Investigating Ladybird Alkaloids as Potential Leads for Novel Insecticides



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Abstract

Ladybird beetles produce a range of alkaloid compounds which are found at high concentration in the haemolymph and released in response to perceived threat. These alkaloids are thought to function as agents of chemical defence and a small number have been shown to antagonise the nicotinic acetylcholine receptor. As intraguild predators, the chemical defence may be under selective pressure from other predatory insects, the alkaloids may therefore have evolved to target insects predominantly. To discover if other ladybird alkaloids show activity at nAChRs and investigate mode of action, alkaloids were extracted from 7-spot, 10-spot, 14-spot, cream spot, pine, orange and harlequin ladybird beetles and purified with acid-base separation. These species are each known to produce a different subset of alkaloids. Their action on nicotinic acetylcholine receptors was investigated by constructing concentration-inhibition plots by co-applying alkaloid extracts with acetylcholine to human embryonic muscle-type of TE671 cells and locust neuronal nAChRs. Peak current, net-charge and late (1 s) current were measured with whole-cell patch-clamp at V_H = -75 mV.

All alkaloid extracts antagonised the ACh responses of both cell types but potency and selectivity varied. The late current was the most strongly affected current parameter and the most potent antagonists of locust 1 s currents were alkaloids produced by 10-spot, 7-spot, pine and harlequin ladybird with IC_{50}s ranging from 0.29–0.76 µg/mL. The 14-spot, cream spot and orange ladybird alkaloids had higher IC_{50}s, ranging from 1.6-4.9 µg/mL. The IC_{50} values calculated for all species with, the exception of harlequin and orange ladybirds were significantly lower for locust than human nAChRs and these ranged from 1.3–47.8 µg/mL. The alkaloid extract that

showed the highest selectivity for locust nAChRs was that of the 7-spot ladybird, with an IC_{50} value 58.4 fold lower. The alkaloids of the 10-spot, 14-spot, cream spot and pine ladybird also showed significantly higher potency for locust nAChRs over human nAChRs with IC_{50}s 10.3–35 fold lower. Orange and harlequin ladybird alkaloids did not show higher potency for locust nAChRs. Harlequin ladybird alkaloids were highly potent to the nAChRs of both cell types whereas orange ladybird alkaloids showed low activity. The differences in potency correspond with differences in ecology, with the species that showed highest insect potency often found in close association with other predatory ladybird species. Those that showed low insect potency are arboreal specialists that are less likely to encounter insect predators.

To investigate mode of action, ACh concentration-response curves in the presence and absence of harlequin ladybird alkaloid extract were constructed using TE671 cells. Increasing ACh concentrations were unable to overcome the inhibition caused by this alkaloid extract, indicating non-competitive action. Fitting the Woodhull equation to IC_{50} values calculated at holding potentials of 50, -50, -75, -100 and -120 mV revealed that the antagonism caused by this alkaloid extract was voltage-dependent ($P = 0.012$), with late current IC_{50}s of 23.1, 15.5 and 6 µg/mL at holding potentials of +50, -50 and -120 mV respectively. These data indicate that the active alkaloid(s) within this extract bind at an allosteric site and the strong voltage-dependence of inhibition suggests mode of action is open-channel block rather than enhancement of desensitisation. The alkaloid harmonine was separated from other components of the alkaloid extract by thin-layer chromatography and the inhibitory activity tested against both human and locust nAChRs at V_H = -75 mV. Both fractions were found to be active and displayed similar inhibitory activity towards human nAChRs with IC_{50}s of 1.68 µg/mL for harmonine and 4.77 µg/mL for other alkaloid

compounds. Harmonine was found to be significantly more potent towards locust nAChRs with an IC_{50} of 0.072 µg/mL as opposed to 3.23 µg/mL. This could indicate that harlequin ladybirds produce several alkaloids with different functions; harmonine may act as a chemical defence compound targeted towards insect predators.

The alkaloid extract of the 10-spot ladybird was found to show significantly higher potency towards locust nAChRs than human. One of two alkaloids produced by this species is (-)-adaline. Synthetic (-)-adaline displayed identical inhibitory activity towards both cell types as the alkaloid extract of the 10-spot ladybird. Potency was significantly higher towards locust nAChRs than human, with IC_{50}s of 10.26–45.83 µM against human and 0.55–2.97 µM against locust nAChRs. ACh concentration-response curves showed that (-)-adaline acts non-competitively and IC_{50} values at different holding potentials reveal that it is strongly voltage-dependent ($P = 0.003$ in human and $P = 0.007$ in locust nAChRs) and inactive at positive holding potentials. As with the alkaloid extract of the harlequin ladybird, this alkaloid acts as a non-competitive negative allosteric modulator which shows higher potency for locust over human nAChRs and strong voltage-dependence of inhibition, consistent with open-channel block.

A structurally diverse range of ladybird alkaloids displayed inhibitory activity towards both human and insect nAChRs. This inhibition was non-competitive and voltage-dependent for both harlequin ladybird alkaloid extract and adaline, indicating that these alkaloids act as negative allosteric modulators, possibly open-channel blockers. The majority of ladybird alkaloid extracts tested were significantly more potent towards insect than human nAChRs. It remains to be seen whether the activity of these compounds is restricted to the nAChRs, or whether they act as non-selective

pore blockers. Ladybird alkaloids appear to represent a library of novel nAChR antagonists, some of which could be further investigated as potential insecticide leads.

