of applications, most notably in reversing bradycardia in emergency resuscitation (Scheinman *et al*, 1975). In contrast, the indole alkaloid reserpine from *Rauwolfia serpentina* has been used to reduce blood pressure (Shamon & Perez, 2009) in cases of hypertension. Reserpine has a number of activities but is used clinically because it causes deamination of catecholamines, blocking transport into synaptic vesicles and leading to a suppression of the sympathetic nervous system because of a reduction in free noradrenaline (Kopin & Gordon, 1962). One of the most widely used pharmaceutical alkaloids is morphine, one of a complex mixture of opiate alkaloids produced by the poppy *Papaver somniferum*. Morphine agonises opioid receptors (Abdelhamid *et al*, 1991) in the central nervous system and has potent analgesic properties. Even the non-specialist is likely to be familiar with some regularly encountered alkaloids for example; stimulants including caffeine and nicotine, narcotics such as cocaine and heroin and psychedelic drugs such as lycergic acid diethylamide (LSD). All of these aforementioned compounds display toxicity if given in a sufficiently high dose and some alkaloids have been exploited as poisons to kill a variety of organisms. Strychnine is an alkaloid produced by members of the genus *Strychnos* and is a competitive antagonist of both glycine-gated Cl- channels and nicotinic acetylcholine receptors (Matsubayashi *et al*, 1998). It causes rapid death in humans at low concentrations and has been widely used in suicide attempts and poisonings. Strychnine, along with nicotine and several other alkaloids has also been used as an insecticide. One of the most widely used groups of insecticides, accounting for roughly 17% of the global market, is neonicotinoids (Jeschke & Nauen, 2008). Neonicotinoids are synthetic alkaloids with structural similarities to nicotine and the frog alkaloid epibatidine.

1.6 The Need for Insecticides:

Global population is expected to reach 9.3bn by 2050 (United Nations, 2011), with the majority of growth being seen in the developing world (GAP Report, 2010). Currently 13.6% of people are considered undernourished with a global population of 6.8bn (FAO, 2010) and 35% of deaths of under five year olds are attributed to starvation (WHO, 2011). If the human population is to have sufficient food, fibre and energy crops, then agricultural productivity per unit area must increase significantly, with fewer petro-chemical inputs (Bruinsma, 2003, Gregory & George, 2011). By 2050 arable productivity will need to increase by at least 25% to maintain current per capita consumption (Bruinsma, 2009; Smith *et al*, 2010) and global production will need to increase by 70% to ensure that the minimum calorie requirement of the human population is met (FAO, 2009). One of the major drains on agricultural output is pest damage. The Oxford English Dictionary defines a pest as "an animal that competes with humans by consuming or damaging food, fibre, or other materials intended for human consumption or use". Insect crop pests may cause direct damage by feeding directly on plants (e.g. locusts), or by acting as parasites and consuming sap (e.g. aphids). They may also act as vectors for diseases such as pea streak virus (PSV) and potato leafroll virus (PLRV) (Robert *et al*, 2000). Estimating the proportion of crops lost to pests is inherently difficult to accurately achieve, but figures between 15-18% have been put forward as a global average (Oerke & Dehne, 2004). This can be as high as 40-50% in developing countries (CABI, 2009). Pesticides are substances used to prevent, destroy, repel or mitigate against pests (Ecobichon, 2003). Those specifically targeted for insect pests are classified as insecticides. Along with improvements in chemical fertilisers, irrigation and selective breeding and genetic modification for higher yielding cultivars, the development of novel synthetic pesticides has lead to a doubling in food production between 1971-2004 (Oerke & Dehne, 2004). If this rate of production increase is to be maintained, it is essential

to develop novel pesticides which are stable, cheap to produce and display the qualities of Paul Ehrlich's 'magic bullet', showing selective toxicity towards pest insects but low toxicity to non-target organisms.

As well as being responsible for crop damage, insects are major vectors for a number of human diseases. *Anopheles*, *Culex* and *Aedes* mosquitoes are found worldwide, are hematophagous and considered a nuisance in much of the developed world. They are also responsible for spreading pathogens which kill millions every year, including; malaria, yellow fever, dengue fever, encephalitis and West Nile virus. In Africa alone, where 90% of cases of malaria occur (Hemingway & Ranson, 2000), malaria accounts for 20% of all childhood deaths and 40% of public health spending (WHO, 2010). Of the World Health Organisation's 'neglected tropical diseases' 65% are caused or transmitted by invertebrates and 30% are spread by insects (WHO, 2011). Other insect disease vectors are; tsetse flies, sandflies, triatomid bugs, fleas and lice (Hemingway & Ranson, 2000). After agriculture, disease vector control accounts for the second largest consumption of insecticides. By virtue of their hematophagous nature, vector insects are found in close association with people, and therefore insecticides used for their control must cause rapid incapacitation and show high specificity. Pyrethroid compounds have been the insecticides of choice for impregnating bed nets and house spraying because, in contrast to many other insecticides, they are comparatively safe, fast acting (Curtis *et al*, 2003) and serve to deter as well as kill mosquitoes (Lindsay *et al*, 2008). Unfortunately these insecticides become less effective over time and it is essential to develop new compounds.

1.7 The Need for New Insecticides:

There will always be a need to develop novel insecticides. Currently there is a drive to discover more target specific replacements for older insecticides such as

organochlorines, organophosphates and carbamates, which can be dangerous to the people that are regularly exposed to them, or exposed to high doses (Zweiner & Ginsburg, 1988; Kamanyire & Karalleidde, 2004). They may also be damaging to the environment (Capkin *et al*, 2006). The major reason to develop new insecticides however is the increasing incidence of insecticide resistance in crop pests and disease vectors (Hemingway & Ranson, 2000). Treating crops with chemical insecticides applies a strong selective pressure on affected pests, leading to the development of insecticide resistance (Brattsten *et al*, 1986). A number of mechanisms responsible for the development of insecticide resistance have been identified; penetration resistance – changes to the cuticle which reduce the amount of insecticide reaching the target (e.g. DeVries & Georghiou, 1981); behavioural resistance, whereby pests avoid crops treated with insecticide (Sparks *et al*, 1989); metabolic resistance – the development or up-regulation of detoxifying enzymes which reduce the efficacy of insecticides (e.g. Daborn *et al*, 2002); and finally, target site resistance – subtle changes such as point mutations to the binding site of an insecticide target (e.g. Liu *et al*, 2005). The two most widespread and economically important mechanisms are metabolic and target site resistance (Brattsten *et al*, 1986).

By 1990 over 500 incidences of insecticide resistance had been reported (Georghiou, 1990) and this number has been increasing steadily since. Some species have developed resistance to all major classes of insecticide (e.g. *Lygus linea* [Snodgrass, 1996]), and because of this these are often treated with combinations of insecticide. In Michigan, the cost of controlling the Colorado potato beetle varied from $35-74/ha in non-resistant populations, to $306-412/ha in areas with high levels of resistance (Grafius, 1997). Even with treatment, up to 12.2-20.5% of the crop was damaged, which caused a $13.3M loss to the industry in 1994 (Grafius, 1997).

For these reasons it is essential to continue developing novel insecticides so that appropriate, safe and effective treatments are available to farmers and those wishing to control disease vectors (Zaim & Guillet, 2002; Liu *et al*, 2006). Since organochlorine, organophosphate and carbamate pesticides fell out of favour in the latter half of the 20th century (Casida, 1980) the search for compounds with insecticidal activity has focussed on natural products of plant origin. Pyrethroid insecticides account for roughly 17% of the global market. They are synthetic analogues of pyrethrum, a compound found in *Chrysanthemum* daisies, which are able to rapidly 'knock down' flying insects. Although overshadowed by plant derived compounds, alkaloids produced or sequestered by frogs and arthropods have recently been put forward as lead structures for insecticides. Imidacloprid, and other neonicotinoid insecticides are alkaloids which possess a 6-chloro-3-pyridyl moiety which is also present in the arrow frog alkaloid epibatidine and known to be a nicotinic acetylcholine receptor (nAChR) agonist (Matsuda *et al*, 2001). The alkaloid nereistoxin produced by *Lumbriconereis heteropoda*, a marine worm, served as the lead for the nAChR modulating insecticide cartap (Tomizawa & Catsida, 2003).

1.8 Ion Channels:

Electrical communication is a fundamental feature of complex organisms, and may also prove crucial for some single-celled organisms (Nielsen *et al*, 2010). The generation of electrical signals enables voluntary movement, involuntary movement such as the expansion and contraction of the muscles responsible for respiration, sensation of the environment and cognition of the information then received. Rapid electrical signalling is achieved by the successive movement of ions across cell membranes, along a neural pathway in the form of a pulse. The speed at which these signals travel is highly variable, and dependent upon a multitude of

factors not limited to; presence/absence of myelination, diameter of nerve fibre or axon, the voltage across the membrane and temperature.

The cytoplasm of a cell is electrically insulated from the extracellular space by the lipid bilayer of the plasma membrane. This acts as a barrier to ions, and enables a voltage to be maintained across the membrane. This membrane potential is essential for rapid electrical communication within the nervous system. The transport of ions across membranes, down an electrochemical gradient, is by means of ion channels; a ubiquitous component of both prokaryotic and eukaryotic organisms (Jan & Jan, 1997; Anderson & Greenberg, 2001). Active transport of ions against an electrochemical gradient is achieved by specific pumps. This process requires energy in the form of ATP (Hodgkin & Keynes, 1955; Caldwell *et al*, 1960) and plays a minimal role in electrical communication. Ion channels serve a number of functions, both in maintaining homeostasis and in the propagation and conduction of action potentials. These transmembrane pores are responsible for maintaining cell turgor and osmotic balance (Martinac *et al*, 1987), in addition to maintaining the membrane resting potential needed for electrical communication by means of action potential propagation and conduction. This resting potential is achieved by ensuring the concentration of ions on either side of the membrane is carefully regulated with the net charge generated by anions and cations necessarily unbalanced on either side of the membrane. The resting potential of a neuron is usually between -40 and -90mV (Levitan & Kaczmarek, 1991) caused by intracellular Cl^-, with an excess of extracellular Na^+. The intracellular concentration of K^+ is much higher than the extracellular, but the positive charge is outweighed by intracellular anions and the net charge on the cell is still negative. The relationship between ion concentration on either side of the membrane and voltage is described by the Nerst equation:

$$E = \frac{RT}{F} \log_e \frac{[\mathrm{X}]\mathrm{o}}{[\mathrm{X}]\mathrm{i}}$$

Where *E* is the membrane potential (mV) inside relative to outside, *R* is the universal gas constant, *T* is the absolute temperature (K), *F* is the charge carried by 1 gram-equivalent of ions (C) and $[X]_{i/o}$ represent the intracellular/extracellular concentration of ions.

Action potentials are the result of changes in membrane permeability to Na^+ and K^+ as a function of time and membrane potential. These changes are exacted by the opening of functionally and structurally different ion channels, in response to a range of stimuli; change in voltage across the cell membrane, the binding of a ligand in the form of a neurotransmitter or hormone, or in response to a physical stimulus such as heat or pressure. Ion channels are classified by ion specificity, mechanism of gating and more recently by sequence homology. Unitary channel conductance and pharmacology are also included in some classifications.

1.9 Ion Channels as Insecticide Targets:

The ubiquity and fundamental importance of ion channels makes them ideal targets for insecticides (Raymond-Delpech *et al*, 2005). For this reason, all five major insecticide classes rely on ion channels to achieve toxicity. Insecticides need to rapidly immobilise pest insects to be effective, and to do this they must target the ion channels found in major circuits of the central nervous system. The most commonly utilised targets are the voltage gated sodium channel (VGSC) and the nicotinic acetylcholine receptor (nAChR), both essential for action potential propagation and transmission in both the central and peripheral nervous systems. These ion channels are common to vertebrates and invertebrates, therefore the insecticides must exploit subtle molecular differences to target insect channels.

Pyrethroid insecticides selectively target the voltage gated sodium channel of arthropods. These channels open in response to a change in membrane potential and transmit a depolarising pulse along an axon. The change in voltage required to

cause a channel opening event is tightly regulated, but the binding of pyrethroid compounds shifts this activation voltage in the hyperpolarising direction (Tatebayashi & Narahashi, 1994). This results in rapid 'knock down' immobilisation with a characteristic twitching (Chen *et al*, 1985).

Nicotinic acetylcholine receptors are targeted by a range of insecticides (Matsuda *et al*, 2001). Organophosphates non-selectively inhibit acetylcholine esterase, preventing acetylcholine (ACh) molecules from disassociating from the receptor. Nitromethylenes agonise nAChR, as do neonicotinoids. The nAChR therefore represents a suitable target for insecticide lead structures.

1.10 The Nicotinic Acetylcholine Receptor:

The nAChR is a ligand gated non-selective cation channel responsible for mediating fast cholinergic synaptic transmission (Alkondon *et al*, 1998; Matsuda *et al*, 2001). This receptor has been the subject of great research attention because it was the first example of a neurotransmitter receptor to be identified (Langley, 1907) and because of the key role it plays in information transfer throughout the nervous system of vertebrates and in the central nervous system of invertebrates (Breer & Sattelle, 1987). The receptors were first characterised as a separate entity from acetylcholinesterase (AChE) by Changeux *et al* (1970), using receptors purified from the electric organ of the electric fish *Electrophorus electricus* using α-bungarotoxin (α-BgTX). Subsequent work has revealed that nAChRs are allosteric membrane glycoproteins of ~300kDa (Changeux & Edelstein, 1998). The length of the protein is approximately 16nm and the diameter roughly 8nm (Changeux & Taly, 2008). The receptor is composed of five subunits displaying pentameric symmetry (Fig. 1.1.9) arranged around a central, funnel shaped pore. Each subunit has four symmetrically arranged membrane spanning α-helices (M1-M4) and the pore is lined by the M2 and N-terminal of M1 (Miyazawa *et al*, 2003; Unwin, 2005).

The channel pore is formed of rings of amino acid residues, most of which are non-polar (Miyazawa *et al*, 2003). When in the closed conformation a hydrophobic ring is formed by the inner M2 helices, this causes the pore to taper and prevents ion permeation (Miyazawa *et al*, 2003). Crystallography has revealed that the α-subunits fall slightly out of symmetry with the non-α subunits when the channel is at rest. Activation of the channel by a ligand causes the β-sheets of these subunits to rotate through 15°, and this interrupts the hydrophobic interactions which form the gate. A concomitant configuration change in the M2 helices, causing the centre of the pore to widen, accompanies rotation of the α-subunits. This opens the pore and allows ions to flow (Miyazawa *et al*, 2003; Unwin, 2005).

There are five distinct subunit families, namely α, β, γ, δ and ε and configuration of these subunits accounts for the known pharmacological diversity of nAChRs (Dani & Bertrand, 2007). The most common arrangement in vertebrate muscle is 2α, β, γ, δ but variations in this configuration cause changes to the functional and pharmacological property of the receptor (Dani & Bertrand, 2007). A distinction can be made between the muscle type and neuronal type nAChR (Corringer *et al*, 2000), the former are composed of α1, β1, γ, δ and ε, whereas the latter can be formed of α2-10 and β2-4 subunit combinations or α7-9 subunit homomers (Tomizawa & Casida, 2003; Dani & Bertrand, 2007). Muscle type nAChR are found only at the neuromuscular junction and the electric organ of *Torpedo* whereas neuronal type nAChR occur on both pre and post-synaptic membranes in the vertebrate brain and ganglia (Brejc *et al*, 2001; Tomisawa & Casida, 2003). Another distinction can be made between foetal and adult muscle nAChR. In foetal muscle and electrocytes, as opposed to adult muscle the ε subunit is replaced by γ (Reynolds & Karlin, 1978; Mishina *et al*, 1986). Both muscle and neuronal nAChR are placed in the superfamily of neurotransmitter-gated ion channels known as Cys-loop receptors along with $GABA_A$, $5\text{-}HT_3$, glycine and γ-aminobutyric acid receptors (Tomizawa & Casida, 2003; Dani & Bertrand, 2007). This is because all

subunits have a pair of cysteine residues connected by a disulphide bond on their extracellular amino-terminals.

The channel exists in three states; closed at rest, open pore and closed desensitised (Dani & Bertrand, 2007). ACh is the endogenous excitatory neurotransmitter released from the pre-synaptic membrane which activates the nAChR. A molecule of acetate combines with one of choline by means of an ester bond. Crystallography of the glial ACh binding protein (AChBP) of *Lymnaea* reveals that the quaternary ammonium group of the ACh molecule interacts covalently with the hydrophobic Arg B104, Val B106 and Leu B112 residues of the binding site (Brejc *et al*, 2001). The binding site of muscle type nAChR is formed by the interface of the extracellular domains of the α-subunits and neighbouring ε/γ or δ subunit of the channel (Silman & Karlin, 1969; Karlin, 2002). At least two molecules of ACh are required to activate the channel (Dani & Bertrand, 2007), and for this reason it is not possible to generate a functional channel with fewer than two α-subunits. Crystallography has revealed that the pore has a diameter of 20Å at both the intracellular and extracellular vestibule, and projects 60Å into the synapse from the membrane surface (Unwin, 2005).

Three structural features appear to be essential for cation selectivity; the presence of an anionic glutamate residue within the opening of the pore, the replacement of a valine with a threonine in TM2 of the channel surface and the absence of a proline between TM1 and TM2 (Galzi *et al*, 2002). Reversal of these parameters changes the selectivity of α7 homomeric nAChR to anionic.

The nAChR of vertebrates is much better studied than that of invertebrates (Tomizawa & Casida, 2003), but they appear to be pharmacologically distinct from one another (Osborne, 1996; Tomizawa & Casida, 2003). Insect nAChR respond weakly to suberyldecholine, a potent agonist of mammalian nAChR. K-bungarotoxin acts as a more potent antagonist of insect nAChR than α-bungrotoxin, and both bind reversibly (Albert & Lingle, 1993). This structural differentiation allows the

development of highly selective insecticides, such as the neonicotinoids. Expression studies using *Xenopus* oocytes have shown that non-α subunits of the nAChR are responsible for the selective affinity of neonicotinoids to insect nAChR (Lansdell & Millar, 2000).

The complexity of nAChR channels allows pharmacologically active compounds to interact with the channel in a large number of different ways. The majority of non-competitive inhibitors are thought to bind to the central lumen of the pore (Jozwiak *et al*, 2007). Whereas the most famous nAChR antagonist α-BgTX binds irreversibly with the nAChR through complex protein-protein and protein-sugar interactions (Dellisanti *et al*, 2007). The polyamine containing wasp philanthotoxins display activity consistent with having two binding sites. Different analogues are able to bind to different parts of the pore and display potencies and voltage dependencies (Brier *et al*, 2003).

1.11 Electrophysiology:

Electrophysiology is the study of the electrical phenomena of cells, nervous systems and whole organisms. Luigi Galvani's 1791 discovery that frog muscles contract in response to an electrical spark triggered research into neurotransmission. However, it wasn't until the 1950s when Hodgkin and Huxley developed the voltage-clamp technique and published their experiments with squid giant axons that an understanding of ionic currents and action potentials was achieved. In order to conduct their experiments Hodgkin and Huxley needed to control the membrane potential. This is because the amplitude of an ionic current changes in direct proportion to the voltage and resistance across the cell membrane in accordance with Ohm's law:

$$I = \frac{V}{R}$$

I is current, *V* is voltage and *R* represents resistance. In order to accurately elucidate the relationships governing ion channel currents and neuronal transmission, it is important to control the voltage across the cell membrane. The advent of electrical amplifiers allows the simultaneous recording of membrane potential and injection of current. A number of techniques have been developed to measure the electrical activity of cells: Voltage clamp utilises two electrodes filled with an electrolyte solution to impale a cell, one to inject current and the other to measure the membrane potential. The experimenter sets the membrane potential at a desired voltage. It is then possible to record the current injected to maintain the membrane potential at the set level. This technique requires cells to be large enough to withstand being pierced with two electrodes and so squid giant axons were used originally. More recently oocytes from the African clawed frog (*Xenopus laevis*) have been utilised. It is possible to use oocytes to express non-native proteins by injecting the corresponding DNA or RNA. This is then transcribed and the protein assembled. In the case of ion channels and receptors these are translocated to the membrane.

The Patch-clamp technique (Fig. 1.1.10) was developed by Neher and Sackmann in the 1980s. Patch-clamp is a technique that requires only a single electrode to simultaneously measure membrane potential and inject current. It is possible to record the electrical activity of the whole cell, or individual ion channels in the membrane. The electrode used is pulled to a very fine tip and filled with an electrolyte solution which closely matches the cytoplasm. In order to record whole-cell currents, the aperture is placed on a section of cell membrane and negative pressure applied. This breaks the membrane and pulls it into the electrode. The seal formed has a very high resistance and effectively blocks ion leakage. Any ions flowing into, or out of the cell can be recorded by a highly sensitive current-to-

voltage converter. To record from a single ion channel or patch of membrane it is possible to place the aperture against the membrane, and apply a small amount of negative pressure which generates a high resistance seal, but does not damage the membrane patch in contact with the electrode. The major advantage of patch-clamp recording is that it can be used on much smaller cells than two electrode voltage-clamp. This removes the need to use an expression system and allows a much wider range of native cells to be used, including the neurons of small insects.

1.12 The TE671 Cell Line:

In order to make electrophysiological recordings it is necessary to have suitable cells to work with. Immortalised lines are widely used because they can be cultured easily, and once characterised, provide a reliable and consistent source of ion channels. The TE671 cell line (Fig. 1.1.11) is one such immortalised culture of human origin. This line was first derived by McAllister *et al* (1977) from a cerebellar medulloblastoma tumour and consists of undifferentiated preneuroglial cells. It was found that TE671 cells have a doubling time of approximately 18hours if kept at 37°C in an atmosphere of 5% $C0_2$ (Synapin *et al*, 1982). Five visibly differentiable cell types were recognised by Synapin *et al* (1982), present during all growth stages. These are: "(1) Large, flat, sometimes multinucleated cells; (2) small, round or polygonal cells; (3) Long, centrinuclear bipolar spindle cells; (4) monopolar, mononuclear cells; and (5) small flat cells, sometimes with branching extensions."

TE671 cells have been shown to respond to ACh, and this response could also be antagonised by α-BgTX, D-tubocurarine or decamethonium, but only weakly by atropine (Synapin *et al*, 1982). For this reason the ACh response of TE671 is thought to be mostly nicotinic. This is further supported by the discovery that the TE671 nicotine response is identical to the ACh response (Grassi *et al*,

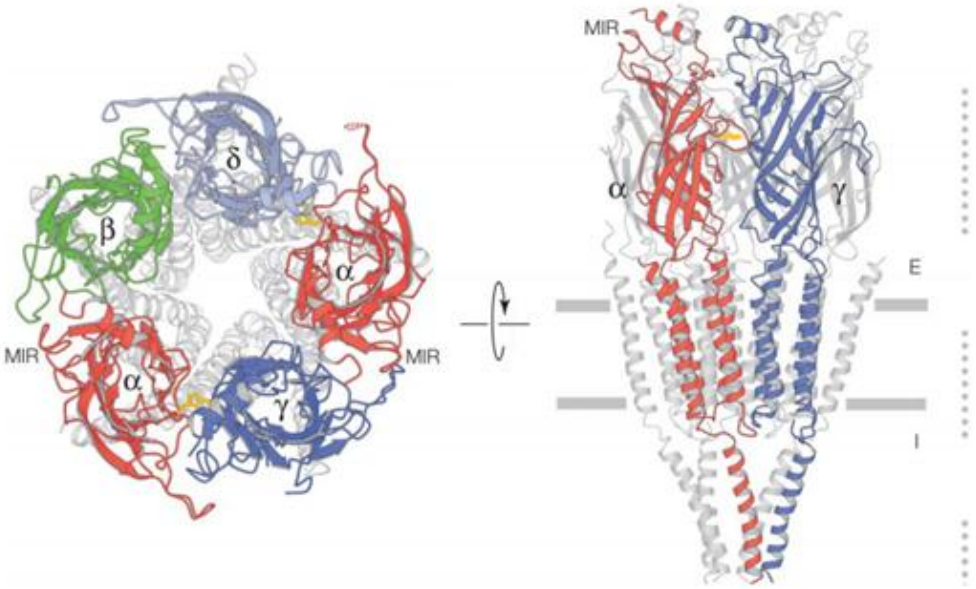


Figure 1.1.9. Ribbon diagram of of muscle type nAChR as viewed from the synaptic cleft (left) and horizontal with the plasma membrane (right). Ligand binding domain is highlighted on the α-subunits. From Unwin (2005).

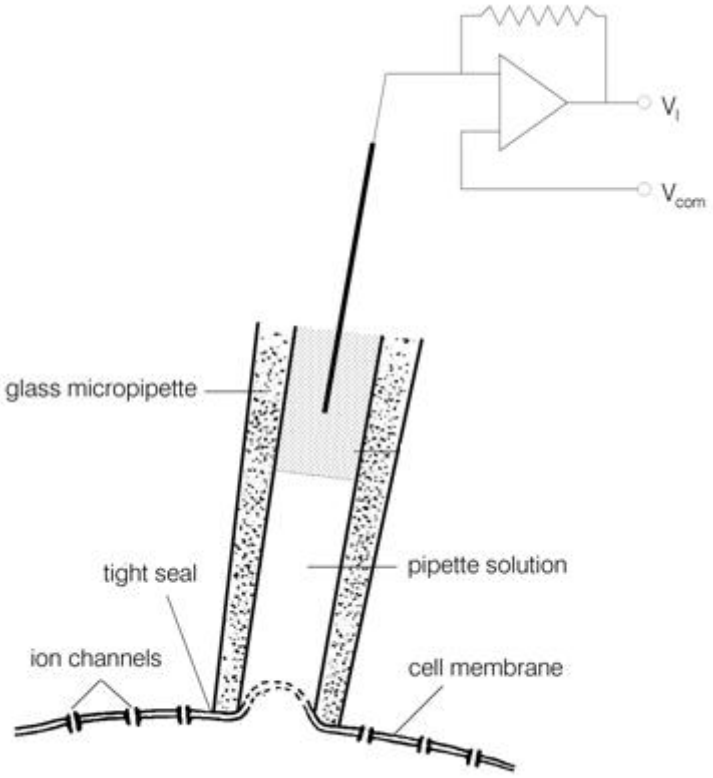


Figure 1.1.10 Simplified diagram showing the patch-clamp technique. From Mergler, 2010.

1993) and although M3 muscarinic receptors are present (Bencherif & Lukas, 1991), they are not detectable using electrophysiology. For this reason they are a good model for human nAChR. Oswald *et al* (1989) and Luther *et al* (1989) both showed that TE671 cells show channel activity to ACh in the μM range, but do not respond to co-application of ACh with α-BgTX.

A binding site for acetycholine (ACh) and α-BgTX was first noted by Syapin *et al* (1982) and the consensus was that TE671 cells express neuronal type ACh receptors because they originated from neuronal tissue. Shoepfer *et al* (1987) demonstrated that the nAChR expressed by TE671 are actually composed of muscle type α-subunits. Further work revealed that the TE671 nAChR has subunit stoichiometry $(\alpha 1)_2\beta 1\gamma\delta$, the presence of γ subunits instead of ε showing that TE671 is an embryonic muscle cell line (Schoepfor *et al*, 1987; Luther *et al*, 1989; Yamamoto *et al*, 1991). The discovery of a rare point mutation at the third base of codon 61 in the N-*ras* gene alerted Stratton *et al* (1989) that TE671 was likely to be a rhabdomyosarcoma rather than medulloblastoma. DNA fingerprinting confirmed that TE671 is a derivative of the same line as RD cells and should be considered to be rhabdomyosarcoma. The presence of muscle-type nAChR and other phenotypic characteristics of TE671 are explained by the fact that this cell line is of muscle rather than neuronal origin (Stratton *et al*, 1989).

1.13 Locust (*Schistocerca gregaria*) Neurons:

Locusts are swarming insects belonging to the order Orthoptera, along with crickets and grasshoppers. They have often been used as model insects in experimental biology (e.g. Osborne, 1996; Bicker, 2007) because they are easy and cheap to acquire and maintain, and they are also large and robust enough to tolerate many experimental procedures (Osborne, 1996). Glutamate is thought to be the main neurotransmitter in insect nervous systems (Usherwood & Machili, 1966; Usherwood & Machili, 1968) but ACh is also a known neurotransmitter in the

insect central nervous system (Suter & Usherwood, 1985; Breer & Satelle, 1987) and insect nervous tissue is particularly rich in nAChR (Matsuda *et al*, 2001). Locust (*Locusta migratoria*) synaptosomes were shown to convert radio-labelled choline into acetylcholine, this was then released into the synaptic cleft in response to a Ca^{2+} influx (Breer & Knipper, 1984). Isolated locust (*Schistocerca gregaria*) neurons have been shown to possess α-BgTX sensitive ACh receptors (Kerkut *et al*, 1969) and activate in response to ACh at a minimum concentration of 10μM (Suter & Usherwood, 1985). Treatment of neurons separately with atropine and δ-tubocurarine revealed that both nicotinic and muscarinic acetylcholine receptors are present (Suter & Usherwood, 1985). The consensus of Osborne (1996) was that receptors with a nicotinic pharmacology are better represented in insect central neurons than muscarinic. The difference in abundance is thought to be at least one order of magnitude for *Drosophila* (Salvatera & Foders, 1979). A protocol for dissecting, culturing, Ca^{2+} imaging and patch-clamping Kenyon cells (Fig. 1.1.12) from the domestic cricket (*Acheta domesticus*) is described by Cayre *et al* (1998). Further experiments have revealed that these cells, found in the paired mushroom bodies express receptors for ACh, GABA, glutamate, octopamine, dopamine (Cayre *et al*, 1999). The ACh induced inward current was mimicked by nicotine, unaffected by atropine and reduced by α-BgTX and mecamylamine (Cayre *et al*, 1999). The mushroom bodies of insects have been implicated as responsible for olfactory and spatial memory in insects (Heisenberg *et al*, 1985; Mizunami *et al*, 1993). Locust brains have also been shown to express nAChR (Hanke & Breer, 1986) and these provide a convenient model insect nAChR for the purposes of electrophysiology.

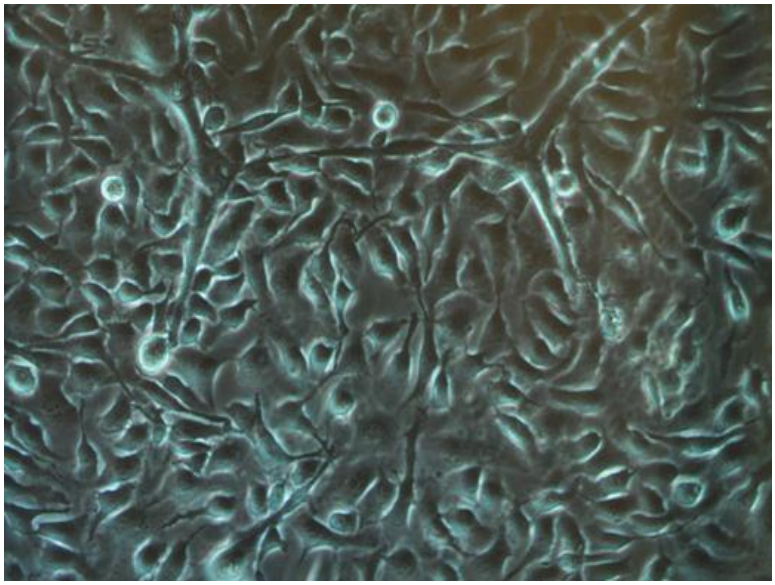


Figure 1.1.11 TE671 cells viewed at 200x magnification. A number of morphologically distinct cell types can be seen.

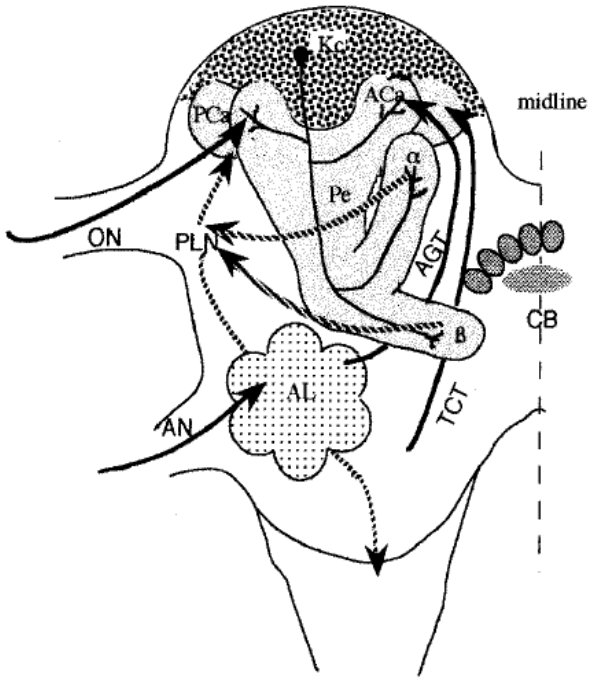


Figure 1.1.12 Schematic of the left half of a brain from *Acheta domestica*. Kc, Kenyon cells; ACa, anterior calyx; PCa, posterior of adult Kenyon cells, calyx; Pe, pedunculus; a, lobe; b, lobe; ON, optic nerve; AN, antennal nerve; AL, antennal lobe; AGT, antennoglomerular tract; TCT, tritocerebral tract; PLN, protocerebral lateral neuropil; CB, central body. From Cayre *et al*, 1999.

1.14 Aims:

The aim of this project is to investigate the effects of ladybird extracts and pure alkaloids on human muscle type nAChR expressed by TE671 cells, and locust (*Schistocerca gregaria*) neuronal nAChR. We wish to discover whether these alkaloid compounds display activity and may be the basis for ladybird toxicity and aposematism. Further to this, if they do display activity we wish to find whether they display selectivity for either human nAChR or those of locusts, and whether they could serve as novel insecticide lead structures.

Two species of commonly encountered British ladybird, the 7-spot (*C. 7-punctata*) and the pine ladybird (*Exochomus 4-pustulatus*) (Fig. 1.1.13) along with the invasive Asian harlequin ladybird (*H. axyridis*) (Fig. 1.1.2) will be wild collected in the UK. Collection of beetles will be followed by solvent and acid/base extraction. This will allow us to quantify the amount of alkaloid produced by these beetles. Comparison of high performance liquid chromatography (HPLC) traces for harlequin and 7-spot ladybird alkaloid extracts will enable us to see whether regular intraguild feeding ladybirds (harlequins) show a greater diversity of alkaloids than the aphidophagous 7-spot ladybird. This could be indicative of alkaloid sequestration in harlequin ladybirds.

Whole cell patch-clamp will be used to elucidate the activity of ladybird compounds when co-applied with Ach. This project aims to build upon previous experiments (Ian Mellor, Ian Duce, Mike Birkett, John Pickett & Francesca Cash, unpublished data) which suggest adaline, precoccinelline and hippodamine show activity against nAChR. The extract used will be from the harlequin ladybird (*H. axyridis*) (Fig. 1.1.2) which is known to produce harmonine, an acyclic amine alkaloid (Fig. 1.1.7). This species was chosen because intraguild feeding experiments have demonstrated that whole beetles are toxic to other ladybirds (Yasuda & Ohnuma, 1999; Sato & Dixon, 2004; Kajita *et al*, 2010). They are also comparatively large in size, and occur in large numbers in many parts of the UK

simplifying the task of collecting enough material for electrophysiology. Gas chromatography has revealed that the mean harmonine content is 106.6µg/beetle and vibrancy of elytral colouration appears to correlate with harmonine content (Bezzerides *et al*, 2007; Fischer *et al*, 2010). If harmonine proves to display toxic activity this will provide further evidence that the bright colouration of harlequin ladybirds is an aposematic warning.

Figure 1.1.13 7-spot ladybird – *C. 7-punctata* (left) and pine ladybird – *Exochomus 4-pustulatus* (right).

Hippodamine is an azaphenalene alkaloid (Fig. 1.1.7) produced by the convergent ladybird (*Hippodamia convergens*) (Fig. 1.1.14). The development of a synthetic route to this alkaloid has meant that it is possible to obtain enough pure material to allow electrophysiology. The tri-cyclic carbon skeleton displayed by this alkaloid is found in a number of ladybird alkaloids (Fig. 1.1.7) and this makes these compounds useful for examining structure/function relationships, allowing us to study whether subtle differences in structure correspond with differences in toxicity. This will allow us to gain a better understanding of how these compounds interact with the nAChR.

Fundamentally, establishing the toxic actions of these compounds will allow us to gain insight into their functional significance, as well as providing a unique opportunity to discover lead compounds for novel pesticides.

Figure 1.1.14 Convergent ladybird (*Hippodamia convergens*). This species naturally produces hippodamine. From González, 2010.

2. Materials and Methods

2.1 Beetles:

Adult beetles of three species (*Coccinella 7-punctata*, *Exochomus 4-pustulatus* and *Harmonia axyridis*) were collected between September and December of 2010 from the grounds of University Park (Nottingham) and Rothamsted Research (Harpenden). Both sexes were collected and these were stored in 20ml universal tubes at -80°C until extraction.

2.2 Alkaloid extraction & purification:

Solvent extraction:

Frozen beetles of the three species were placed in separate conical flasks containing reagent grade methanol (~50ml/100beetles) with a glass bung covering the aperture. This was placed on a magnetic stirrer and left over night at room temperature. The methanol was decanted into a round bottom flask and the methanol evaporated using a Rotavapor evaporator (BUCHI, Switzerland). This process was repeated with fresh methanol and the precipitate from both extractions combined. Methanol extract from *H. axyridis* can be seen in figure 2.2.1.

Acid-base extractions:

The dried methanol extract was reconstituted with 50ml 1M hydrochloric acid (HCl) and decanted into a glass separation funnel. The flask was then washed with ether and poured into the separation funnel. The contents were then mixed and left to separate. The two layers were decanted into separate flasks and the bottom, aqueous layer was returned to the separating funnel. The top, organic layer was discarded. The flask was once again washed with ether and the contents placed in the funnel. This process was repeated three times. The resulting aqueous solution was adjusted to ~pH 10-12 using sodium hydroxide (NaOH) and returned

to the separating funnel. The flask was washed with dicholoromethane (DCM) and then added to the funnel. The contents were mixed and left to separate. The bottom, organic layer returned to the funnel and washed again with DCM. The top, aqueous layer was retained in a conical flask. The process was repeated three times. The final wash was made using reagent grade water. Magnesium sulphate ($MgSO_4$) was added to the organic layer to remove any water from solution. The remaining liquid was then passed through a filter paper into a clean round bottom flask and stored at 4°C. This solution was evaporated and the distillate dissolved in a minimal volume of DCM. A small sample of this extract was blot tested with Dragendorff's reagent to confirm the presence of alkaloids. The remaining extract was transferred to a clean glass vial and dried under a stream of nitrogen. The resulting precipitate was stored at 4°C.

High Performance Liquid Chromatography (HPLC):

Dried extracts were dissolved in a running solvent (H_2O + 0.1% HCOOH + 1% tetrahydrofuran [THF]) to a concentration of either 10mg/ml (*H. axyridis*) or 20mg/ml (*C. 7-punctata*) and then put through preparative scale, reverse phased HPLC (Shimadzu SCL-10AVP, Shimadzu Intruments, Japan). The stationary phase (250x4.6mm, ACE HPLC Columns) was packed with C18 and the injection volume was 100µl. The organic solvent was acetonitrile (CH_3CN), the concentration of which increased from 5% to 15% over forty five minutes, the concentration then remained at 15% for ten minutes before the initial 5% concentration was resumed and maintained for a further five minutes. All peaks were collected in centrifuge tubes, and the process repeated.

Figure 2.2.1 A 10 ml universal tube containing harlequin ladybirds which have been washed with methanol. The yellow organic extract can be seen at the bottom of the tube.

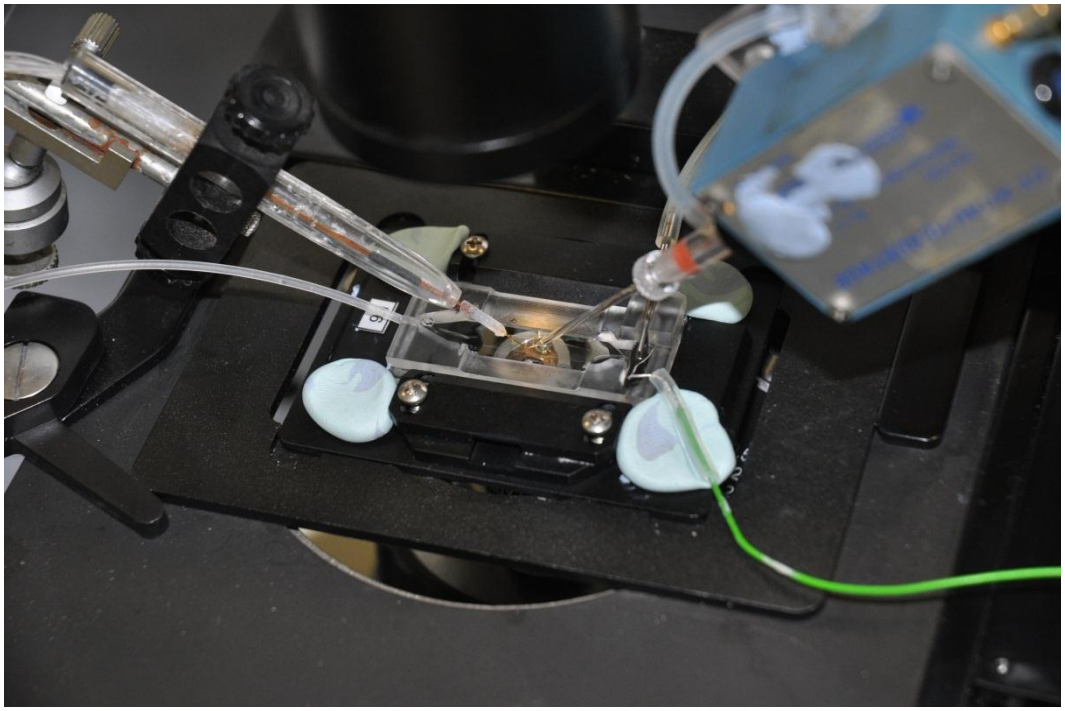


Figure 2.3.2 Perfusion chamber with micromanifold (left) and patch-clamp head stage with patch pipette electrode (right). Bath electrode can be seen leaving perfusion chamber to the right. Cover-slips with cells were placed in this set-up to make whole cell recording.