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Abbreviations

ACh	Acetylcholine
AUG	Area under graph
DCM	Dichloromethane
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
d-tbc	Delta tubocurarine
EC_{50}	Half maximal effective concentration
FCS	Foetal calf serum
HPLC	High-performance liquid chromatography
HCl	Hydrochloric acid
IC_{50}	Half maximal inhibitory concentration
M-nAChR	Muscle-type nicotinic acetylcholine receptor
N-nAChR	Neuronal-type nicotinic acetylcholine receptor
NaOH	Sodium hydroxide
Pen/Strep	Penicillin/Streptomycin
PDL	Poly-D-lysine
PLL	Poly-L-lysine
TLC	Thin-layer chromatography
V_H	Holding potential
4PL	Four-parameter logistic equation

Ladybird species

2-spot	*Adalia bipunctata*
7-spot	*Coccinella septempunctata*
10-spot	*Adalia decempunctata*
14-spot	*Propylea quatuordecimpunctata*
Cream spot	*Calvia quatuordecimguttata*
Harlequin	*Harmonia axyridis*
Pine	*Exochomus quadripustulatus*
Orange	*Halyzia sedecimguttata*

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Chapter 1: Introduction

1.1 Ladybird Beetles

Ladybird beetles (Coleoptera: Coccinellidae) are found on every continent with the exception of Antarctica (Roy & Majerus, 2010), and inhabit a huge range of habitats, from sea level to at least 4260 m (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; O'Connell *et al*, 2012) 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 feed exclusively upon pollen and fungus (Minelli & Pasqual, 1977). Coccinellids are holometabolous and go through four larval instars before pupating into adult beetles (Hodek, 1973). 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 ladybird 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 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 (Figure 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 production of a signal, such as warning colouration to indicate that the bearer is an unprofitable prey item. This is usually associated with a chemical defence in the form of toxins or other antifeedant compounds (Poulton, 1890; discussed in Mappes *et al*, 2005). This is supported by the fact that there are few accounts of vertebrate 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

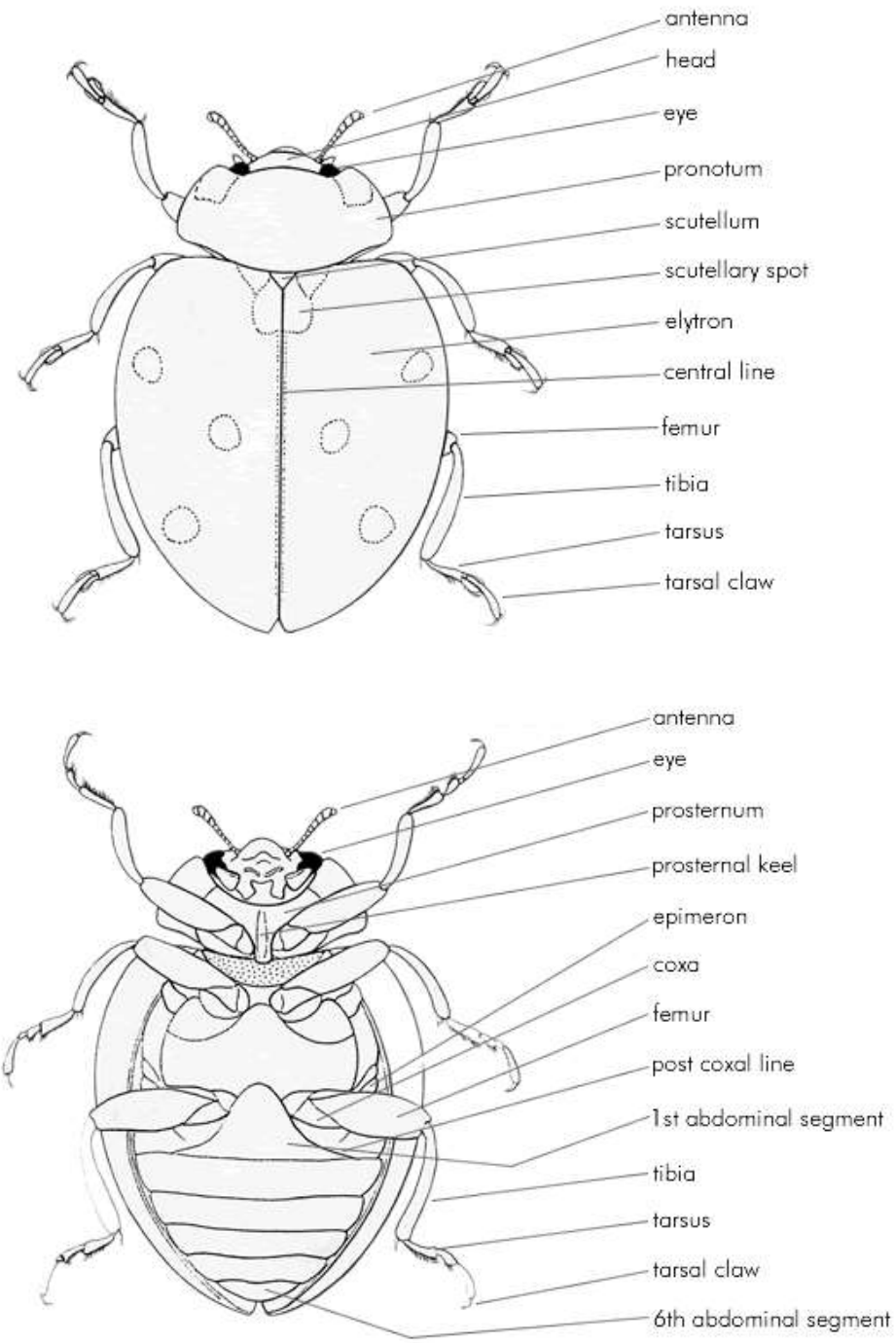


Figure 1.1.1 Diagrams showing the generalised anatomy of ladybird beetles as shown from above (top) and below (bottom). Diagrams courtesy of the Cambridge Ladybird Survey (www.ladybird-survey.org).