2.3 Cell culture

TE671 human muscle cells:

Cells were cultured in a Dulbecco's modified Eagles Medium (DMEM) with 10% foetal calf serum (FCS), 2mM glutamine (Gln) and 0.5% penicillin/streptomycin (pen/strep). Cultures were passaged once per week and plated in 35mm Petri dishes over glass coverslips (5x20mm) in 2ml of DMEM. These were kept in a 36.5°C incubator with 5% $C0_2$. After 20 passages the culture was re-derived from stock stored in liquid nitrogen. For recordings, confluent coverslips were transferred to a perfusion chamber (Fig. 2.3.2) with mammalian ringer (135mM NaCl, 5.4mM KCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 5mM HEPES, 10mM D-glucose adjusted to pH 7.4 with NaOH) flowing at 5ml/min.

Cultured locust neurons:

Gregarious forms of the desert locusts (*S. gregaria*) were kept in locust breeding cages measuring 50x40x32cm (Fig. 2.3.3). These were maintained at an ambient temperature of 26-28°C with a 40W incandescent bulb used to provide a basking spot of 38°C and a 12:12 light cycle. Locusts were fed *ad libitum* with a combination of leaves from ryegrass (*Lolium sp.*), dandelion (*Taraxacum officinale*) and plantain (*Plantago sp.*). Four adult locusts were used to harvest neurons to cover a 35mm Petri dish. Prior to dissection, locusts were cold anaesthetised at 4°C for ten minutes and then dipped in 70% ethanol before being decapitated with a pair of dissection scissors. The heads were kept in cooled Ca^{2+}/Mg^{2+} free Rinaldini's saline (135mM NaCl, 25mM KCl, 0.4mM $NaHCO_3$, 0.5mM glucose, 5mM HEPES adjusted to pH 7.2 with NaOH) over ice until dissection. The head was pinned through the mandibles (Fig. 2.3.4) with 25G hypodermic needles (Microlance, BD UK) into a 35mm Petri dish with a 4mm layer of Sylgard® (Dow Corning, USA). An incision was made from each eye to the frontal ridge using Vanna's microscissors (Agar scientific, UK) and a line cut along the frontal ridge to

connect the two incisions. The antennal nerves were severed and the front of the head capsule removed to expose the brain (Fig. 2.3.3 and Fig. 2.3.4). The optic nerves were then cut and the brain removed using forceps, and scissors to remove any connective tissue. Air sacs were discarded and the brain placed in cooled Rinaldini's saline (Fig. 2.3.5). When all four brains were removed, the mushroom bodies, located above the optic nerve, were separated from the rest of the brain with scissors. The mushroom bodies were opened with forceps and placed in 1.5ml Eppendorfs with 500µl Rinaldini's saline containing 2mg/ml collagenase and 0.5mg/ml dispase. They were then incubated for 1hr at 18°C on a shaking platform (Luckham, Sussex). Following enzyme treatment, the neurons were centrifuged at 6000rpm for 3 minutes, the supernatant removed and replaced with 500µl of culture medium (5:4 DMEM[10% FCS, 2mM Gln] : Schneider's insect medium, with 2% pen/strep). This was repeated to ensure that no collagenase or dispase were present. The mushroom bodies were then gently triturated through a 1ml pipette tip before being distributed on to coverslips (5x20mm) which had been placed into a 35ml Petri dish containing 2ml of culture medium. Dishes were incubated at 36.5°C in a 5% CO_2 atmosphere. Locust neuron preparations yielded ~200-300 neurons/cm^2 and remained viable for up to two days. To make recordings, coverslips were transferred to the perfusion chamber and perfused with locust Ringer (180mM NaCl, 10mM KCl, 2mM $CaCl_2$, 10mM HEPES made up to pH 7.2 with NaOH) at a rate of 5ml/min.

Figure 2.3.3 Locust breeding cage in which locusts (*Schistocerca gregaria*) were kept.

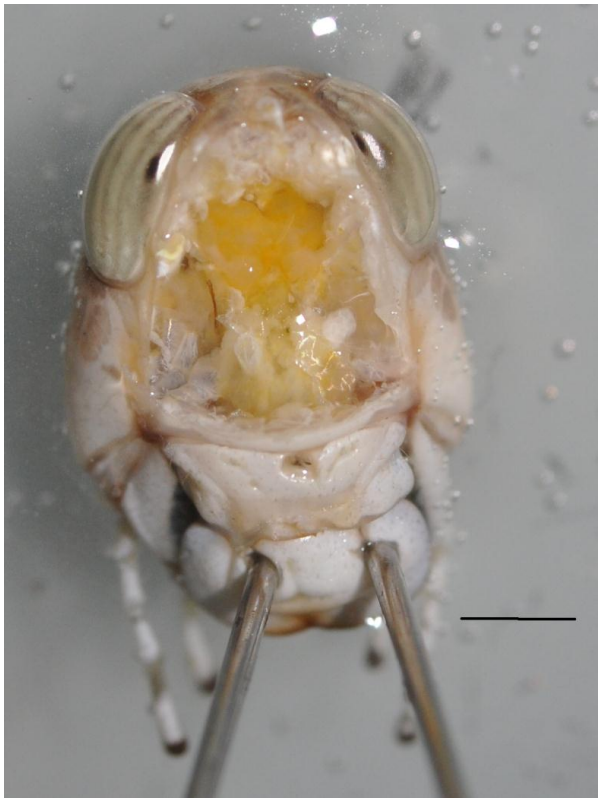


Figure 2.3.4 Locust head pinned through mandibles. Front of head capsule has been removed to expose brain. Scale bar represents 2mm.

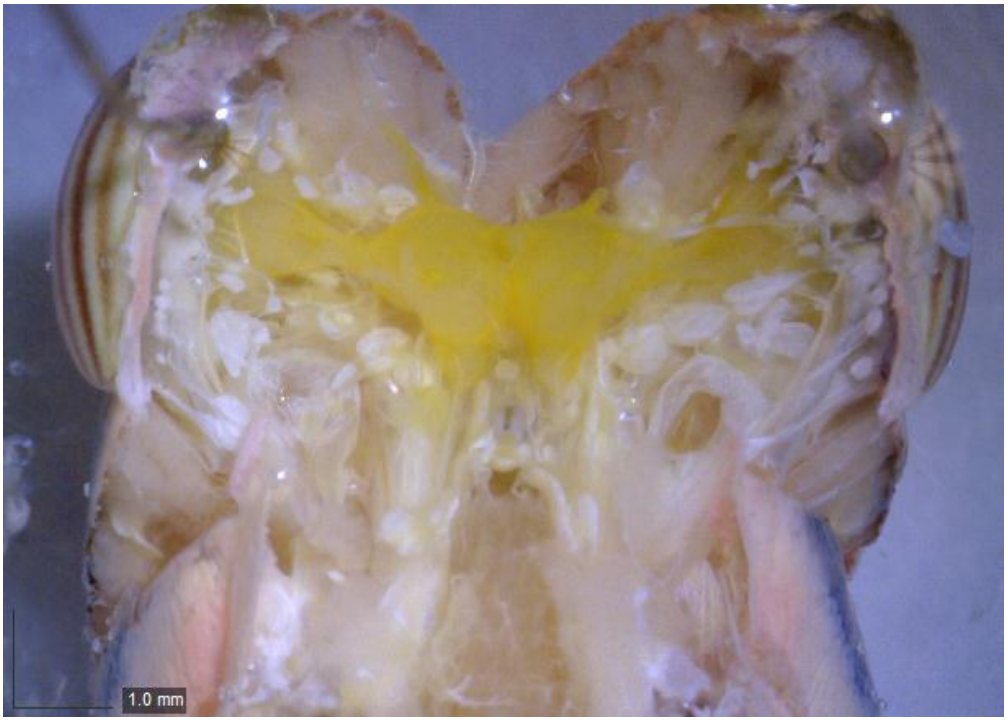


Figure 2.3.5 Locust brain *in situ*, optic nerves can be seen connecting to the eyes on either side of the head, antennal nerves have been severed but antennal lobes are visible below optic nerves.

Figure 2.3.6 A locust brain after removal from head capsule. The mushroom bodies are located at the top of the picture, above the optic nerves.

Coating Petri dishes:

Prior to plating locust neurons it was necessary to treat coverslips so that cells were sufficiently well adhered to allow patch-clamp and perfusion. Two compounds were tested for efficacy of adhering cells without interfering with whole-cell ACh currents. Glass coverslips (~5x20mm) were positioned on the bottom of 35mm Petri dishes. The first treatment was poly-l-lysine, a homopolymer of L-lysine produced commercially by fermenting *Streptomyces* bacteria. 2ml of 0.02% poly-l-lysine (Sigma, UK) was placed in each dish using a Pasteur pipette. This was left at room temperature for 2hr, the liquid was then removed and dishes left to dry in a sterile cabinet (SC). The second compound tested was concanavalin A (ConA), a carbohydrate binding lectin isolated from the jack-bean plant (*Canavalia ensiformis*). Petri dishes and coverslips were covered in 2ml ConA at a concentration of $1x10^{-5}$mg/ml and incubated at 36.5°C for two hours, the liquid was removed and dishes left to dry in a SC. In order to test the efficacy of the two treatments, whole cell currents in response to 100μM ACh application were recorded.

2.4 Whole-cell patch-clamp:

Boroscilicate glass patch pipettes (World Precision Instruments, USA) were pulled to ~5MΩ using a P-97 Flaming/Brown micropipette puller (Sutter Instrument Co., USA) and filled with solutions of either: Cesium pipette solution (140mM CsCl, 1mM $MgCl_2$, 11mM EGTA and 5mM HEPES, pH 7.2) for recording from TE671 cells, or low potassium locust pipette solution (180mM NaCl, 10mM KCl, 2mM $CaCl_2$, 10mM HEPES, pH 7.2) for recording from locust neurons. These solutions were used to minimise the impact of potassium channel currents on whole cell activity. Whole-cell currents were monitored using an Axopatch 200 (Axon instruments, USA) patch-clamp amplifier and recorded to the hard disk of a Dell computer using WinWCP V4.1.3 software (University of Strathclyde). Alkaloid concentrations

ranging from $1x10^{-4}$ – $1x10^{-12}$M for hippodamine and $1x10^{-1}$ – $1x10^{-9}$ mg/ml for *H. axyridis* extract were co-applied with ACh in 1s pulses using a DAD-12 Superfusion system (Adams and List Associates, USA) fitted with a 100µm polyamide coated quartz output tube. To perfuse cells, the output tube was manoeuvred so that the aperture was facing the cell so that test solutions flowed over and round the cell (Fig. 2.3.8). Both the amplifier and perfusion system were controlled by WinWCP V4.1.3 software. A wash-off of either mammalian or locust Ringer was constantly flowing from the tip of the perfusion manifold at ~3ml/hr. ACh and test compounds were applied at 400mm/Hg for one second with a 30s wash-off between applications. All recordings were taken at room temperature (22°C-25°C). Alkaloids were dissolved in dimethyl sulfoxide (DMSO) to create stock solutions for further dilution with the appropriate Ringer's solution. The minimum dilution volume used was 100 fold. Controls were conducted to ensure that DMSO was not interfering with ACh induced currents by co-applying ACh with DMSO at a concentration of 1% and measuring the whole-cell electrical response of both TE671 cells and locust neurons.

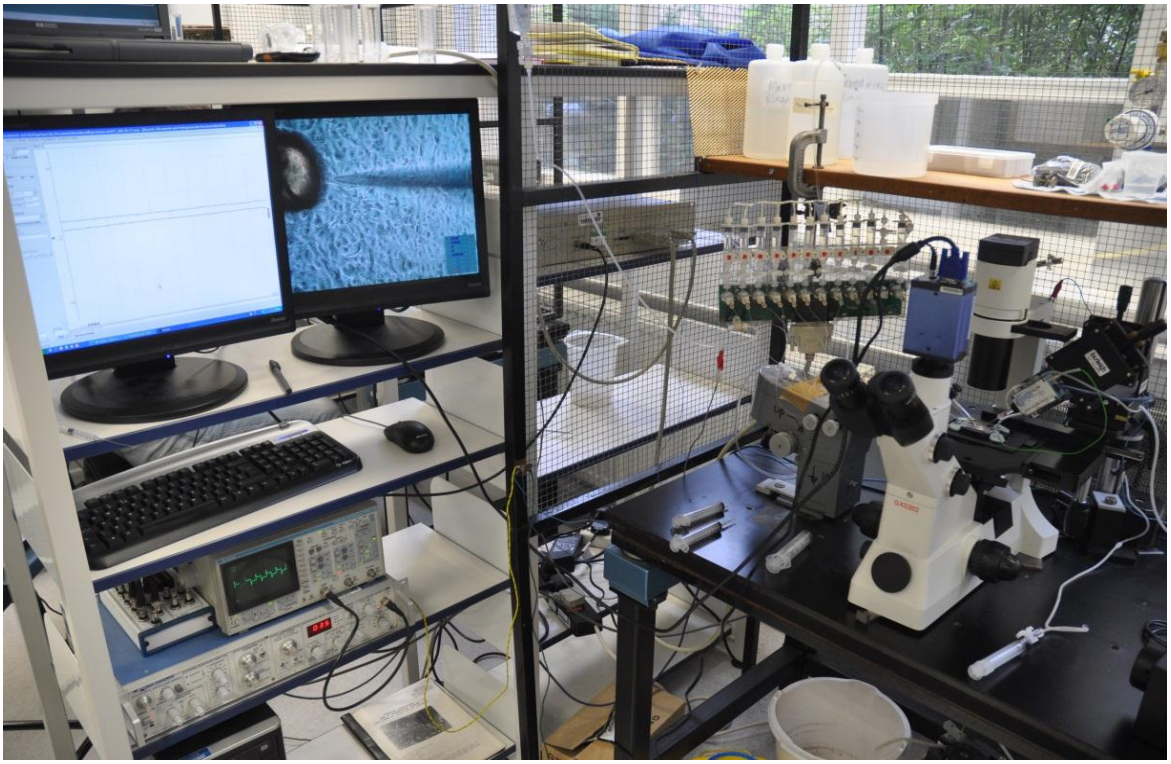


Figure 2.3.7 Whole-cell patch-clamp rig with single cell perfusion system.

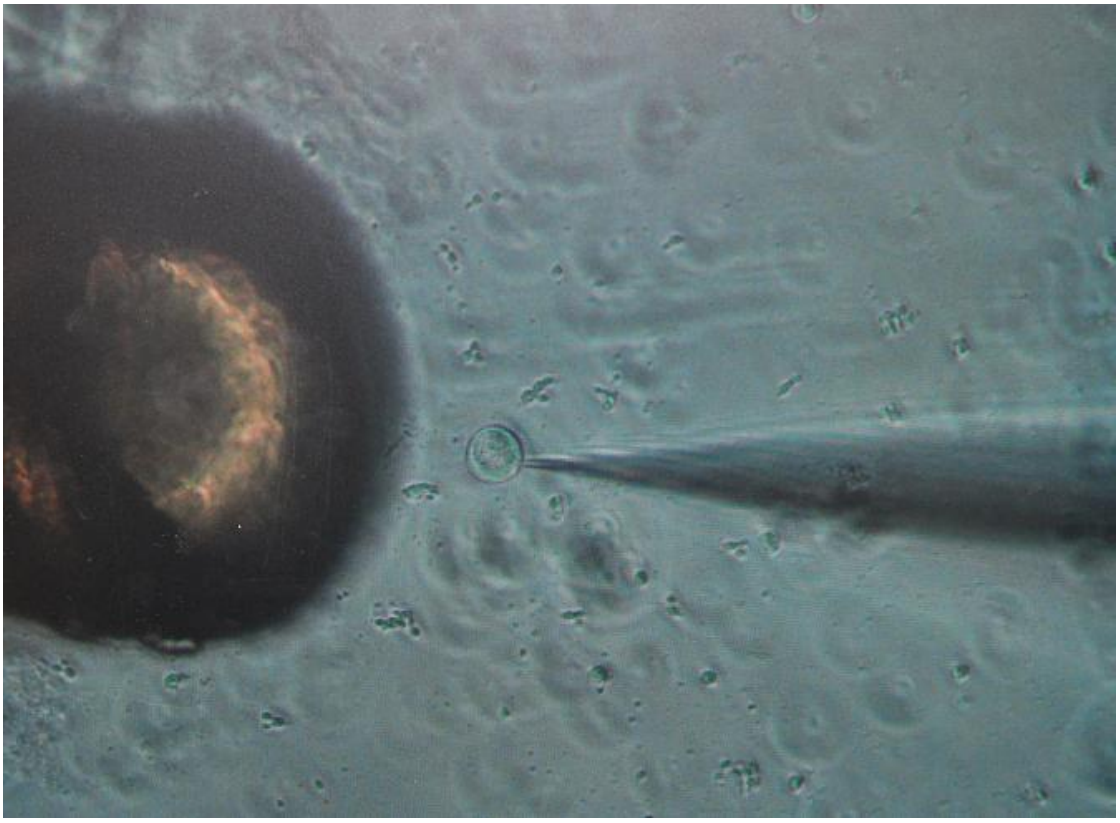


Figure 2.3.8 Locust neuron clamped (centre and right) with 100µm manifold tip (left). Compounds were applied to cells at 400mm/Hg. 200x magnification.

2.5 Analysis:

IC_{50}:

Late current was measured at one second post application and transposed to GraphPad Prism 5 (GraphPad Software, USA). All values were normalised against the ACh control current taken at the same time point. The mean and standard error of the normalised current measurements were plotted against concentration to generate concentration inhibition plots. A log[inhibitor] v.s. normalised response regression curve was fitted to these points and the IC_{50} value taken from this. To detect any voltage dependence and evaluate the differences in potency between TE671 cells and locust neurons, sum of squares F-test comparisons were conducted using Graphpad Prism 5. The two data sets being compared were plotted on the same axis. Two models were fitted to the data and the sum-of-squares of deviations of the data points from the models is quantified. An F ratio is calculated using the following calculation.

$$F = \left(\frac{SSnull - SSalt}{SSalt}\right) / \left(\frac{DFnull - DFalt}{DFalt}\right)$$

Where SS_{null} is the sum-of-squares for the null hypothesis model, SS_{alt} is the sum-of-squares for the alternative model. DF_{null} is the degrees of freedom for the null hypothesis and DF_{alt} is the degrees of freedom for the alternative hypothesis. From the F ratio and two degrees of freedom values a P value is then calculated.

3. Ladybird Collection and Alkaloid Extraction

3.1 Ladybirds:

Adult ladybird beetles were wild collected between September and December of 2010. Three species were found in sufficient numbers to allow alkaloid extraction, these were; the harlequin ladybird (*Harmonia axyridis*), the seven-spot ladybird (*Coccinella 7-punctata*) and the pine ladybird (*Exochomus 4-pustulatus*). Number collected, mean weights and lengths are shown in table 3.1.1.

	Harmonia axyridis	***Coccinella 7-punctata***	***Exochomus 4-pustluatus***
Number	314	355	55
Mean weight (mg)	28.94 (N=50)	41.06 (N=50)	9.8 (N=50)
Mean length (mm)	6.75 (N=50)	7.25 (N=50)	
Total alkaloid (mg)	28.41	19.06	1.62
mg/g beetles	3.13	0.46	3.01

Table 3.1.1 The numbers of beetles collected between September and December of 2010, along with mean weight, length and the total weight of acid-base extract yielded.

The three extracts tested positive for alkaloids using a Dragendorff reagent spot test. After being dried under nitrogen the resulting precipitates were each a different colour. The extract from *H. axyridis* was dark orange/brown, that from *C. 7-punctata* was orange and finally, that from *E.4-pustulatus* was yellow.

3.2 High Performance Liquid Chromatography (HPLC) profiles:

To ascertain the complexity of extracts the whole beetle acid-base extracts of *H. axyridis* and *C. 7-punctata* underwent HPLC. It was decided that the yield from *E. 4-pustulatus* (1.62mg) was too small to allow HPLC and further testing, it was therefore stored frozen to be supplemented at a later date.