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 & Kimura, 2001 but see Sloggett, 2010).

Ladybird colour and patterns were found to be more effective feeding deterrents to Japanese quails (*Coturnix coturnix japonica*) than either taste or smell in the 7-spot ladybird – *Coccinella septempunctata* (Marples *et al*, 1994). In aposematic ladybird species, all life stages including eggs are brightly coloured (Winters *et al*, 2014), but a predation bioassay has demonstrated that adult beetles appear to be more effectively defended than either the eggs or larvae of the Mexican ladybird *Epilachna paenulata* (Camarano *et al*, 2006).

Under laboratory conditions, feeding trials have shown that many ladybirds are considered unpalatable and unacceptable as prey items by many predators including; birds, hedgehogs, beetles, lizards and toads (Frazer & Rothschild, 1960). They are also able to deter ants protecting aphids and coccids (Marples, 1993a; Majerus *et al*, 2007; Deyrup *et al*, 2014). 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).



Figure 1.1.3 Eyed ladybird (*Anatis ocellata*) reflex bleeding from the femoro-tibial joints and depositing haemolymph on the elytra.

When disturbed, ladybirds 'reflex bleed' (autohemorrhage) (Figure 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), releasing viscous yellow haemolymph 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. japonica*) (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 azamacrolide alkaloids (Attygalle *et al*, 1993) (Figure 1.1.4).

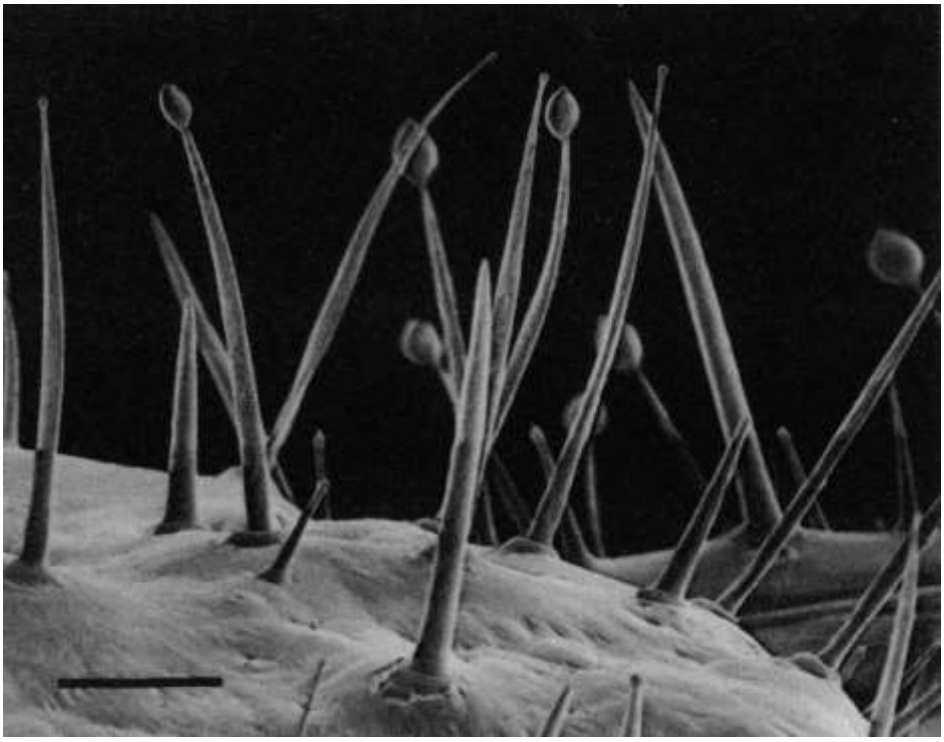


Figure 1.1.4 SEM of glandular hairs from a larva of the Mexican bean beetle *Epilachna varivestis* with droplet of alkaloid containing fluid. Scale bar shows 0.1 mm. From Attygalle *et al* (1993).

1.3 Ladybird Toxicity

A number of compounds have been isolated from ladybird haemolymph, including; acetates, acids, alkanes, alcohols, aldehydes, benzaldehydes, benzopyroles, monoterpenes, sesquiterpenes, polyketides, pyrazines and alkaloids (Durieux *et al*, 2010; Deyrum *et al*, 2014). 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 shown that precoccinelline, an alkaloid produced by the 7-spot ladybird acts as a kairomone, attracting the parasitoid wasp *Dinocampus coccinellae* which then oviposits within the beetle (Figure 1.1.5) (Majerus *et al*, 1997; Al Abassi *et al*, 2000) and deterring the aphid parasitoid *Aphidius ervi* (Nakashima *et al*, 2004).

The strong implication that the reason ladybirds are considered unpalatable by many species may be due to the alkaloids found in the haemolymph (Figure 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) has never been established experimentally. A study conducted by Marples *et al* (1989) demonstrated that feeding 7-spot ladybirds at a mass equivalent to 5.3 beetles per day to nestling blue tits (*Parus caeruleus*) caused death within two days. Autopsy indicated 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*

decempunctata) and water ladybirds (*Anisostica 19-punctata*). Pine and kidney spot ladybirds (*Exochomus quadripustulatus* 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. septempunctata*) that had been parasitised by the wasp *Dinocampus coccinellae* (right). The wasp paralyses the beetle but still allow it to reflex-bleed. The larva then spins 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 *Daphnia*, and are mainly concerned with the ecological impacts of toxicity on intraguild predation. Intraguild predation is the phenomenon whereby 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 (*Harmonia axyridis*), the 7-spot ladybird and the 2-spot ladybird. It was discovered that larvae of each species were able to safely consume conspecific eggs and *C. septempunctata* could safely consume *A. bipunctata* eggs. However, larvae of *H. axyridis* suffered 65% and 15% mortality when fed eggs from *A. bipunctata* and *C. septempunctata* respectively.

Both *A. bipunctata* and *C. septempunctata* suffered 100 % mortality when fed eggs from *H. axyridis*. Finally, *A. bipunctata* suffered 46 % mortality when fed eggs from *C. septempunctata* (Sato & Dixon, 2004). These findings are largely consistent with those of Yasuda & Ohnuma (1999), who found that *H. axyridis* appear to be fatally toxic to *C. septempunctata* but not *vice versa*. A similar study which focussed just on *C. septempunctata* and *A. bipunctata* found that not only are *C. septempunctata* reluctant to feed on eggs from *A. bipunctata*, but they also fail to complete larval development when they do so. *A. bipunctata* appeared to be less reluctant to feed on eggs from *C. septempunctata* 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* (Figure 1.1.6) and *C. septempunctata*, also found that *H. axyridis* is toxic to *C. septempunctata*. It was demonstrated that *C. septempunctata* 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 they fail to identify a causative agent of apparent toxicity. 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, became incapacitated within 24-36 hours and did 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). Whole beetle extracts of *H. axyridis*, *A. bipunctata* and *C. septempunctata* were all found to be toxic to *Daphnia magna* (Nedvěd *et al*, 2010). The extract from *H. axyridis* was found to be 67 times more potent than that of *C. septempunctata*, and ten times more potent than *A. bipunctata*. However, this experiment only took into consideration the weight of beetles from which the extract originated, not the weight of extract itself or alkaloid content. The different toxicities observed may therefore represent different quantities of alkaloid produced and stored rather than actual differences in potency. This methodology was also used by Arenas *et al* (2015) against *Daphnia pulex* to compare potency of five UK native ladybirds. These were all found to cause *Daphnia* mortality above control level and were, in order of decreasing potency; orange, 2-spot, pine, 14-spot and larch ladybird (Arenas *et al*, 2015). Again, because alkaloids were not isolated and quantified, these findings can not be considered firm indications that ladybird alkaloids are indeed the basis of ladybird chemical defence.

Egg feeding trials reveal that harlequin ladybirds are often able to consume the eggs of other ladybird species without ill effect, but when this situation is reversed, the predator is often killed (Kajita *et al* 2010). The assumption has been





Figure 1.1.6 Harlequin ladybird eggs (left) and larvae (right). Both eggs and larvae contain alkaloids. Many species of ladybird are intraguild predators and the larvae are particularly ferocious cannibals.

that harmonine, an alkaloid produced by *H. axyridis* which is found at high concentrations in eggs, is responsible for this toxic activity (Agarwala & Dixon, 1992; Daloze *et al*, 1995; Sato & Dixon, 2004; Dolenska *et al*, 2009; Sloggett, 2009a; Sloggett, 2009b; Sloggett, 2010), but this has not been shown experimentally and another explanation has been put forward by Vilcinskas *et al*, 2013. Recent work has shown that the injection of harlequin ladybird haemolymph into adult 7-spot ladybirds results in mortality within 13 days, but the injection of the equivalent quantity of harmonine alone, and heat-sterilised haemolymph does not. Microscopy of and DNA isolation from *H. axyridis* haemolymph samples revealed the presence of endosymbiont microsporidia which were subsequently identified as a novel strain with high homology to *Nosema thomsoni*. Transcriptome analysis revealed very low levels of microsporidia gene expression, suggestive that microsporidia were present in a physiologically inactive spore stage and thus non-damaging to host beetles, which presumably possess an evolved resistance (possibly harmonine itself). The authors argue that it is pathogenic microsporidia acting as biological weapons that make harlequin ladybirds, their eggs and larvae dangerous prey items for other ladybirds, rather than chemical defence provided by harmonine, but this has been widely disputed and methodology questioned (Solter *et al*, 2013). Subsequent feeding trials reveal that the majority of 7-spot ladybirds are able to tolerate feeding upon harlequin larvae (88.6 %) and that neither microsporidia or harmonine appear to give harlequin ladybirds a competitive advantage (Gegner *et al*, 2015).

1.4 Alkaloids Isolated From Ladybirds

Alkaloids are a structurally and functionally diverse group of natural products (Harborne, 1993) and synthetic compounds. They can be broadly defined as organic molecules which contain basic nitrogen usually 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 most alkaloids contain at least one ring structure which is usually heterocyclic. 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 are a logical starting point in understanding ladybird toxicity.

Alkaloids are produced by a diverse array of organisms including: bacteria, fungi, plants and animals. Plants produce a huge number of alkaloid compounds, many of which are utilised by man as medical drugs (e.g. atropine from *Atropa belladonna*, morphine from *Papaver*), 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*) 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 (Wink *et al*, 1998), or they may serve to deter predators by tasting and smelling unpleasant (Glendinning & Gonzalez, 1995).

The first alkaloids to be isolated from ladybirds were coccinelline and its free base precoccinelline (Figure 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 (Figure 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 radiolabelled 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 from the mealworm ladybird

(*Cryptolaemus montrouzieri*) which is an intermediate between the two structural groups (Brown & Moore, 1982).

A recent incorporation study has shown that stearic acid is the biosynthetic precursor to both coccinelline and harmonine and that myristic acid provides the carbon skeleton for adaline (Haulotte *et al*, 2012). A further labelling experiment has shown that dietarily acquired 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 rather than ladybird biosynthesis but no other alkaloids found in ladybirds appear to be the result of sequestration from plants (Hartmann & Toppel, 1987; Hartmann *et al*, 1989). Ladybirds that feed upon heterospecific beetles, eggs and larvae accumulate alkaloids to levels detectable by gas chromatography-mass spectrometry, but it is not known whether these are truly sequestered or simply metabolised (Sloggett *et al*, 2009a).