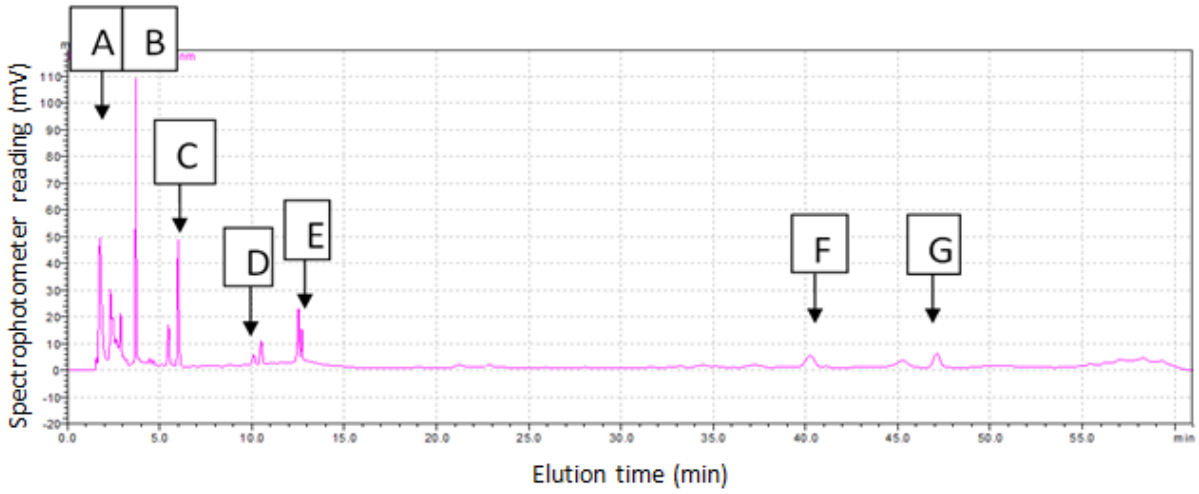


Figure 3.2.1 HPLC profile for *H. axyridis* whole acid-base extract. A 50µl injection volume at a concentration of 10mg/ml.

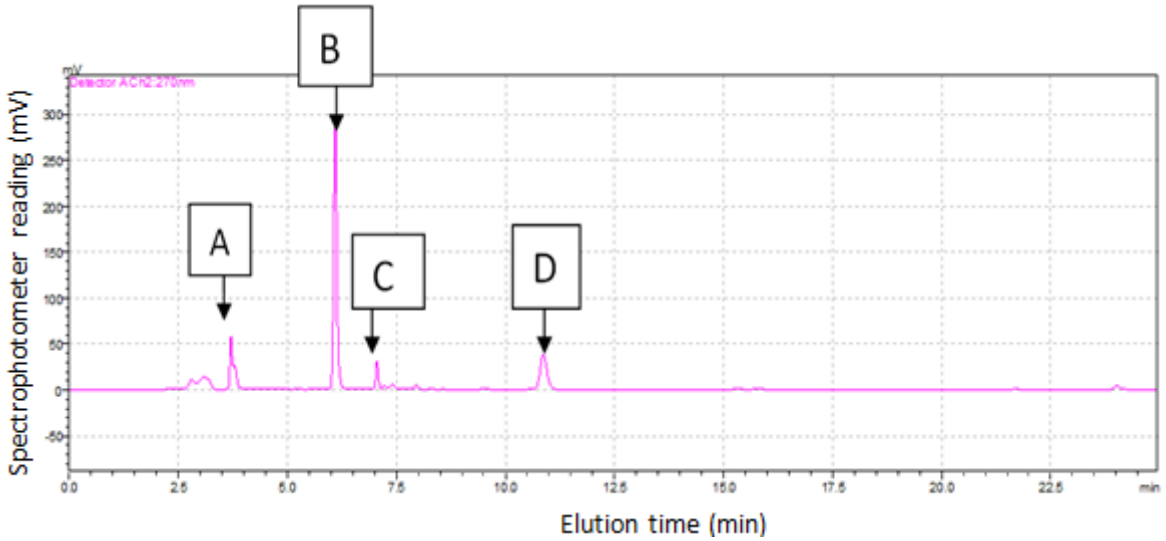


Figure 3.2.2 HPLC profile for *C. 7-punctata* whole acid-base extract. A 50µl injection volume was used at a concentration of 20mg/ml.

It can be seen that the HPLC profile of *H. axyridis* contains a larger number of major peaks, seven as opposed to the four present in the profile for *C. 7-punctata*. All major peaks for both figures have elution times below 12 minutes, as would be expected for alkaloids. The extract of *H. axyridis* contains two minor peaks with elution times at 40 minutes and 46 minutes, whereas none of these hydrophobic compounds appear to be present in the extract of *C. 7-punctata*.

4. Whole-cell patch clamp of TE671 human muscle cells

4.1 Introduction:

Preliminary investigation suggested that ladybird alkaloids possibly target nAChR. Therefore TE671 human muscle cells expressing muscle type nAChR were subjected to applications of ACh and increasing concentrations of *H. axyridis* extract or hippodamine to ascertain the effect of these compounds on whole-cell nAChR currents generated in response to ACh. Low vertebrate toxicity is a fundamental requirement of pesticide lead structures and it was therefore essential to establish how potent ladybird alkaloids are to a vertebrate model system.

4.2 Controls:

Repeated ACh Application:

To test whether repeated exposure of TE671 cells to ACh has an effect on size or duration of ion currents generated, cells were subjected to six successive 1s applications of 10μM ACh at 400mm/Hg. 10μM was shown to be sufficient to activate nAChR activity without causing complete ACh desensitisation (Shao, 1997). A 30s wash off was running between applications with a flow rate of 3ml/hr. All subsequent results presented in this chapter were subject to the same application protocol. Example traces from cells held at -50mV (A) and -100mV (B) can be seen in figure 4.2.1 and a column chart showing the mean normalised size of currents generated by repeated applications is shown in figure 4.2.2. Current measurements were taken 1s post application. This was because preliminary investigation suggested that ladybird alkaloids affect channel closing activity rather than peak current. 1s post application current is a more representative measurement of this activity. It can be seen that the magnitude of 1s current diminishes slightly, but plateaus at >80% that of the initial ACh response.

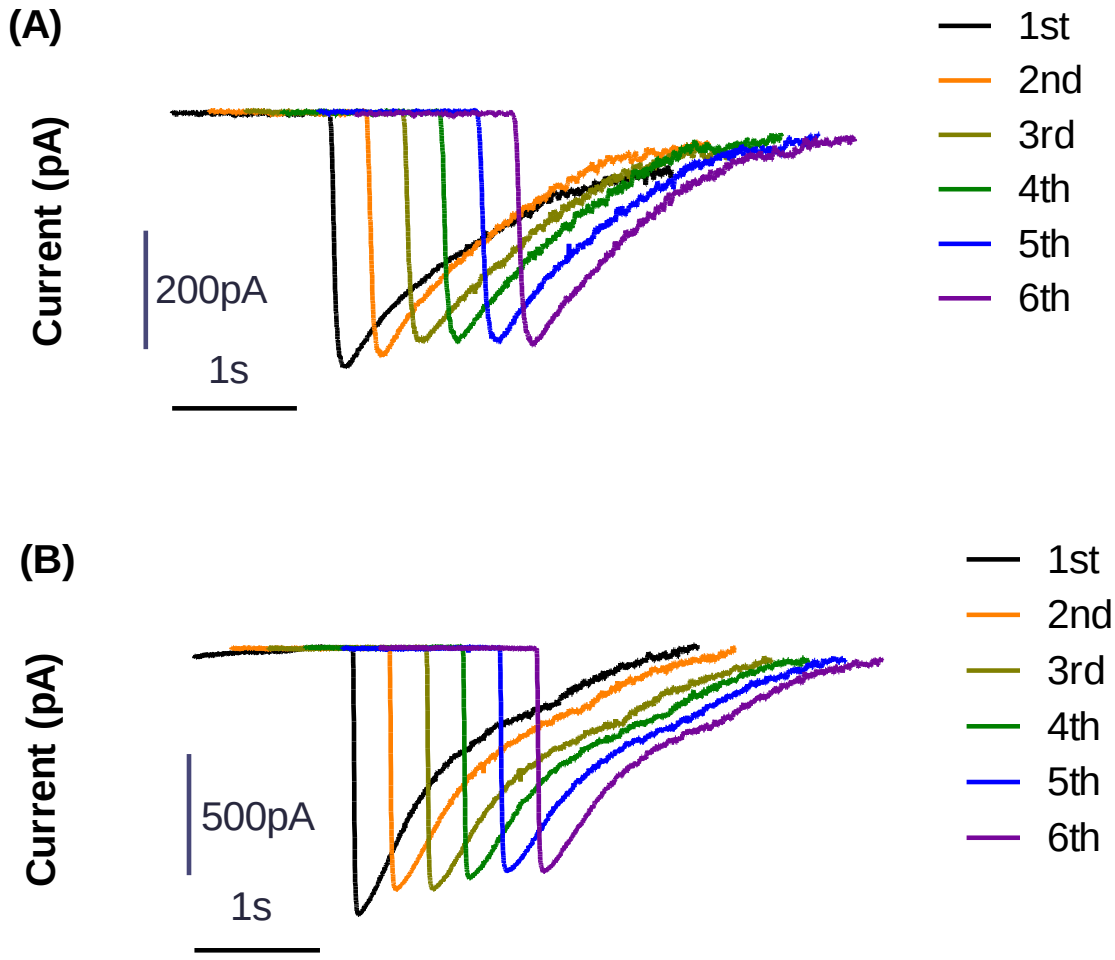


Figure 4.2.1 Traces showing the result of six repeated 1s exposures to 10μM ACh with a period of 30s between applications. The trace shown in (A) was held at V_H = -50mV, that shown in (B) was held at V_H = -100mV.

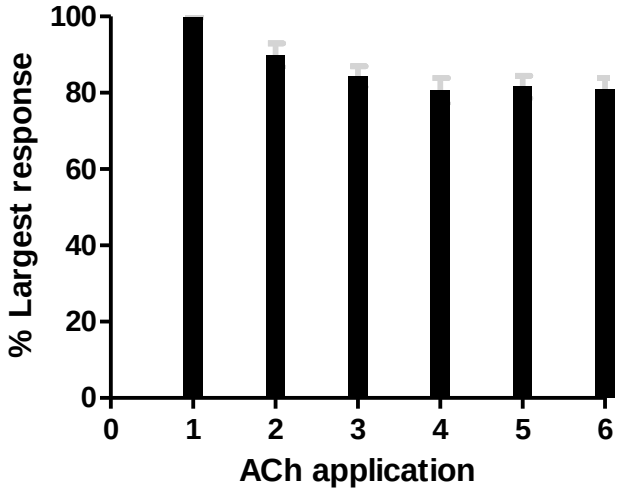


Figure 4.2.2 Column chart showing the magnitude of 1s post application ion currents generated in response to repeated ACh application to TE671 cells clamped at -50mV. The largest current measurement in each series was given a value of 100% and the others normalised against it. N=6. The size of the second response diminished to a mean of 90% of the first application, the third to 85%, and following current measurements reached a plateau at 80% of the initial application. Error bars show standard error.

DMSO Control:

To ascertain whether the solvent in which test compounds were dissolved to make up stock solutions, DMSO, had any effect on ACh-induced currents, TE671 cells were perfused with a 1s pulse of ACh and after 30s were perfused with an identical ACh solution containing 1% DMSO. This is the highest concentration of solvent used in the following experiments. From the results it was decided that the impact of 1% DMSO was negligible and was suitable for the subsequent experiments. Example traces of TE671 cells perfused with ACh and ACh containing 1% DMSO can be seen in figure 4.2.3.

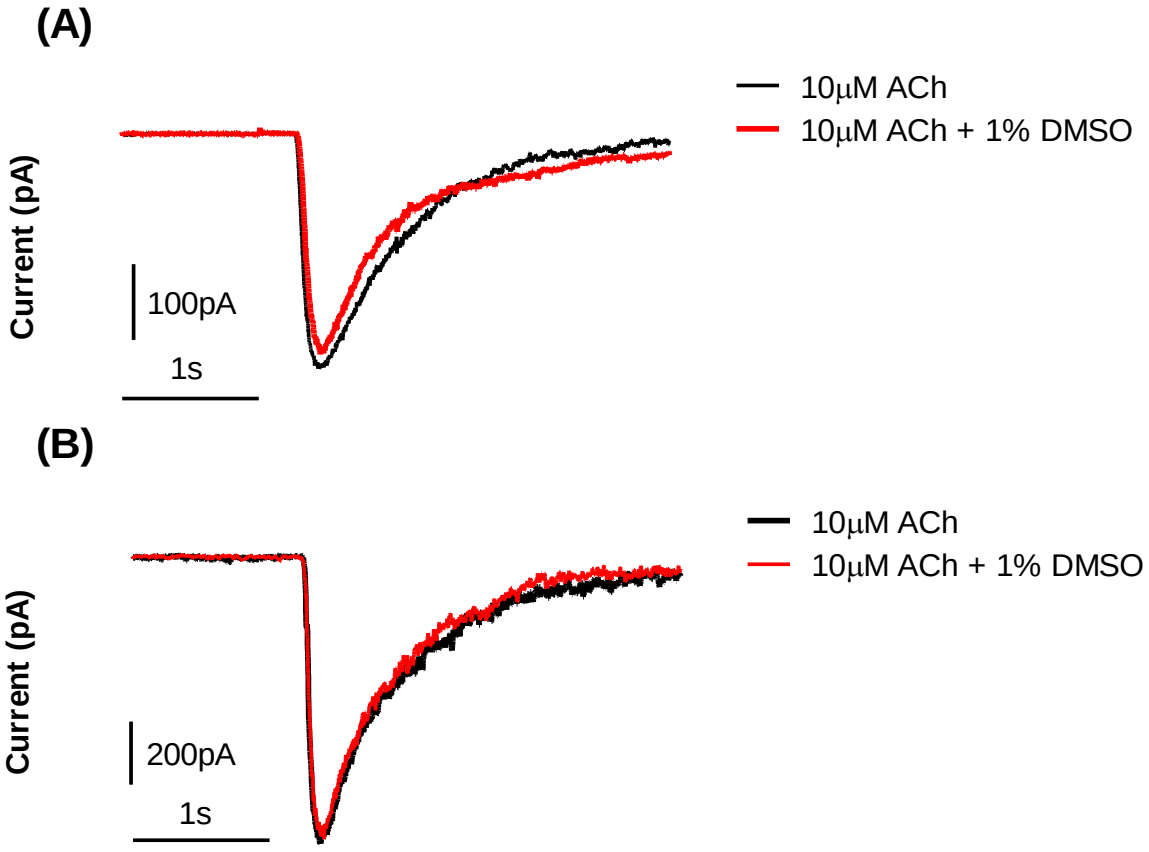


Figure 4.2.3 Example traces showing the effect of 1% DMSO on channel ion currents generated in response to ACh application in TE671 cells clamped at (A) -50mV and (B) -100mV.

4.3 Harlequin Ladybird Whole Alkaloid Extract:

The acid-base extract of the harlequin ladybird (*H. axyridis*) was dissolved in DMSO to make up a stock solution of 10mg/ml and co-applied with 10μM ACh at the concentrations shown in figures 4.3.1 and 4.3.2. Whole-cell responses were recorded using patch-clamp. A 4s recording sweep was used with a 1s application (shown with a red line). Figures 4.3.1 and 4.3.2 show example traces (A) and concentration-inhibition curves (B) for TE671 cells held at -50mV and -100mV respectively. Cells were perfused with increasing concentrations of *H.axyridis* extract and a final application of ACh (not shown) was used to demonstrate that inhibitory activity was caused by the test compounds rather than ACh desensitisation or loss of seal between electrode and cell. Results were discarded if the 1s current of the final ACh application was equal to, or lesser in magnitude than the current generated by the highest concentration of extract.

It can be seen from the concentration-inhibition plots and traces shown in figures 4.3.1 and 4.3.2 that *H. axyridis* extract displays little activity at concentrations below $1x10^{-3}$mg/ml but has an inhibitory effect on nAChR currents at concentrations greater than this. The traces show that at a concentration of 100 μg/ml *H. axyridis* extract can cause outward currents, but the concentration-inhibition curves show that this is not always the case.

Application of *H. axyridis* extract alone at a concentration of 10 μg/ml (figure 4.3.3) failed to demonstrate agonism and did not generate any whole-cell electrical activity at V_H -50mV (A) or -100mV (B).

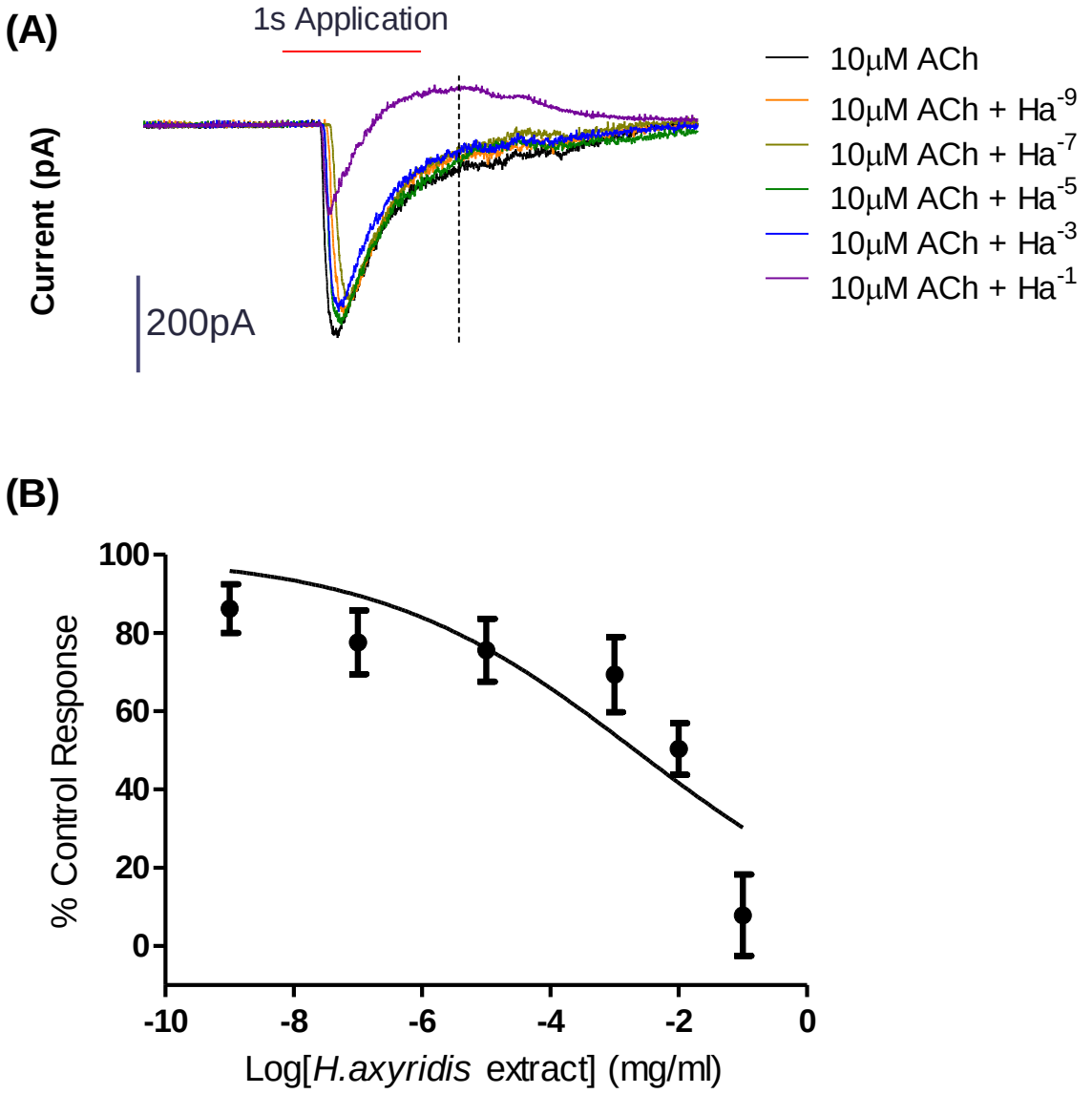


Figure 4.3.1 (A) Whole-cell responses of a TE671 cell after application of 10μM ACh and then co-application of 10μM ACh with increasing concentrations ($1x10^{-x}$mg/ml) of *H. axyridis* alkaloid extract. V_H was -50mV and the dotted line shows one second post onset of ACh response, the point at which current measurements were taken for IC_{50} calculation. (B) A concentration-inhibition non-linear regression curve showing the effect of increasing concentrations of *H. axyridis* on 1s post application currents elicited by ACh when V_H = -50mV. The IC_{50} for this curve is 2.12μg/ml. N=10 (N=12 for $1x10^{-2}$ mg/ml). Error bars show standard error.

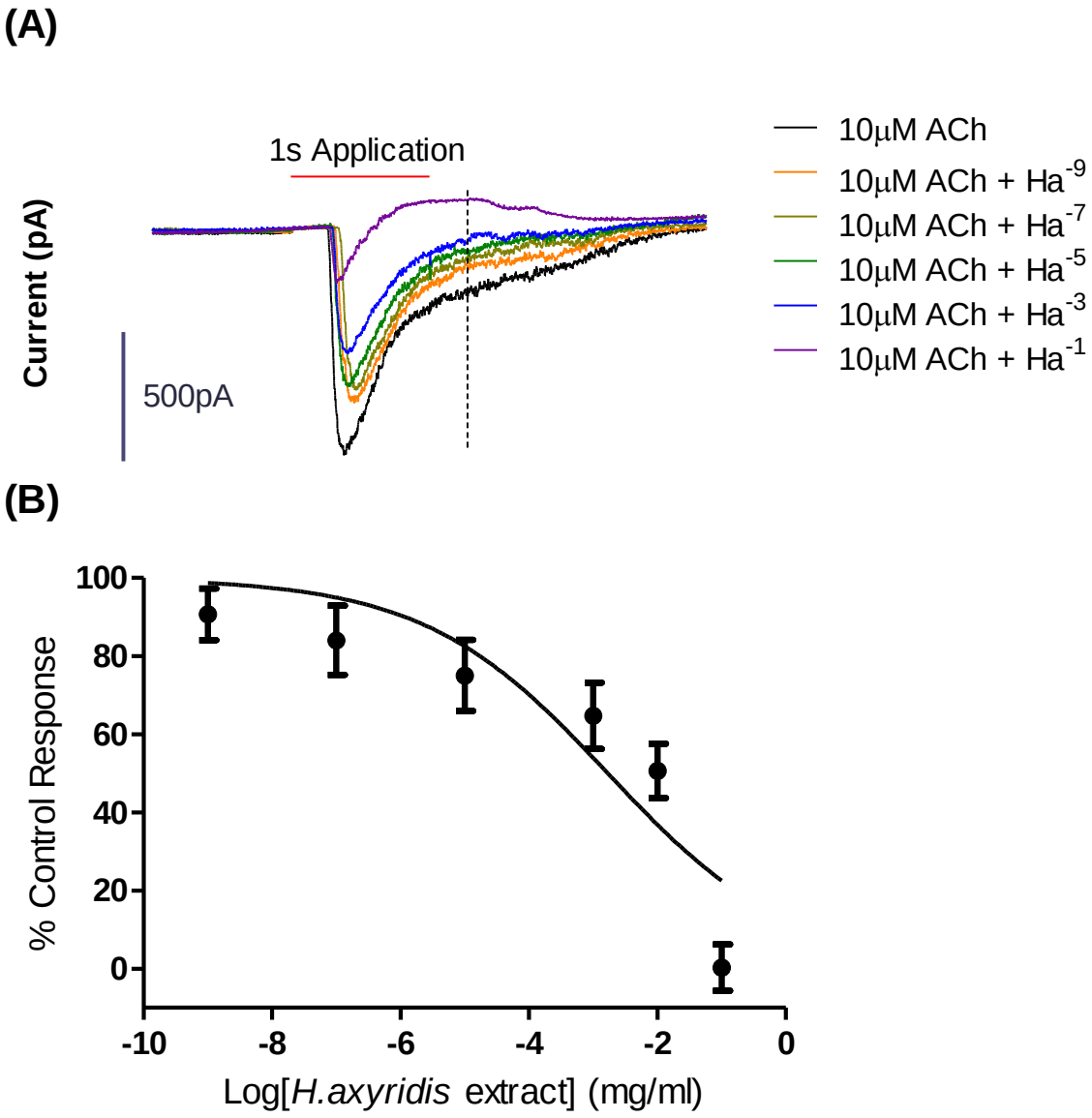


Figure 4.3.2 (A) Whole-cell responses of a TE671 cell after application of 10μM ACh and then co-application of 10μM ACh with increasing concentrations ($1x10^{-x}$mg/ml) of *H. axyridis* alkaloid extract. V_H was -100mV and dotted line shows one seconds post onset of ACh response, the point at which current measurements were taken for IC_{50}. (B) A concentration-inhibition non-linear regression curve showing the effect *H. axyridis* alkaloid extract has on size of currents elicited by ACh when V_H = -100mV. Current readings were taken 1s after application to measure activity affecting channel current. The IC_{50} for this curve is 1.70 μg/ml. N=12 (N=11 for $1x10^{-2}$). Error bars show standard error.

F-test Comparison:

When the curve fitting parameters of figures 4.3.1 and 4.3.2 are compared with an F-test, there is no significant difference (P= 0.6847) indicating that the compound/s in *H. axyrdis* do not display a voltage dependent relationship when targeting the nAChR of TE671 cells.

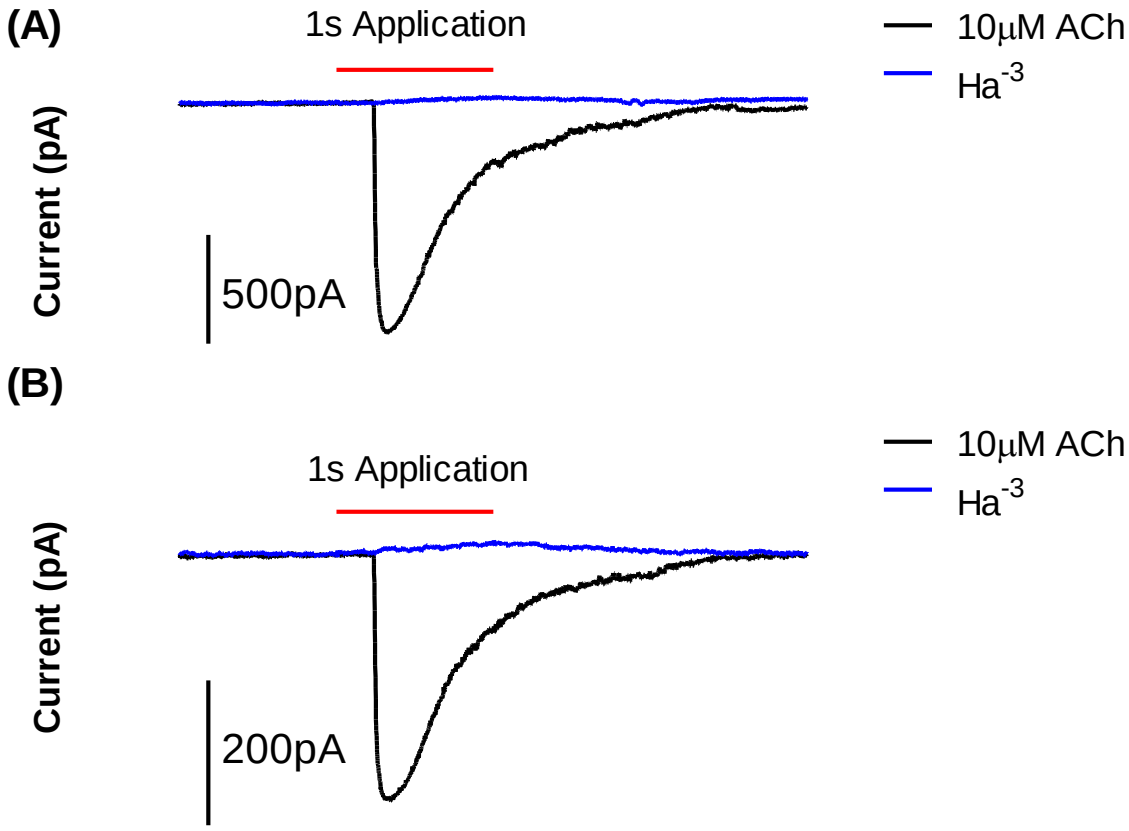


Figure 4.3.3 Example traces showing that *H. axyridis* extract applied in the absence of ACh generates no electrical activity in TE671 cells at a V_H of either -50mV (A) or -100mV (B).

4.4 Hippodamine, Synthetic Analogue of an Alkaloid Produced by *Hippodamia convergens*:

An analogue of the alkaloid hippodamine, naturally produced by the convergent ladybird, was synthesised by Dr. Rob Stockman (University of Nottingham). This was made into a stock solution of 10mM in DMSO and diluted to the concentrations shown in figures 4.4.1 and 4.4.2. Increasing concentrations of hippodamine were co-applied with 10μM ACh . The traces (A) and concentration-inhibition curves (B) clearly show that increasing concentrations of hippodamine cause greater inhibition of ACh currents.

Application of hippodamine alone at a concentration of 10μM (Figure 4.4.3) generated a slight inward current upon application. This could be indicative of agonism or could be caused by residual ACh from the output tube or a response to pressure.

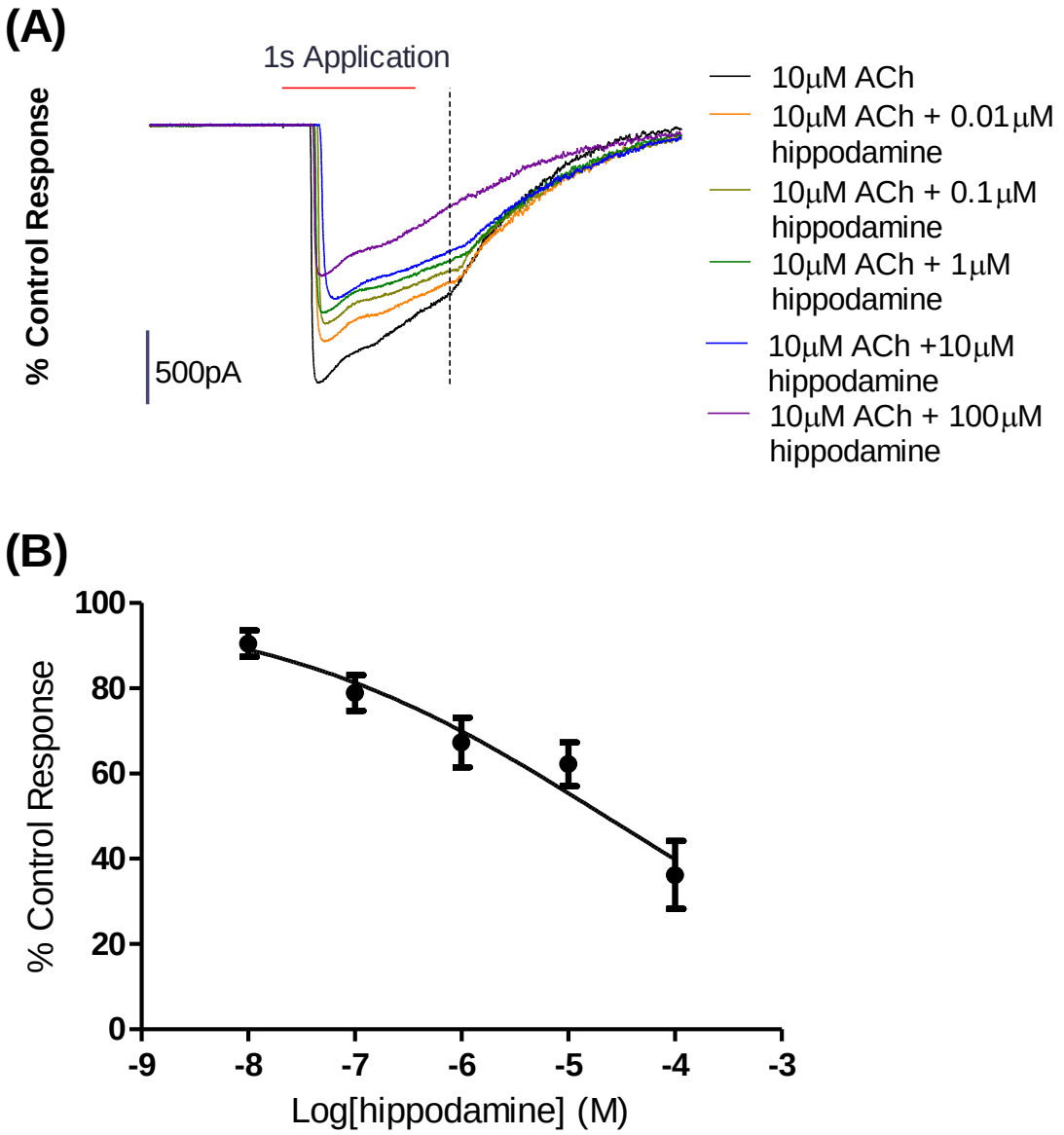


Figure 4.4.1 (A) Whole-cell responses of a TE671 cell after application of 10μM ACh and then co-application of 10μM ACh with increasing concentrations of synthetic hippodamine (M), an alkaloid produced by the convergent ladybird (*Hippodamia convergens*). The holding potential was -50mV and applications were for 1s. The dotted line shows one seconds post onset of ACh response, the point at which current measurements were taken for IC_{50}. (B) Shows the concentration-inhibition plot fitted with a non-linear regression, for locust neurons held at -50mV and subjected to applications of increasing concentrations of hippodamine in conjunction with 10μM ACh. The IC_{50} for this curve is 22.3μM. N=13 and error bars show standard error.

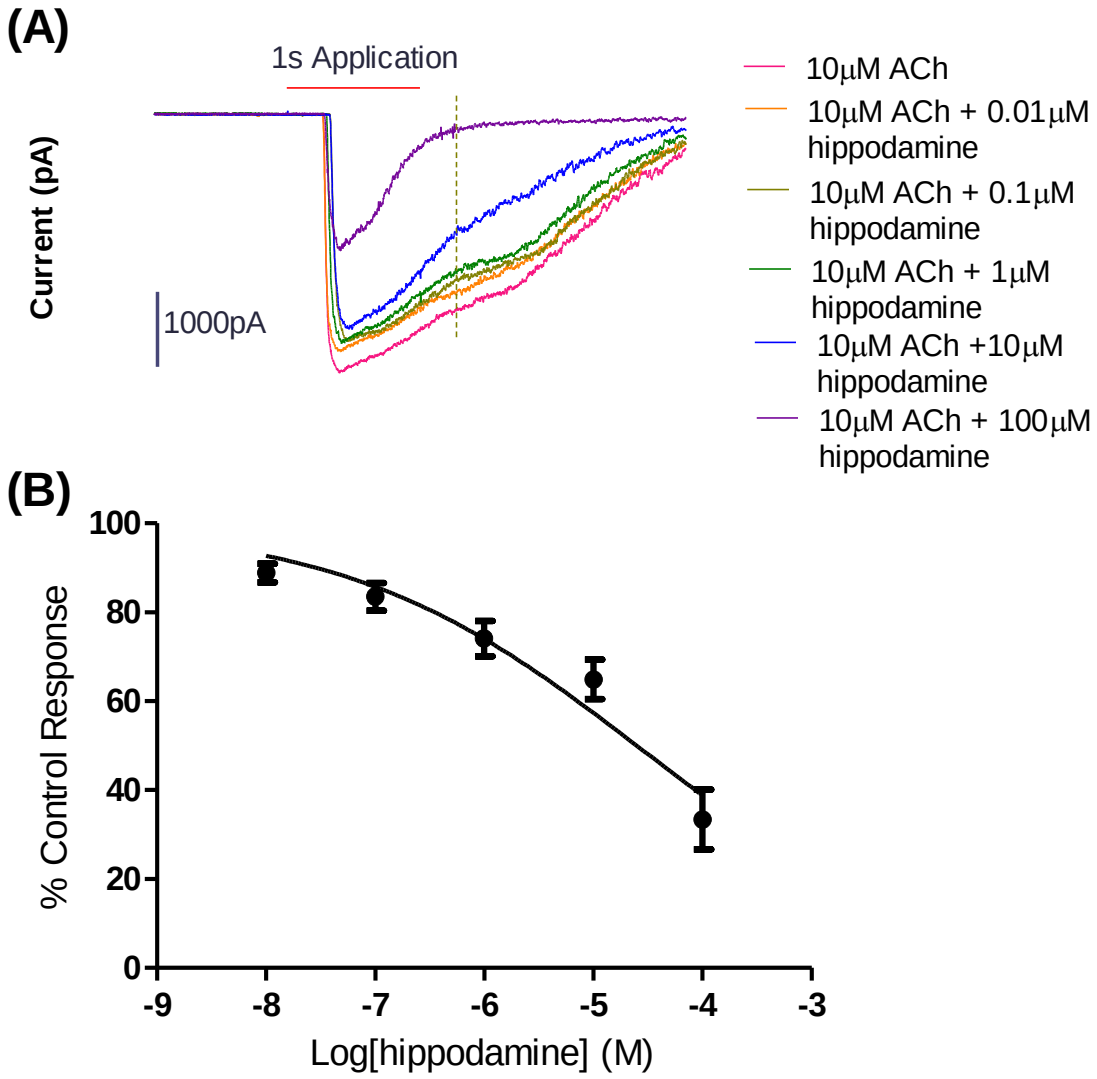


Figure 4.4.2 An example trace (A) and concentration-inhibition slope fitted with a non-linear regression curve (B) showing the affect of synthetic hippodamine when co-applied to TE671 cells held at -100mV. Current readings were taken at 1s post onset of ACh response (dotted line) to record activity on channel closing activity. The IC_{50} generated by this curve is 25μM. N=12 (N=11 for 1μM hippodamine) and error bars show standard error.

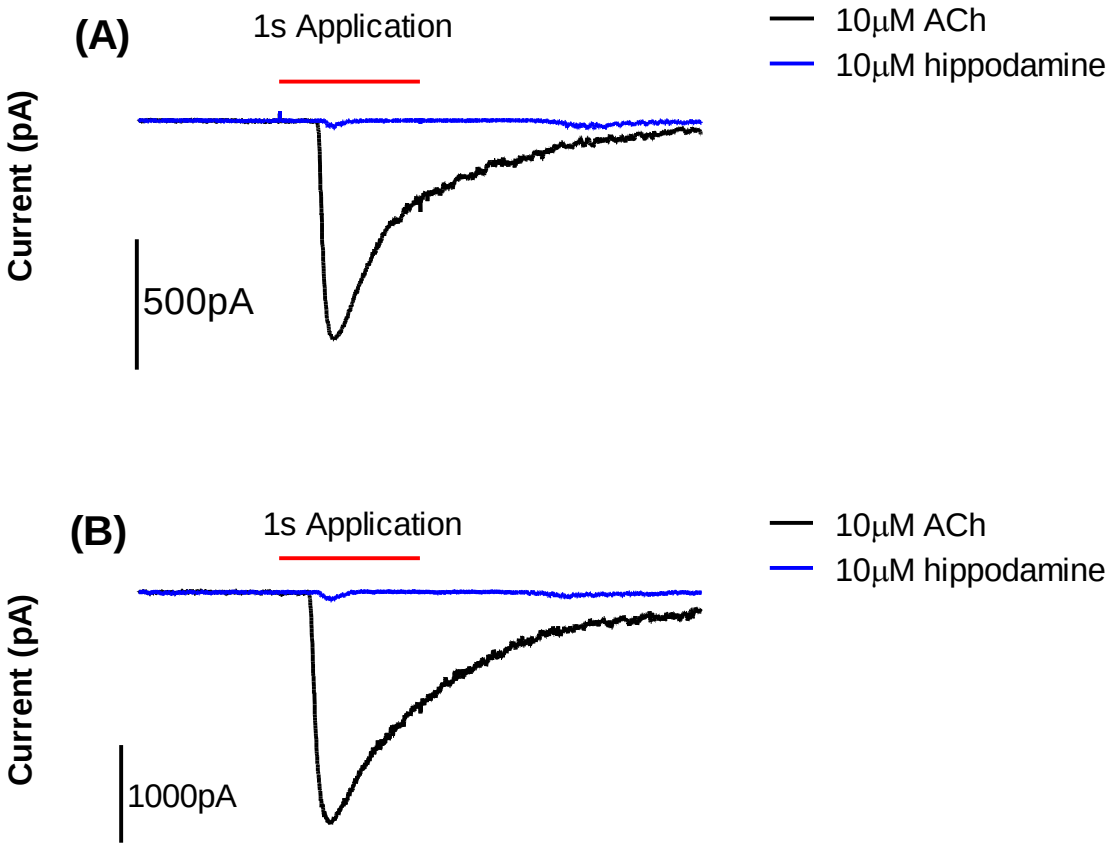


Figure 4.4.3 Example traces showing that hippodamine applied in the absence of ACh generates minimal electrical activity in TE671 cells at a V_H of either -50mV (A) or -100mV (B). Slight inward currents are visible at the start of application and wash-off, but these are likely a result of residual ACh or a response to the pressure of the application rather than agonism of the nAChR.

F-test comparison:

When the curve fitting parameters of figures 4.4.1 and 4.4.2 were compared with an F-test, there was no significant difference (P= 0.8549) indicating that hippodamine does not display a voltage dependent relationship when targeting the nAChR of TE671 cells.

5. Whole-cell Patch-clamp of Cultured Locust (*Schistocerca gregaria*) Neurons

5.1 Introduction:

Cultured locust neurons were used as a model to test the inhibitory activity of *H. axyridis* extract and hippodamine against invertebrate nAChR currents generated by the application of ACh. Any compound being investigated as a potential pesticide must show potent toxicity towards insects, and the nAChR is a suitable target which is utilised by a number of currently available pesticides. Initial attempts to elicit nAChR activity with 1s pulses of 10μM ACh at 400mm/Hg proved unsuccessful, so the concentration was increased to 100μM leading to consistent activation of nAChR (Fig 5.2.2). All results presented below were conducted using the aforementioned protocol and 100μM ACh. Unlike TE671 cells, locust neurons do not naturally adhere to glass surfaces and must be attached to allow patch-clamp and perfusion. The coverslips onto which they were plated therefore needed to be coated to allow adhesion.