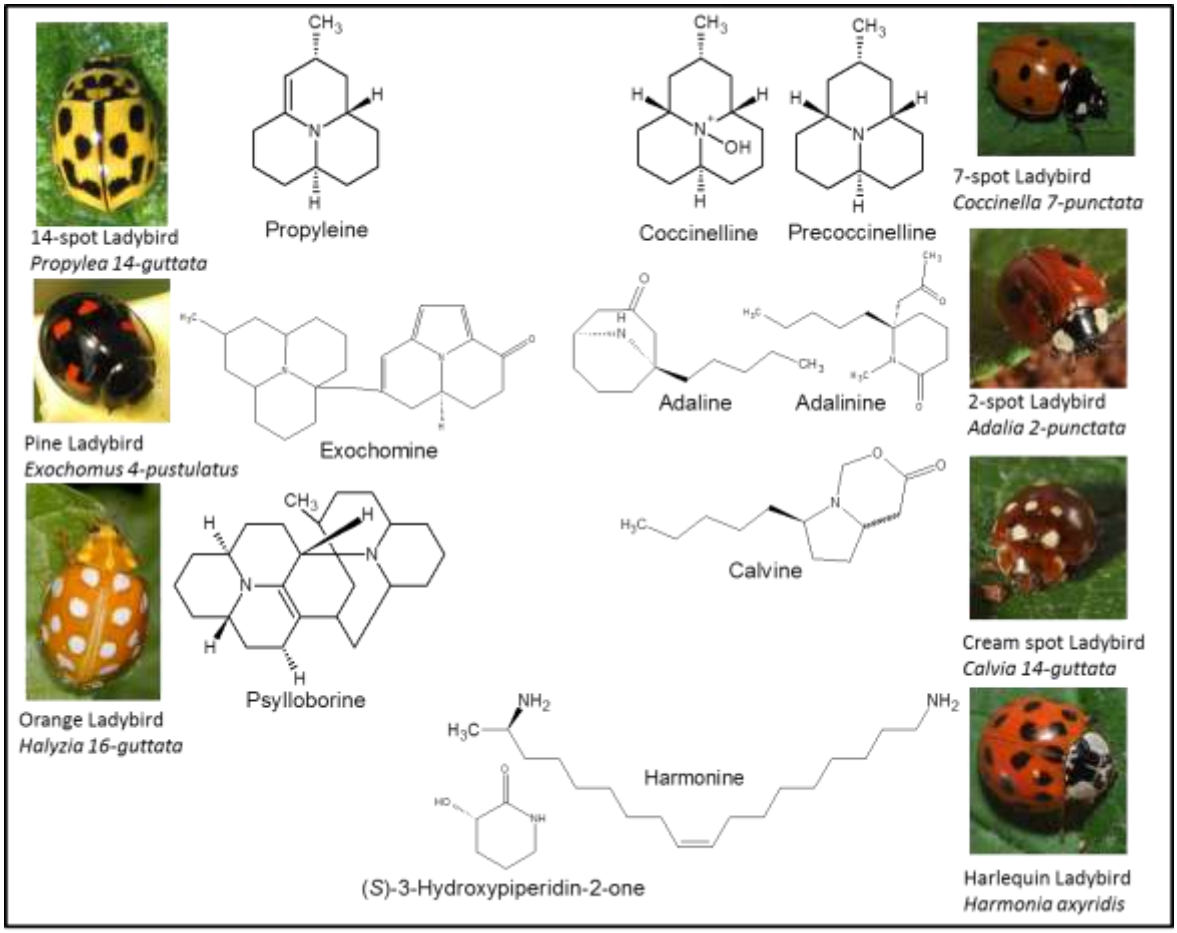
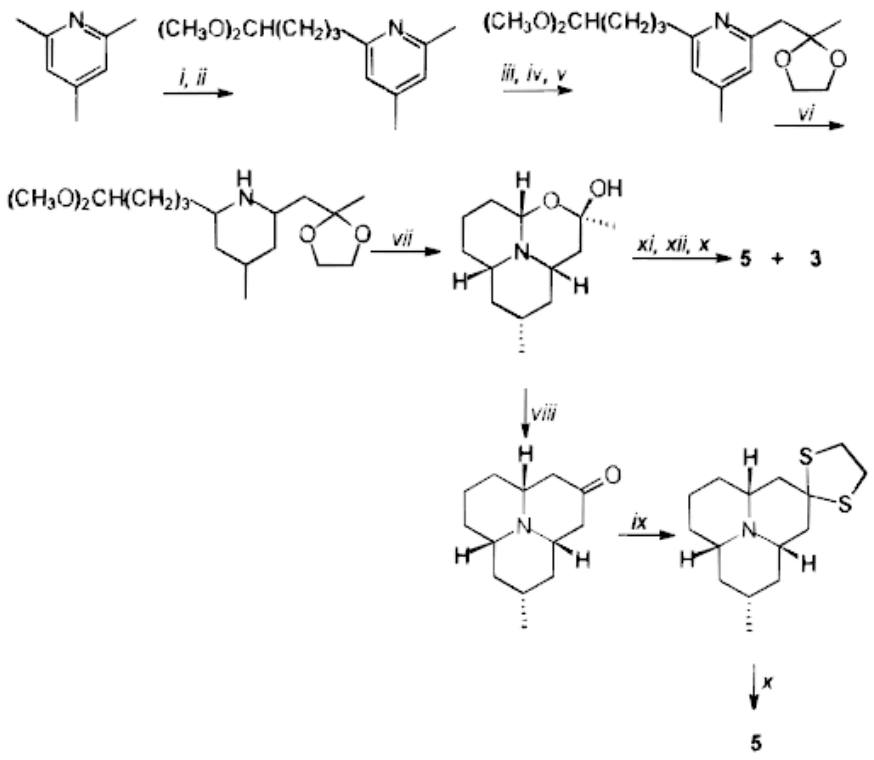


Figure 1.1.7. Commonly encountered UK ladybirds and the alkaloids they are known to produce.