5.2 Coating Coverslips:

The efficacy of two compounds were tested to adhere locust neurons in place to allow patch-clamp and perfusion, these were concanavalin-A and poly-L-lysine. Their capability to attach cells to coated coverslips was tested by conducting whole cell patch-clamp while perfusing cells with 100μM ACh. The electrical responses to ACh were recorded and results presented in figures 5.2.1 and 5.2.2.

Conconavalin A:

Con-A proved very effective at holding neurons in place for patch-clamping and perfusion, it was however not possible to elicit an ACh response in cells which had been stuck down in this manner. Figure 5.2.1 shows an example trace from a locust neuron plated onto a coverslip treated with con-A at a concentration of 10 μg/ml and then exposed to 100μM ACh for 1s.

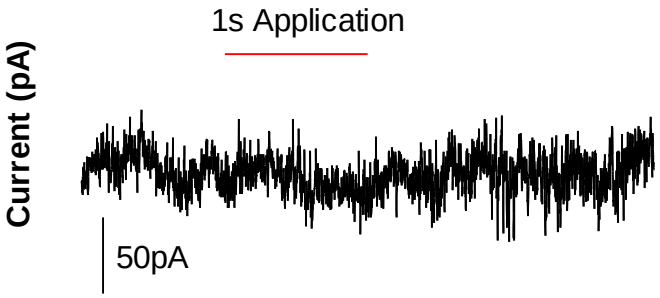


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Figure 5.2.1 Trace showing elimination of ACh induced current to locust neurons adhered with conconavalin-A. The recording sweep shown is 4 seconds and the red line indicates the 1s ACh application period. No indication of electrical activity could be detected from any locust neurons adhered with con-A.

Poly-L-lysine:

Locust neurons plated over 0.02% poly-L-lysine coated coverslips were stuck down sufficiently well to allow patch clamp and perfusion. Figure 5.2.2 shows an example trace of an inward current triggered by applying 100μM ACh to locust neuron plated onto a coverslip treated with poly-L-lysine. All subsequent tests were conducted on locust neurons plated over poly-L-lysine coated coverslips.

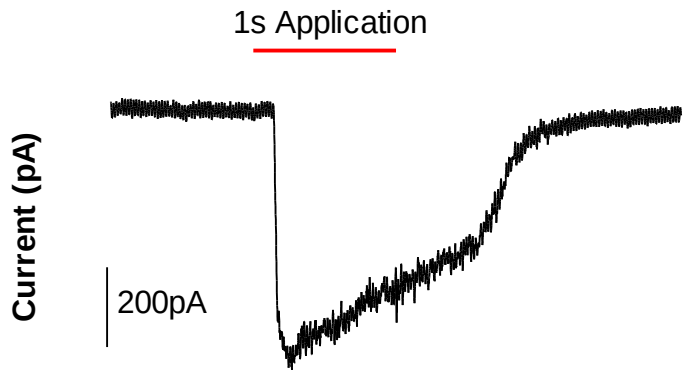

Figure 5.2.2 Trace showing the response of a cultured locust neuron plated onto a coverslip treated with 0.02% poly-L-lysine, to application of 100μM ACh. The subsequent inward current has a peak amplitude of 620pA at 229ms after ACh application begins.

5.3 Controls:

Repeated ACh exposure:

To test if repeated exposure to ACh has any effect on size or duration of nAChR currents elicited in response to ACh; six successive 1s applications of 100μM ACh were made to individual locust neurons. Figure 5.3.1 shows that the 1s current measurement diminishes by less than 10% between the first application and the 6th. Figure 5.3.2 shows traces from locust neurons held at -50mV and -100mV with a period of 30s between ACh applications. A wash-off of locust ringer was running in between applications with a flow rate of 3ml/hr.

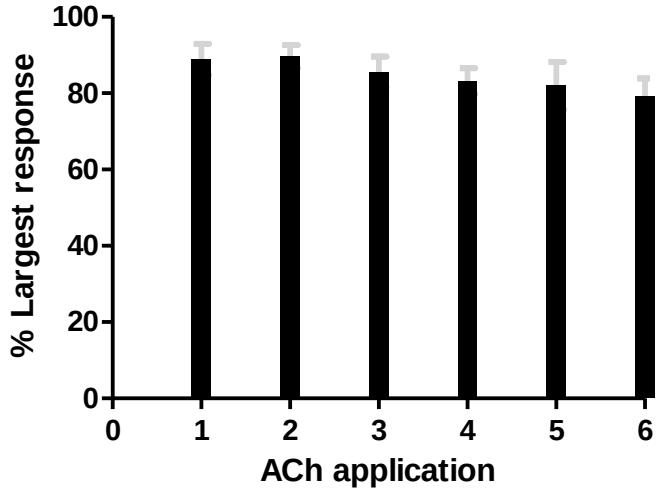

Figure 5.3.1 Column chart showing the magnitude of 1s post application ion currents generated in response to repeated ACh (100μM) application to locust neurons clamped at -50mV. The largest current measurement in each series (usually the first application) was given a value of 100% and the others normalised against it. N=9. The 1s post application current magnitudes can be seen to diminish slightly for each subsequent application, the difference in mean current is less than 10% between the first and 6th application. Error bars show standard error.

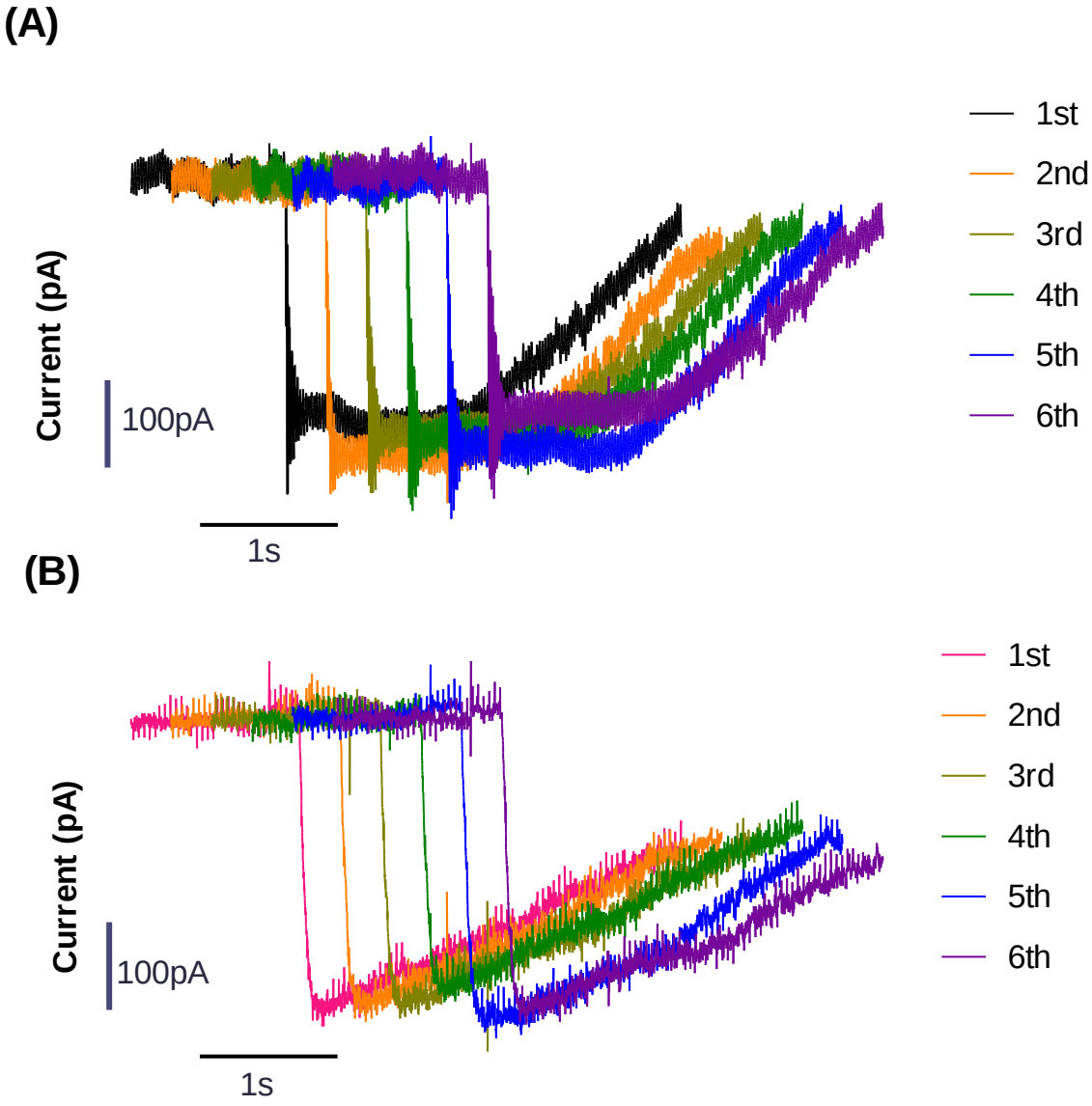

Figure 5.3.2 Traces showing the whole-cell electrical activity of a locust neuron held at (A) -50mV and (B) -100mV and repeatedly exposed to 100μM ACh six times. The size of currents generated can be seen to vary slightly, but the variance is less than 10%.

DMSO Control:

To test whether the solvent used to make up stock solutions, DMSO, had any effect on ACh-induced currents, locust neurons were exposed to 100μM ACh and an identical ACh solution containing 1% DMSO (figure 5.3.3). This is the highest concentration of solvent used in the following experiments. From these results it was decided that the impact of DMSO on results was negligible and therefore it was suitable for subsequent experiments.

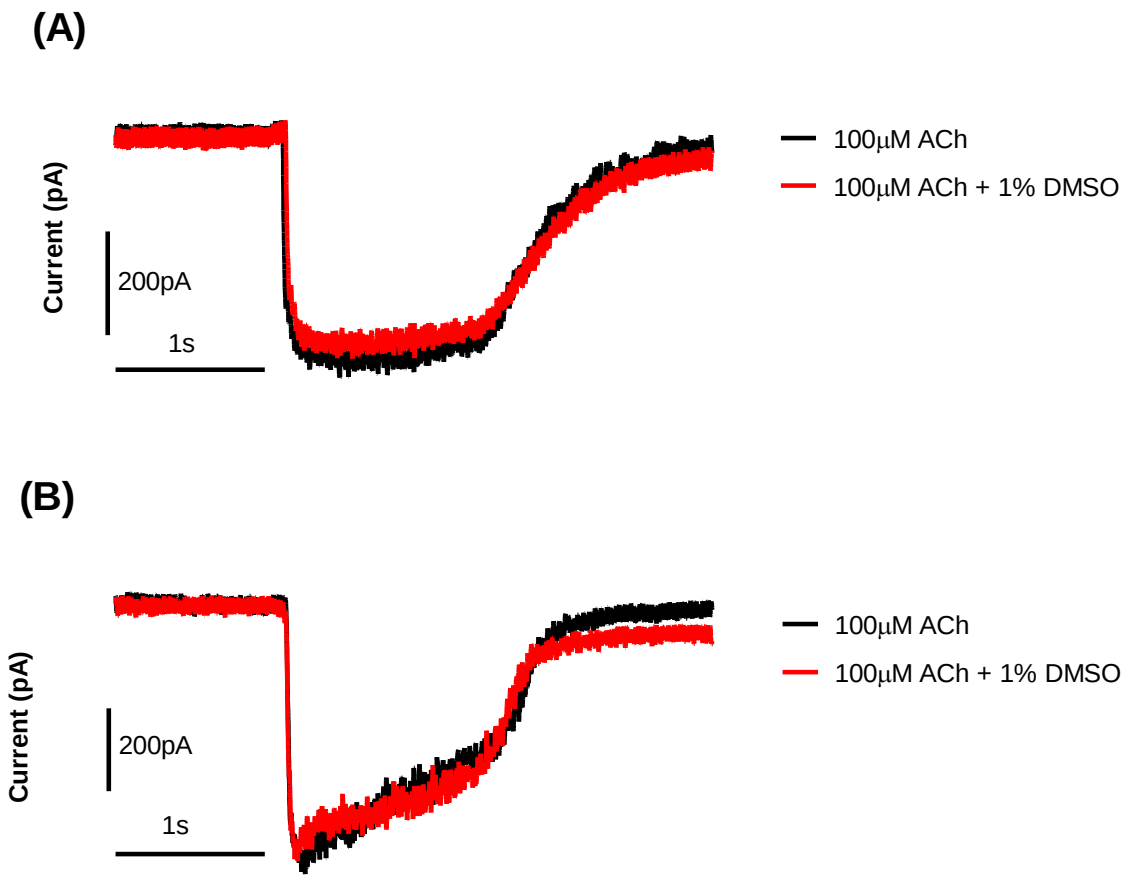

Figure 5.3.3 Trace showing the inward current of a locust neuron induced by application of 100μM ACh (black) and 100μM ACh with 1% DMSO (red) with a V_H of (A) -50mV and (B) -100mV.

5.4 Harlequin Ladybird Whole Alkaloid Extract:

Acid-base extract of the harlequin ladybird (*H. axyridis*) was dissolved in DMSO to make up a stock solution of 10mg/ml and co-applied with 100μM ACh at the concentrations shown in figures 5.4.1-5.4.2. Whole-cell responses were recorded using patch-clamp. A 4s recording sweep was used with a 1s application made at 1s into the recording period. Figures 5.4.1 and 5.4.2 show example traces (A) of, and concentration-inhibition curves (B) for locust neurons held at -50mV and -100mV respectively. Neurons were exposed to increasing concentrations of whole alkaloid extract and a final application of ACh (not shown) was used to demonstrate that inhibitory activity was caused by the test compounds rather than ACh desensitisation or loss of seal between electrode and cell. Results were discarded if the 1s current of the final ACh application was equal to, or lesser in amplitude than the highest concentration of extract applied.

Both concentration-inhibition plots suggest that the extract shows little activity at low concentrations but displays the ability to cause effective inhibition of the nAChR at concentrations greater than $1x10^{-3}$ mg/ml and causes an outward current 15% (-50mV) and 16% (-100mV) of the control inward current at a concentration of 0.1 mg/ml.

To investigate whether *H. axyridis* extract acts as an agonist to nAChR expressed by locust neurons, applications of extract at a concentration of $1x10^{-2}$ mg/ml were made to individual ACh responsive locust neurons with a V_H of either (A) -50mV or (B) -100mV. Example traces from neurons which showed an inward current response are shown in figure 5.4.3. Only 45% (N=5 of 11) of cells clamped at -50mV, and 50% (N=6 of 12) of cells held a -100mV showed any activity upon application of *H. axyridis* extract. Mean current size, normalised against ACh response is shown in figure 5.4.4. The timings of the two responses coincide with the time at which the extract is perfused across the cell, and the time the wash-off begins.

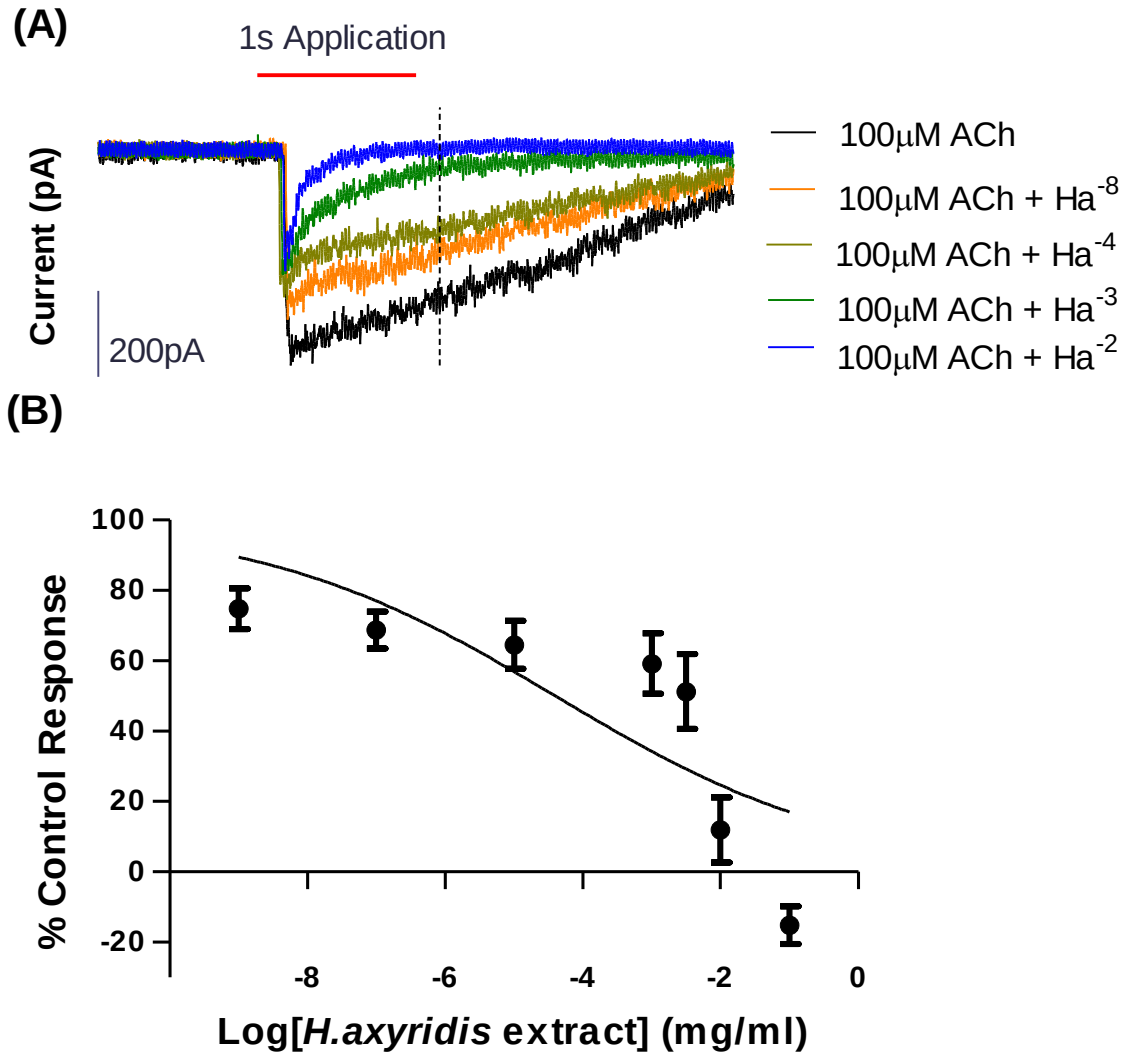

Figure 5.4.1 (A) Whole-cell responses of a locust neuron after application of 100μM ACh and then co-application of ACh with increasing concentrations ($1x10^{-x}$ mg/ml) of *H. axyridis* alkaloid extract. V_H was -50mV and dotted line shows one second post onset of ACh response, the point at which current measurements were taken for IC_{50}. Traces for $1x10^{-10}$ mg/ml and $1x10^{-6}$ mg/ml are not shown for clarity. (B) A concentration-inhibition non-linear regression curve showing the effect *H. axyridis* alkaloid extract had on size of currents elicited by 100μM ACh when held at -50mV. The IC_{50} for this curve is 39.9ng/ml, N=14 (N=7 for $1x10^{-2}$ mg/ml). Error bars show standard error.