The only other species known to contain ladybird alkaloids are the cantharid beetle *Chauliognathus pulchellus* from Australia (Moore & Brown, 1978) and the poison dart frog *Dendrobates auratus* (Daly *et al*, 1993). Moore and Brown (1978) isolated precoccinelline, hippodamine and propyleine from *C. pulchellus*. It is not known whether this species produces these alkaloids *de novo* or sequesters them from ladybirds. Precoccinelline is found on the skin of wild collected *D. auratus* but not their captive counterparts, so is assumed to be sequestered. Synthetic routes to a number of ladybird alkaloids have been discovered (e.g. Ayer & Browne, 1977; Glisan King & Meinwald, 1996; Yamazaki *et al*, 1999). 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 & Browne (1977) and described in detail by Glisan King & Meinwald (1996).

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).



1.1.8 The synthetic route to azaphenalene ladybird alkaloids asdeveloped by Ayer & Browne (1977). Figure from Glisan King & Meinwald (1996). 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.

1.5 Bioactivity of Ladybird Alkaloids

The first ladybird alkaloid to be isolated was coccinelline (Figure 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 activity (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 (*E. 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 lowered for older, more alkaloid rich beetles (Eisner *et al*, 1986). Several studies have found a positive correlation between alkaloid content and elytral pigmentation, suggestive of aposematic signal honesty (Bezzerides *et al*, 2007; Blount *et al*, 2012; Winters *et al*, 2014; Wheeler *et al*, 2015). The azamacralide alkaloid epilachnene was shown to act as a deterrent 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).

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 3 ng/L and 11 ng/L for Riesling when using 3-isopropyl-2-methoxypyrazine (Kögel *et al*, 2011).

Other than these crude feeding, 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 treated with caution, but the implication that ladybirds possess pain killing properties is possibly suggestive of pharmacological action, and conceivably ion channel blocking activity.

So far only one target and mechanism of action has been proposed for the azaphenalene alkaloids, which are produced by a range of ladybird species (Leong *et al*, 2015). A radioligand binding assay of *Torpedo californica* electric organ membranes with the nicotinic acetylcholine receptor (nAChR) ligands [piperidyl-3,4-^{3}H(N)]-N-(1-(2-thienyl)cyclohexyl)-3,4-piperidine ([^{3}H]-TCP) and [^{3}H]-cytisine was conducted with the five naturally occurring alkaloids; coccinelline, precoccinelline, convergine, hippodamine and the dimeric azaphenalene, exochomine (Figure

1.1.7). The N-Methyl derivatives of precoccinelline (N-Methyl-precoccinelline) and hippodamine (N-Methyl-hippodamine) were also included. All alkaloids displaced [^{3}H]-TCP with similar IC_{50}s ranging from 1.3 to 9.03 µM with the exception of exochomine which had a considerably higher IC_{50} value of 71.7 µM. When the procedure was repeated with [^{3}H]-cytisine but with a fixed coccinellid alkaloid concentration of 30 µM, radioligand binding was unaffected. Cytisine is known to occupy the ACh binding site of nAChRs (Papke & Heinemann, 1994) whereas TCP binds to the chlorpromazine binding site (Leong *et al*, 2015). The fact that alkaloids were able to displace [3H]-TCP but not [3H]-cytisine suggests that alkaloids are binding to an allosteric site and therefore display non-competitive action. A fluorescence assay of membrane depolarisation was then carried out whereby TE671 cells which express human foetal muscle-type nAChRs (M-nAChRs) were exposed to precoccinelline and coccinelline alone and also in combination with ACh concentrations. Neither alkaloid caused membrane depolarisation alone but both caused concentration-dependent inhibition of ACh induced depolarisation with IC_{50}s of 14 and 50 µM for precoccinelline and coccinelline respectively. This inhibition was not affected by ACh concentration, again suggestive of non-competitive inhibition. This result was corroborated by demonstrating the inhibition of ACh induced currents by precoccinelline, in voltage-clamped *Xenopus* oocytes expressing human α7 nAChRs.

The harlequin ladybird has been the focus of several recent studies which have sought to establish the basis of this species' invasive success (Roy & Brown, 2015). The bioactivity of harmonine, the predominant alkaloid found in *H. axyridis*

haemolymph may be an effective weapon driving the invasion. Radial diffusion assays revealed that the whole beetle acetonitrile extract of *H. axyridis* inhibits the growth of *E. coli* but this activity is not displayed by extracts of either 7-spot or 2-spot ladybird. The active component was found to be harmonine, which showed broad-spectrum antibacterial activity against twelve strains, including; *Pseudomonas*, *Bacillus*, *Staphylococcus* and *Mycobacterium* as well as the yeast, *Candida albicans* (Röhrich *et al*, 2011). It was also shown that exposure to harmonine at µM concentrations inhibits growth of *Plasmodium falciparum* asexual blood stages and kills gametocytes, reducing subsequent infection of *Anopheles stephensi* by half. Incubation of harmonine with six immortalised human cancer lines has revealed that this compound displays potent cytotoxicity, with ED_{50}s between 1.03 and 3.04 µg/mL (Alam *et al*, 2002) and anti-proliferative activity towards three human cell lines with IC_{50} values between 18 and 38 µM and two lepidopteran cell lines with IC_{50} values of 53 and 57 µM. Harmonine was cytotoxic to HeLa cells, causing 50 % monolayer destruction at 37 µM (Röhrich *et al*, 2011).