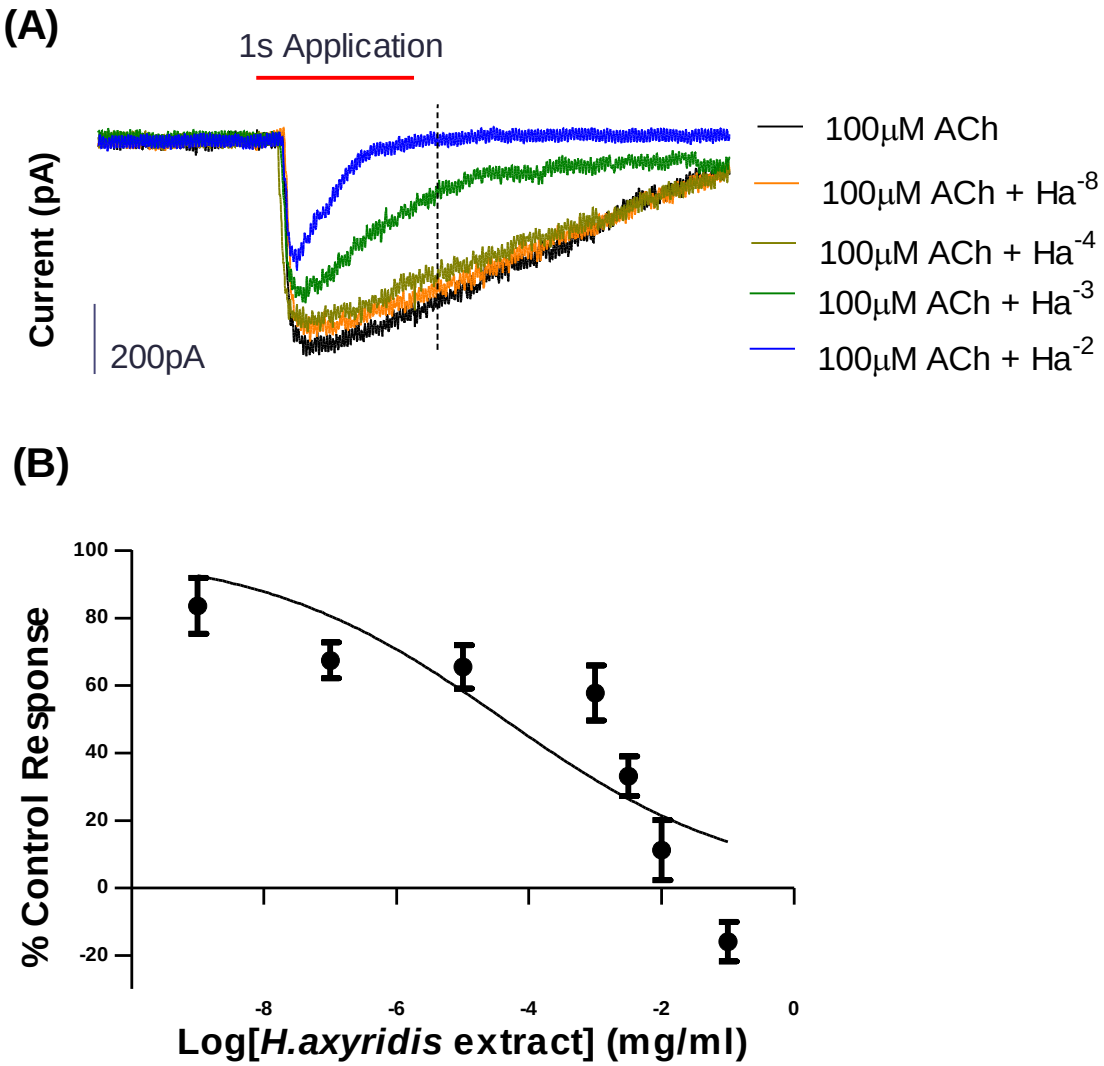

Figure 5.4.2 (A) Whole-cell responses of a locust neuron after application of 100μM ACh and then co-application of 100μM ACh with increasing concentrations ($1x10^{-x}$ mg/ml) of *H. axyridis* alkaloid extract. V_H was -100mV and dotted line shows one second post onset of ACh response, the point at which current measurements were taken for IC_{50}. Traces for $1x10^{-10}$ mg/ml and $1x101^{-6}$ mg/ml are not shown for clarity. (B) A concentration-inhibition non-linear regression curve showing the effect *H. axyridis* alkaloid extract has on size of currents elicited by 100μM ACh when V_H = -100mV. Current readings were taken 1s after onset of ACh response to measure activity affecting channel inactivation. The IC_{50} for this curve is 41.4ng/ml. N=15 (N=6 for $1x10^{-2}$ mg/ml). Error bars show standard error.

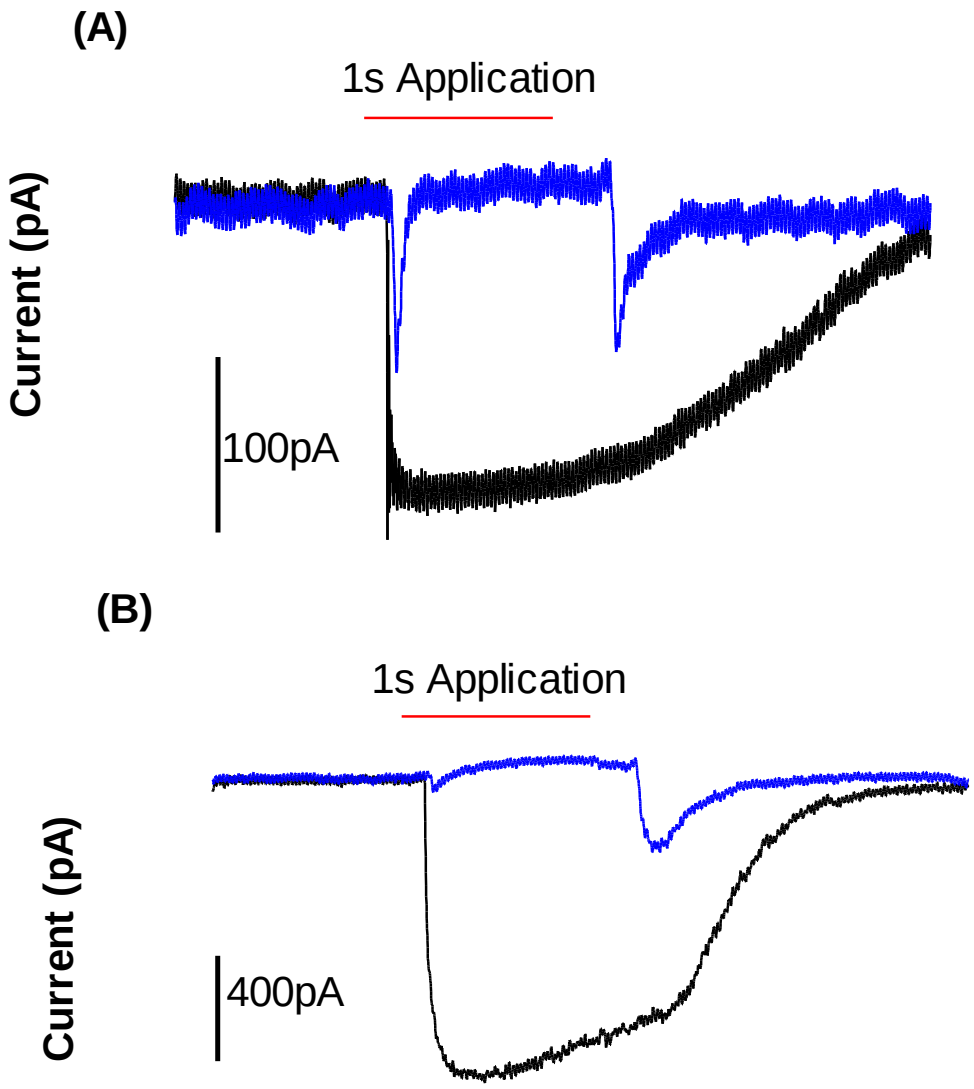

Figure 5.4.3 Example traces of whole-cell currents from individual locust neurons perfused with ACh (black) and harlequin ladybird extract at a concentration of $1x10^{-2}$ mg/ml (blue). The trace in (A) had a V_H of -50mV whereas that shown in (B) had a V_H of -100mV. Two inward currents can be seen on both traces, these coincide with the perfusion of extract (red line) and with the beginning of the wash-off, which began immediately after the application finished.

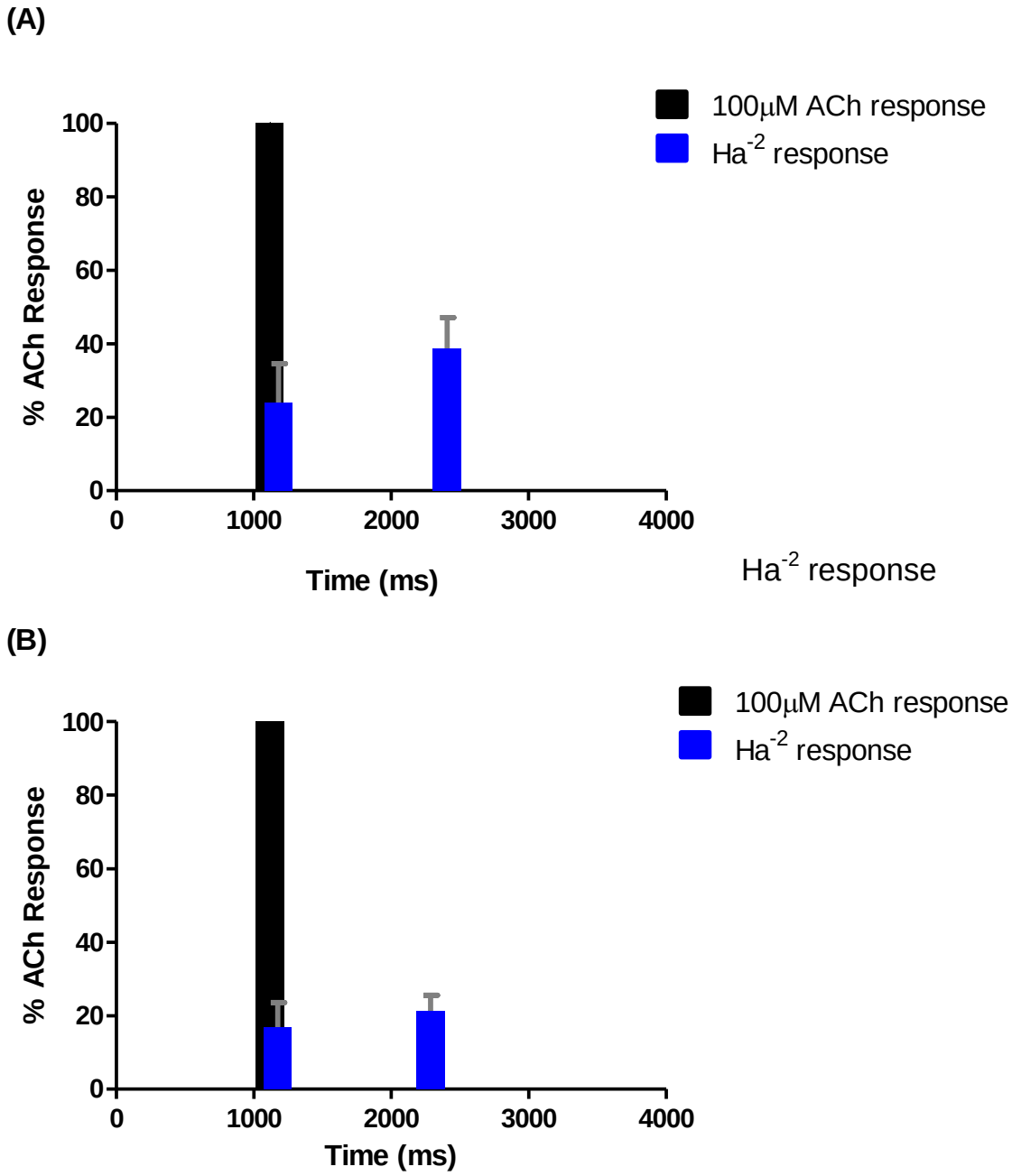

Figure 5.4.4 These column charts show the mean magnitude of the two inward currents (blue) normalised against the ACh response (black). (A) shows the results from neurons held at -50mV and (B) shows the results from neurons held at -100mV. The mean value for the first current in (A) is 24% and for the second is 39%. The mean value for the first current in (B) is 17% and for the second is 21%. Error bars show standard error.

5.5 Hippodamine, Synthetic Analogue of An Alkaloid produced by *Hippodamia convergens*:

An analogue of the alkaloid hippodamine, naturally produced by the convergent ladybird, was synthesised by Dr. Rob Stockman (University of Nottingham). This was made into a stock solution of 10mM in DMSO and diluted to the concentrations shown in figures 5.5.1 and 5.5.2. An identical protocol to that outlined in section 5.4 was used and the results for neurons held at -50mV and -100mV are shown in figures 5.5.1 and 5.5.2 respectively. A clear inhibition of ACh induced currents can be seen in concentrations above 10nM at both holding potentials used. Concentrations above 100μM caused an outward current in cells held at -50mV, and those above 10μM caused inward currents in neurons held at -100mV. This can clearly be seen in the trace shown in figure 5.5.2.

Application of hippodamine alone at a concentration of 10μM failed to generate any electrical activity (figure 5.5.2) at either V_H = -50mV or -100mV.

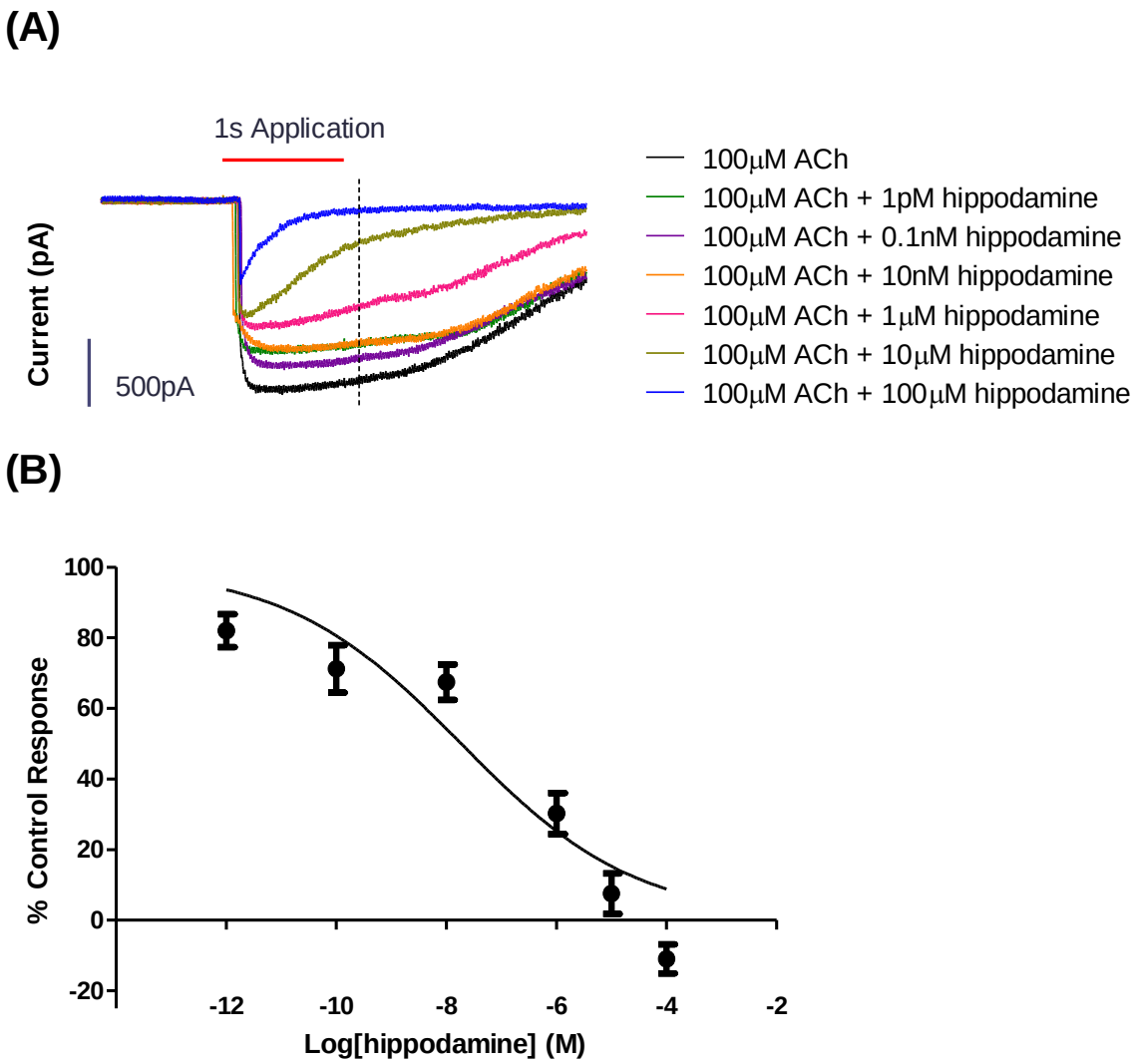

Figure 5.5.1 (A) Whole-cell responses of a locust neuron after application of 100μM ACh and then co-application of 100μM ACh with increasing concentrations of synthetic hippodamine (M), an alkaloid produced by the convergent ladybird (*Hippodamia convergens*). The holding potential was -50mV and applications were for 1s. The dotted line shows one second post onset of ACh response, the point at which current measurements were taken for IC_{50}. (B) Shows the concentration-inhibition plot fitted with a non-linear regression, for locust neurons held at -50mV and subjected to applications of increasing concentrations of hippodamine in conjunction with ACh. The IC_{50} for this curve is 18.5nM. N=14 (N=8 for 10μM) and error bars show standard error.

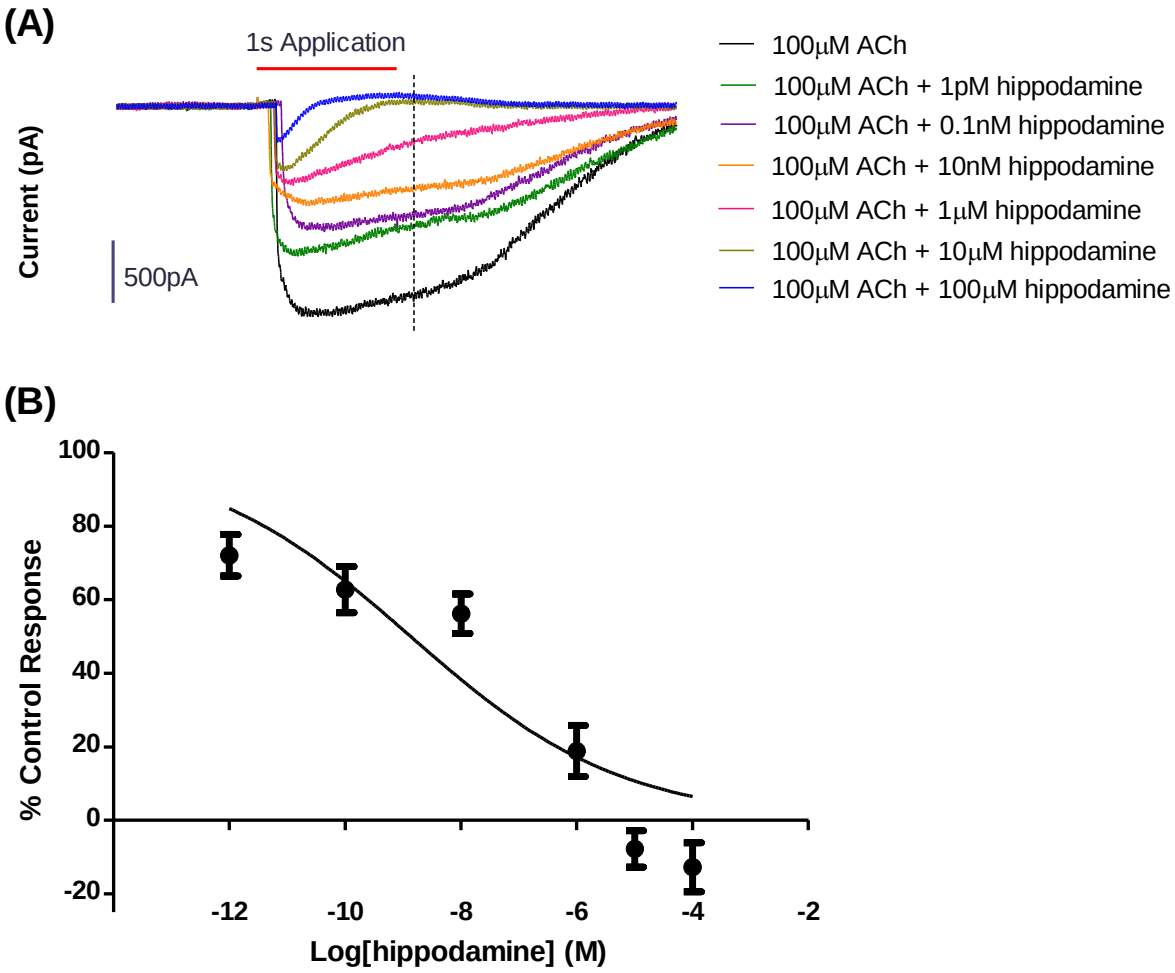

Figure 5.5.2 An example trace (A) and concentration/inhibition slope fitted with a non-linear regression curve (B) showing the affect of synthetic hippodamine when co-applied with 100μM ACh to locust neurons held at -100mV. Current readings were taken at 1s post onset of ACh response (dotted line) to record activity on channel closing activity. The IC_{50} generated by this curve is 1.35nM. N=17 (N=11 for 10μM) and error bars show standard error.

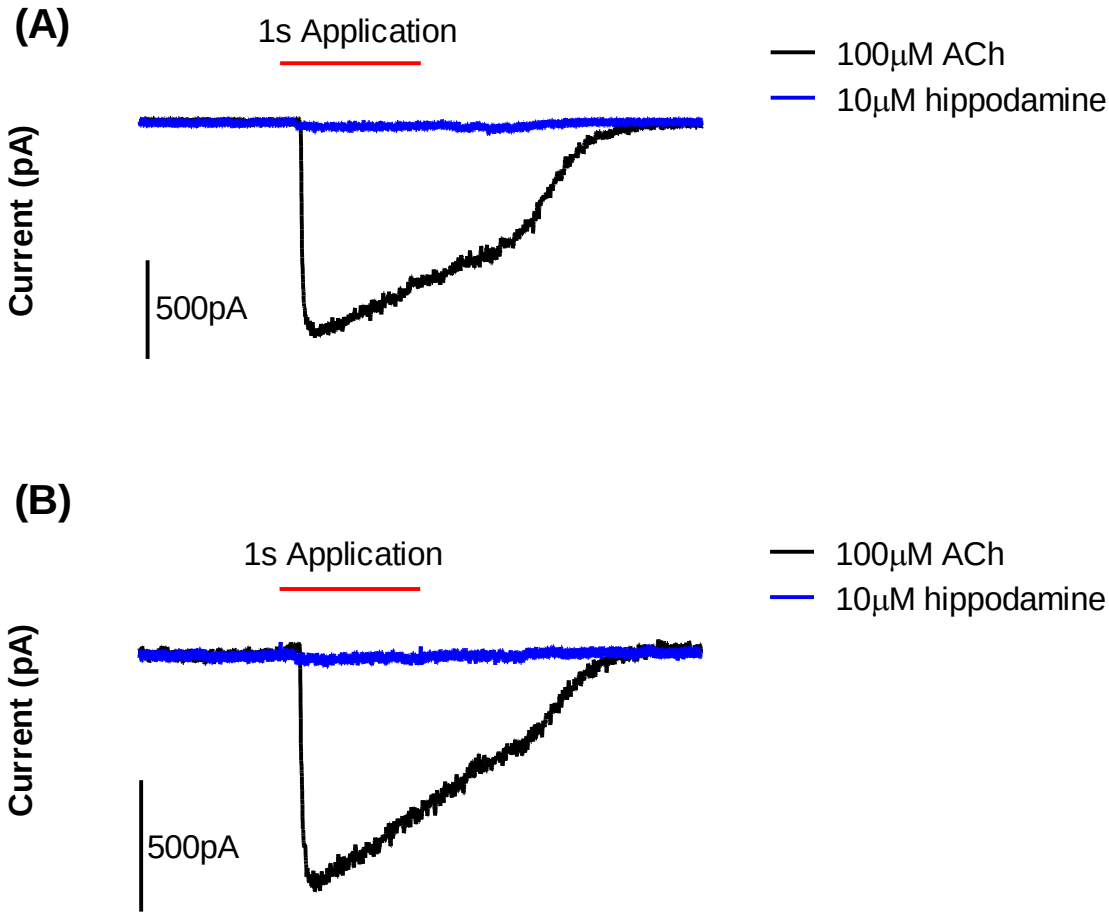

Figure 5.5.3 Whole-cell electrical activity of locust neurons clamped at -50mV (A) and -100mV (B) when hippodamine at a concentration of 10μM was applied to cells in a 1s pulse. No locust neurons displayed any change in electrical activity upon application of hippodamine.

5.6 F-test comparisons:

H. axyridis Extract:

To ascertain whether concentration-inhibition curves and IC_{50}s were significantly different, the following pair-wise comparisons were made using F-tests. The comparisons show that there was no significant difference between the fitting parameters for curves generated from locust neurons at V_H -50mV and -100mV subjected to increasing concentrations of harlequin ladybird extract (A). Comparisons between curves generated using TE671 cells and locust neurons revealed a significant difference in IC_{50}s at V_H -50mV (B) and -100mV (C) with greater inhibition of ACh induced currents in locust neurons.

	Pair-wise comparisons		**P value**
(A)	Locust neuron: -50mV (Fig 5.5.2)	Locust neuron: -100mV (Fig 5.5.3)	0.9798
(B)	Locust neuron: -50mV (Fig 5.5.2)	TE671: -50mV (Fig 4.3.1)	0.0015*
(C)	Locust neuron -100mV (Fig 5.5.3)	TE671: -100mV (Fig 4.3.2)	0.0005*

Table 5.6.1 Results of F-tests comparing the fitting parameters of concentration inhibition curves generated from TE671 cells and locust neurons when subjected to increasing concentrations of *H. axyridis*. Significant results are marked with '*'.

Hippodamine:

Comparisons between concentration-inhibition curves generated by subjecting both TE671 cells and locust neurons to increasing concentrations of hippodamine revealed significant differences between all IC_{50}s.

	Pair-wise comparisons		**P value**
(A)	Locust neuron: -50mV (Fig 5.6.2)	Locust neuron: -100mV (Fig 5.6.3)	0.0153*
(B)	Locust neuron: -50mV (Fig 5.6.2)	TE671: -50mV (Fig 2.4.1)	< 0.0001*
(C)	Locust neuron: -100mV (Fig 5.6.3)	TE671: -100mV (Fig 2.4.2)	< 0.0001*

Table 5.6.2 Results of F-tests comparing the fitting parameters of concentration inhibition curves generated from TE671 cells and locust neurons when subjected to increasing concentrations of hippodamine.

6. Discussion:

The results shown in chapter three, table 3.1.1, show that of the three species collected, 7-spot ladybirds yielded the lowest mean quantity of alkaloid per gram of beetles. The whole alkaloid extract of harlequin and pine ladybirds was 6.8 and 6.5 times as much respectively as was extracted from 7-spot ladybirds. A number of explanations could be put forward for this observation; prior to freezing 7-spot ladybirds seem to be more inclined to reflex bleed than other species (personal observation) and possibly exhaust their supply of alkaloid containing haemolymph which is known to be finite (Holloway *et al*, 1991). Furthermore the HPLC traces shown in figures 3.1.1 and 3.1.2 show that the haemolymph of *H. axyridis* is far more complex, with seven major clusters of peaks, than *C. 7-punctata* which had just four. This lack of alkaloid components identified in the haemolymph could be the reason that total alkaloid content was comparatively so low in 7-spot ladybirds. Peaks A-E in figure 3.1.1 and all peaks in figure 3.1.2 are consistent with alkaloids. A pure reference of precoccinelline had a HPLC elution time of 2.5 minutes (Mike Birkett, personal communication). Peaks F and G in figure 3.1.1 are highly hydrophobic, with elution times over 40minutes. It is difficult to speculate what alkaline compounds other than alkaloids may be present in the haemolymph of ladybirds, and therefore present in the acid-base extract. Further work with these peaks will reveal whether they display any activity and will allow us to identify what they might be.

Six haemolymph components have previously been identified from *H.* axyridis (Durieux *et al*, 2010), these include the alkaloids harmonine and S-3-hydroxypiperidin-2-one, as well as pyrazines and sesquiterpenes. 7-spot ladybirds are the most well studied ladybird species in terms of haemolymph components. 23 compounds have been identified (Durieux *et al*, 2010), including the alkaloids coccinelline, its free base precoccinelline and myrrhine. Other components include

acetates, acids, alkanes, pyrazines, alcohols, monoterpenes, sesquiterpenes, aldehydes and benzopyrroles. The comparative complexity observed for *H. axyridis* extract could be because this species naturally produces a greater diversity of alkaloids than *C. 7-punctata* but there is no evidence to suggest this is the case. Alternatively this could be caused by sequestration of alkaloids from other ladybirds upon which *H. axyridis* is known to feed (Cottrell & Yeargan, 1998; Ware & Majerus, 2008), whereas *C. 7-punctata* are thought to turn to ladybirds only when aphid prey is scarce (Majerus, 1994; Sato *et al*, 2005). If this habit of feeding upon other ladybirds leads to sequestration of ladybird alkaloids, as demonstrated by Sloggett *et al* (2009a) then the alkaloid content of harlequin ladybirds would be expected to be higher than that of 7-spot ladybirds. The jaw structure of pine ladybirds suggests that they are predatory and probably feed upon aphids, coccids and possibly other arthropods (Minelli & Pasqual, 1977). Radwan & Lövei (1983) however conclude that they feed on both aphids and coccids, but make no suggestion that they feed on other ladybirds. It therefore seems unlikely that sequestration of alkaloids from other ladybirds is the sole reason that harlequin and pine ladybirds contained much larger quantities of alkaloid than 7-spot ladybirds.

The results shown in chapter four (figs 4.3.1, 4.3.2, 4.4.1 and 4.4.2) clearly show that both *H. axyridis* alkaloid extract and the convergent ladybird alkaloid hippodamine potently inhibit nAChR whole-cell currents in TE671 cells clamped at both -50mV and -100mV. We can conclude from this that harlequin and convergent ladybirds possess toxic alkaloids as has been previously suggested (Pasteels *et al*, 1973; Agarwala & Dixon, 1992; Daloze *et al*, 1995; Al Abassi *et al*, 1998; Al Abassi *et al*, 2001; Sato & Dixon, 2004; Dolenska *et al*, 2009; Sloggett, 2009a; Sloggett, 2009b; Sloggett, 2010) and these toxic alkaloids target the nAChR. It also seems plausible that the bright colouration displayed by the harlequin and convergent ladybird is likely to serve as an aposematic warning (Daloze *et al*, 1995; Glisan-

King & Meinwald, 1996; Grill & Moore, 1998; Al Abassi *et al*, 2001; Majerus, 2006, Sloggett, 2010). F-test comparisons revealed that differences between concentration inhibition curves generated for the two holding potentials were not significant. Therefore neither harlequin ladybird extract, nor hippodamine display voltage dependent activity towards mammalian nAChR. The IC_{50}s for *H. axyridis* extract were; 2.12 µg/ml (V_H = -50mV) and 1.70 µg/ml (V_H = -100mV). The molecular weight of the *H. axyridis* extract is not known but that of hippodamine is 193.37g/mol. When the IC_{50} values for hippodamine are converted into mg/ml they become 115 ng/ml (Vh = -50mV) and 129 ng/ml (Vh = -100mV). Comparison of the IC_{50} values for *H.* axyridis extract and hippodamine reveals that the latter is 14-18 fold more potent to mammalian nAChR than the former. This is likely to be because the active components of *H. axyridis* extract are in solution and diluted by inactive compounds. The alkaloid which has been isolated from harlequin ladybirds, harmonine is structurally very different from hippodamine (Fig 1.1.7). The potency of harmonine when tested alone is not known, but further work with the fractions identified in figure 3.1.1 will elucidate whether this alkaloid is responsible for the activity observed in these experiments.

The shape of traces shown in figures 4.3.1, 4.3.2, 4.4.1 and 4.4.2 suggest that these compounds may cause desensitisation of the nAChR or act as open channel blockers. The late current, recorded 1s after application appears to be affected more than the peak current. This suggests that activation of the channel is necessary for the alkaloids to exert their channel blocking activity. Application of 10µM ACh results in an inward current which decays to a plateau within roughly two seconds. When 10µM ACh is co-applied with increasing concentrations of harlequin ladybird extract or hippodamine, the inward current decays much more rapidly than the control response. At *H. axyridis* concentrations of 0.1 mg/ml an outward current is visible. This may be caused by activation of K^+ channels. Application of increasing concentrations of ladybird compounds resulted in nAChR

currents which rapidly diminished in a concentration dependent manner. Application of 10μM ACh 30s after application of the highest concentration of harlequin ladybird extract and hippodamine resulted in an inward current comparable with the control response. This shows that the desensitisation caused by these ladybird alkaloids is reversible.

The nAChR activity displayed by both treatments resembles that of philanthotoxin-(12) when co-applied with ACh to TE671 cells. This toxin belongs to a group of polyamine containing wasp toxins which acts as potent noncompetitive antagonists of cation selective ionotropic receptors (Usherwood, 2000; Usherwood & Mellor, 2004; Tikhonov *et al*, 2004). This compound shows weak voltage dependence and is thought to bind to the extracellular surface of the nAChR (Brier *et al*, 2003), interacting with hydrophobic Equatorial Valine and Outer Leucine rings which can be accessed from within the pore (Tikhonov *et al*, 2004). This results in reversible desensitisation. It is possible that the ladybird alkaloids tested are interacting with the same hydrophobic amino acid residues resulting in very similar pharmacology.

The application of ladybird extract at a concentration of 1 μg/ml and hippodamine at a concentration of 10μM in the absence of ACh (figures 4.3.3 & 4.4.3) failed to elicit any channel activity in TE671 cells at either holding potential tested. We have therefore concluded that the tested ladybird compounds do not agonise vertebrate nAChR. Again, this is consistent with the pharmacology of philantotoxin-(12) which doesn't agonise the nAChR of TE671 cells (Brier *et al*, 2003).

The development of a method for making whole-cell recordings from locust neurons enabled us to draw direct comparisons between ladybird compound activity on both vertebrate and invertebrate nAChR. Once optimised it was possible to gather consistent data from the locust preparation. The results shown in chapter five clearly show that both harlequin ladybird extract and hippodamine display

potent antagonism of locust whole-cell nAChR currents. The shape of the traces (figures 5.4.1, 5.4.2, 5.5.1 and 5.5.2) is consistent with those gathered for vertebrate nAChR, with an apparent desensitisation which increased with increasing concentration of extract / alkaloid. F-test comparisons revealed no voltage dependence for *H. axyridis* extract (table 5.6.1) but the comparison between IC_{50} curves for hippodamine at V_H = -50mV (IC_{50} = 18.5nM) and V_H = -100mV (IC_{50} = 1.35nM) (table 5.6.2) yielded a significant difference (p = 0.153). This is surprising because none of the other voltage comparisons proved significant and the same compound doesn't display voltage dependence in vertebrate nAChR. It may be the case that like philanthotoxin-(12), these compounds do show slight voltage dependence but there is not sufficient data to reveal these subtle differences in the other three holding potential comparisons. It is also possible that the noted difference is caused by the point for 10μM hippodamine which is the mean of a smaller number of repeats (N = 8 for V_H = -50mV and N = 11 for V_H = -100mV). These points differ markedly between the two holding potentials and the standard error bars do not cross. More repeats will be needed to assess whether this is the result of actual voltage dependence, or a recording artefact. No other points differ as considerably.

When comparing the locust neuron derived IC_{50} values for *H. axyridis* extract and hippodamine, converted to mg/ml we find that hippodamine is at least 42 fold more potent than harlequin ladybird extract. Again this is likely to be caused by the dilution of active compounds in the ladybird extract, further testing with the HPLC fractions identified in figure 3.1.1 will allow us to identify the active components and the active fractions are expected to show higher potency than whole extract.

When the harlequin ladybird extract was perfused over locust neurons at a concentration of 10 μg/ml in the absence of ACh two separate inward currents occur (fig 5.4.3). Only 45% (N=5 of 11) of ACh sensitive cells clamped at -50mV,

and 50% (N=6 of 12) of cells held a -100mV showed this rapidly desensitising activity. The timings of the two responses coincide with the time at which the extract is perfused across the cell, and the time the wash-off begins. While this might be expected from a rapidly desensitizing agonist concerns that it is caused by either the pressure of the application, or residual ACh in the output tube need to be dispelled. This requires further clarification before we can draw firm conclusions about agonism of locust nAChR by this extract. No channel activity was detectable upon application of hippodamine at a concentration of 10μM in the absence of ACh.

When comparing the concentration inhibition curves for TE671 cells with locust neurons (tables 5.6.1 and 5.6.2) we find that all comparisons are significant, for both *H. axyridis* extract and hippodamine. In every instance the compounds were significantly more potent to invertebrate nAChR than to vertebrate. The difference is clearly reflected in the IC_{50} values and the shapes of the concentration inhibition curves. Extract from *H. axyridis* was at least 41 times more potent to locust neurons than TE671 cells. Hippodamine was at least 1205 times more potent to nAChR of locust neurons than TE671 cells. This is clear evidence of selective toxicity towards invertebrate nAChR. The potent nature of hippodamine towards locust neuron nAChR meant that lower concentrations were needed to get an accurate IC_{50} value. Although hippodamine shows reversible antagonism, it is possible that hippodamine was not completely removed during the 30s wash off in between applications. This may skew the data in favour of locust neurons, but it is very unlikely that this accounts for the huge disparity between the IC_{50} values collected for vertebrate and invertebrate nAChR.

It is surprising that the difference in toxicity is so great when considered in the context of the visual warning that ladybirds display. The targeting of compounds towards invertebrates would suggest that ladybirds are under a selective pressure to develop protection against invertebrate predators. It has long been assumed that the bright, aposematic colouration displayed by many ladybirds

(including harlequin and convergent ladybirds) serves as a warning to vertebrate predators, particularly birds (Dolenská *et al*, 2009). Feeding studies have shown that some ladybird species are fatally toxic to nestling blue tits (Marples *et al*, 1993a; Marples *et al*, 1993b) and ladybird colour is a more effective deterrent to Japanese quails than either taste or smell (Marples *et al*, 1994). The most well studied predatory interactions between ladybirds and other invertebrates, apart from aphids, are between ladybirds and ants (Tursch *et al*, 1971; Majerus *et al*, 1994) and intraguild predation within ladybirds (e.g. Sloggett *et al*, 2010). Ants are known to have good eyesight (Reiskind, 1977) and the ability to discriminate between colours (Kretz, 1979), so it is possible that aposematic colouration is aimed at ants. The alkaloid coccinelline and a small number of other ladybird alkaloids have been shown to repel ants (Tursch *et al*, 1971; Tursch *et al*, 1975) but no work has been carried out to assess the efficacy of visual aposematic warnings to ants. Many species of ladybird are known to prey upon other ladybird species (Hemptinne *et al*, 2000; Sato & Dixon, 2004; Ware *et al*, 2008; Sloggett, 2009a; Sloggett, 2009b; Kajita *et al*, 2010). Consumption of some other ladybirds or ladybird eggs, notably harlequin ladybirds is known to cause death to other species of ladybird (Yasuda & Ohnuma, 1999; Sato & Dixon, 2004). From this it can be concluded that compounds within the haemolymph confer protection against a range of different organisms, both vertebrate and invertebrate. Ladybirds possess an arsenal of warnings; visual, scent, taste and toxicity which enable them to avoid predation. The fact that the alkaloids display much greater toxicity towards invertebrates suggests that these apply the greatest predatory selective pressure and other ladybirds probably apply the greatest predation pressure of all.

The mechanism that allows ladybird alkaloids to selectively target insect nAChR is not known. Insect nAChR have different pharmacological characteristics to those of vertebrates (Tomizawa & Casida, 2003). Two subtypes of nAChR with different α-bungarotoxin sensitivity have been identified in the dorsal unpaired

median neuron of *Periplaneta americana* cockroach. The α-bungarotoxin insensitive receptors can be further subdivided based on the affinity they have for imidacloprid (Buckingham *et al*, 1997; Courjaret & Lapied, 2001). Imidacloprid and other neonicotinoid insecticides possess a negatively charged nitro or cyano group. This interacts with a nAChR subsite which isn't present in vertebrate nAChR, consisting of cationic amino acid residues (Tomizawa & Casida, 2003). This is the basis for the selectivity which neonicotinoids display. It is possible that ladybird alkaloids interact with the same subsite when an agonist is present, but we have no evidence to suggest that this is the case.

The differences in potencies displayed by harlequin ladybird extract and particularly hippodamine between vertebrate and invertebrate nAChR make them promising leads for novel insecticides. The neonicotinoids represent the current 'gold standard' of insect selective insecticides which target the nAChR. Imidacloprid and thiacloprid have IC_{50}s of 4.6 and 2.7nM respectively for *Drosophila* nAChR whereas those for mammalian α4 β2 are 2.6μM and 860nM respectively (Tomizawa & Casida, 2003). This compares with hippodamine that has an IC_{50} of 18.5nM (V_H = -50mV) and 1.3nM (V_H = -100mV) in locust neurons. The human IC_{50} was found to be 22.3μM (V_H = -50mV) and 25μM (V_H = -100mV). The currently available 'gold standard' are between 319-565 times as potent to *Drosophila* as they are to vertebrate nAChR. Hippodamine is at least 1205 times as potent to locust nAChR as it was to human muscle nAChR. It is not known what effect hippodamine, or any other ladybird alkaloids have on other receptors or ion channels. This, along with many other qualities such as thermo and photostability, lipid and water solubility need to be assessed before any attempt is made to develop insecticides from these compounds.

In the future we intend to classify which of the harlequin ladybird fractions identified by HPLC are responsible for the activity against nAChR. Once the compound has been identified we will conduct more concentration inhibition

experiments in both TE671 cells and locust neurons to establish definitive IC_{50} values for this toxin. It will also be important to study the insecticidal effects of these alkaloids *in vivo* to discover how this compares with their observed *in vitro* toxicity. The apparent agonism of locust neuron nAChR by *H.* axyridis extract also warrants further research. Application of this extract to different subtype conformations of nAChR will reveal whether the observed channel activity is the result of rapidly desensitising agonism, or an artefact of residual ACh. Further to this, the vast majority of ladybird alkaloids remain unstudied in terms of biological activity. A huge structural diversity exists and this provides a fantastic potential source of useful compounds, and will also allow us to study the structure-function relationships of these natural products. Studying how these alkaloids interact with receptors and ion channels could give us a deeper understanding of how these complex and dynamic proteins operate and could enable the development of more effective insecticides or therapeutics.

Summary:

The results we have gathered demonstrate that ladybird alkaloids are toxic and they appear to target the nAChR. Comparison of whole-cell IC_{50} values collected for vertebrate (TE671) and invertebrate (locust neurons) nAChR revealed that alkaloid extract of the harlequin ladybird is 41 times as potent to invertebrates as it is to vertebrates. Hippodamine is at least 1205 times as potent to invertebrates as it is to vertebrates. The selectivity displayed by both, and particularly hippodamine makes these promising leads for insecticidal compounds. The effect of these alkaloids on other receptors and ion channels is not known. It also remains to be seen how these electrophysiological results translate into *in vivo* insecticidal activity.

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