1.6 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 causes deamination of catecholamines, 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 morphine and psychedelic drugs such as lysergic 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. Neonicotinoids are synthetic alkaloids with structural similarities to nicotine and the frog alkaloid epibatidine, developed by Shell and Bayer in the 1980s. They now constitute the most widely used insecticides worldwide, representing 24 % of total insecticide sales in 2007 and 80 % of seed treatment sales in 2008 (Jeschke *et al*, 2010). There are currently seven neonicotinoid

structures on the market. The first to be released, imidacloprid remains the most commonly used (Nauen *et al*, 2008). They are employed predominantly against sucking pests as seed and soil treatments or topically on agricultural crops, as well as domestically in flea treatments for pets and household ant and termite control. They act as partial agonists of nAChRs and are classified in category 4A "Nicotinic acetylcholine receptor (nAChR) competitive modulators" of the Insecticide Resistance Action Committee (IRAC) classification (IRAC Mode of Action Classification Scheme, 2015). Neonicotinoids display strong selectivity for insect over vertebrate receptors (Tomizawa & Casida, 2003; Tomizawa & Casida, 2005) exploiting a novel mode of action and overcoming resistance evolved to other nAChR targeting insecticides. Neonicotinoids have several favourable physicochemical characteristics. They remain un-charged at physiological pH, rendering them unable to cross the vertebrate blood-brain barrier but able to effectively perfuse and access the insect CNS. Differences in lipophilic and hydrophobic properties make neonicotinoids highly versatile and suitable for a range of applications. They are also relatively photo-stable and non-volatile, increasing their effectiveness in the field.

1.7 The Need for Insecticides

The global human population is expected to reach 9.73 billion by 2050 and 11.2 billion by 2100 (United Nations, 2015), with the majority of growth being seen in the developing world (GAP Report, 2010), particularly Africa. Provisional estimates reveal that in 2014-2016, 10.9 % of people are considered undernourished globally (up to 41.3 % in Middle Africa) with a population of 7.3

billion (FAO, 2015) and 35 % of deaths of under five year olds are attributed to starvation (WHO, 2011a). 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). The growth of biofuels is likely to further reduce the amount of agricultural land available for food production (Popp *et al*, 2013). 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 physical 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 and potato leaf roll virus (Robert *et al*, 2000). Estimating the proportion of crops lost to insect pests is inherently difficult to accurately achieve, but figures between 15-18 % have been put forward as a global average (Oerke & Dehne, 2004; Oerke, 2005) and are likely to be higher in the developing world. Pesticides are substances used to prevent, destroy, repel or mitigate against pests (Ecobichon, 2003). Those specifically targeted towards 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 led to a doubling in food production between 1971 and 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, agricultural workers and consumers.

As well as being responsible for crop damage, insects are major vectors for a number of human and livestock 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, 2011b). Other invertebrate disease vectors are: tsetse flies, sand flies, triatomid bugs, fleas, lice and ticks (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 due to the evolution of resistance mechanisms and it is therefore essential to develop new compounds.

1.8 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 people regularly or acutely exposed to them (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 pest populations, 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. Snodgrass, 1996), and these often need to be treated with combinations of insecticides. 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 $13.3 M 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 for the control of 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 (www.foodsecurity.ac.uk). 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, the insecticidal activity of animal derived natural products is increasingly being investigated (e.g. Fitches *et al*, 2004; Tedford *et al*, 2004; King & Hardy, 2013). 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 highly potent nicotinic nAChR agonist (Badio & Daly, 1994; 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 & Casida, 2003).

1.9 Ion Channels

Electrical communication is a fundamental feature of complex species and also crucial for some single-celled organisms (Nielsen *et al*, 2010, Lo *et al*, 2013). 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 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 feature 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 such as sodium/potassium-ATPase. 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 huge 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. This resting potential is maintained by ensuring the distribution 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 -90 mV (Levitan & Kaczmarek, 1991) with anionic proteins and an excess of intracellular Cl^- and extracellular Na^+ generating this membrane voltage. The cytoplasmic concentration of K^+ is much higher than the extracellular, but the positive charge is offset by intracellular anions. The relationship between ion concentration on either side of the membrane and voltage is described by the Nernst equation (Nernst, 1888):

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

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 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 including: 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 species transported, mechanism of gating and sequence homology.

1.10 Ion Channels as Insecticide Targets

The ubiquity and fundamental physiological importance of ion channels makes them ideal targets for insecticides (Raymond-Delpech *et al*, 2005). For this reason, many major insecticides interact with ion channels to achieve toxicity. Targeting ion channels within major circuits of the nervous system allows insecticides to rapidly immobilise pest insects. The most commonly utilised targets are the voltage gated sodium channel (VGSC), the nicotinic acetylcholine receptor (nAChR), ligand gated chloride channels ($GABA_A$) and glutamate-gated chloride channels (GluCl). These are essential for action potential propagation and transmission, or for regulating neuronal excitability, in both the central and peripheral nervous systems. With the exception of GluCl these ion channels are common to vertebrates and invertebrates, therefore the insecticides must exploit subtle molecular differences to selectively 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 membrane 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 and delays inactivation (Tatebayashi & Narahashi, 1994). This results in rapid 'knock down' immobilisation with characteristic twitching (Chen *et al*, 1985).

Nicotinic acetylcholine receptors are targeted by a range of insecticides (Matsuda *et al*, 2001; Millar & Denholm, 2007). Organophosphates and carbamates non-selectively inhibit acetylcholine esterase, preventing the termination of ACh action in the synapse leading to neuronal hyperexcitability. Several insecticides

interact directly with nAChRs to modulate channel activity: spinosyns act as positive allosteric modulators, butenolides act as agonists (Nauen *et al*, 2015), neonicotinoid insecticides act as partial agonists (Nagata *et al*, 1997) and nereistoxin analogues (cartap) act as antagonists of nAChRs (Nagata *et al*, 1997; Lee *et al*, 2003).

γ-aminobutyric acid (GABA) acts as an inhibitory neurotransmitter within both vertebrate and invertebrate CNS, gating a ionotropic chloride channel ($GABA_A$) and a metabotropic receptor ($GABA_B$). Fipronil is a phenylpyrazole $GABA_A$ receptor antagonist which causes neuronal hyperexcitability (Cole *et al*, 1993). Lindane and endosulfan also employ this mode of action to achieve excitotoxicity (Ratra & Casida, 2001).

GluCl represent a family of inhibitory chloride channels activated by glutamate (Arena *et al*, 1992) and found in the nervous systems and muscle cells of protostome and pseudocoelomate invertebrates (Cleland, 1996). The avermectin insecticides and anthelminthics act by irreversibly activating GluCl (Duce & Scott, 1985; Scott & Duce, 1985; Arena *et al*, 1992; Wolstenholme & Rogers, 2005) and potentiating the action of glutamate (Cully *et al*, 1994), resulting in membrane hyperpolarisation and paralysis (Wolstenholme & Rogers, 2005).

1.11 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 by Dale and Loewi (Dale, 1914; Loewi, 1921), 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 acetylcholine esterase by Changeux *et al* (1970), using receptors purified from the electric organ of the electric fish *Electrophorus electricus* with a protein derived from snake venom, α-bungarotoxin (α-BgTX). Subsequent work has revealed that nAChRs are allosteric membrane glycoproteins of ~300 kDa (Changeux & Edelstein, 1998). The length of the protein is approximately 16 nm and the diameter roughly 8 nm (Changeux & Taly, 2008). The receptor is composed of five subunits displaying pentameric symmetry (Figure 1.1.9) arranged around a central, funnel shaped pore. Each subunit has a bundle of four membrane spanning α-helices (M1-M4) and the pore is lined by M2 and the N-terminal of M1 (Miyazawa *et al*, 2003; Unwin, 2005). The pore is formed of the radially arranged alpha-helical M2 regions contributed by each subunit. Discrete rings of homologous hydrophilic and hydrophobic amino acids face into the pore (Miyazawa *et al*, 2003) and serve as key determinants of ion selectivity and as binding sites for non-competitive antagonists, such as chlorpromazine, phencyclidine, QX-222 and philanthotoxin-343 (Giraudat *et al*, 1986; Charnet *et al*, 1990; Arias, 1998; Brier *et al*, 2003). When in the closed conformation, an equatorial leucine ring formed by the inner M2 helices, causes the pore to taper and prevents ion permeation (Galzi *et al*, 1992; Miyazawa *et al*, 2003; Tikhonov *et al*, 2004).

There are five distinct families of nAChR subunits, namely α, β, γ, ε and δ and 17 individual subunits within these families are known in vertebrates (Millar & Gotti, 2009) 16 of which are found in mammals (Millar, 2003). The subunit

arrangement in vertebrate muscle (M-nAChR) is 2α, β, γ/ε, δ whereas vertebrate neuronal receptors (N-nAChR) are composed of just α and β subunits. Variations in this configuration determine the functional and pharmacological properties of the receptor (Tomizawa & Casida, 2003; Dani & Bertrand, 2007; Millar & Gotti, 2009). Several distinctions can be made between muscle type and neuronal type nAChRs (N-nAChR) (Corringer *et al*, 2000), the former are composed of α1, β1, γ, ε and δ, whereas the latter can be formed of α2-10 and β2-4 subunits in various combinations or α7-9 subunit homomers (Tomizawa & Casida, 2003; Dani & Bertrand, 2007). Specific N-nAChR subtypes are located in particular regions (Reviewed by Millar & Gotti, 2009), for example α4β2 is predominantly expressed in the central nervous system (CNS) whereas α3β4 is found in the peripheral nervous system (PNS). Muscle-type nAChRs are found only at the neuromuscular junction and the electric organs of several electric fishes, including *Torpedo* and *Electrophorus,* whereas neuronal type nAChRs occur on both pre and post-synaptic membranes in the vertebrate brain and ganglia (Brejc *et al*, 2001; Tomizawa & Casida, 2003). Another distinction can be made between foetal and adult muscle nAChRs. In foetal muscle and electrocytes, as opposed to adult muscle, the ε subunit is replaced by γ (Reynolds & Karlin, 1978; Mishina *et al*, 1986). nAChRs are often classified by the sensitivity they display to α-BgTX, with both M-nAChRs and homomeric N-nAChRs highly sensitive, and heteromeric N-nAChRs comparatively insensitive (Tomizawa & Casida, 2001). Both muscle and neuronal nAChRs are placed in the superfamily of neurotransmitter-gated ion channels known as Cys-loop receptors along with serotonin ($5\text{-}HT_3$), glycine and γ-aminobutyric acid ($GABA_A$) receptors (Tomizawa & Casida, 2003; Dani & Bertrand, 2007). A

phylogenetically conserved feature of Cys-loop receptors is a pair of cysteine residues connected by a disulphide bond on their extracellular amino-terminals.

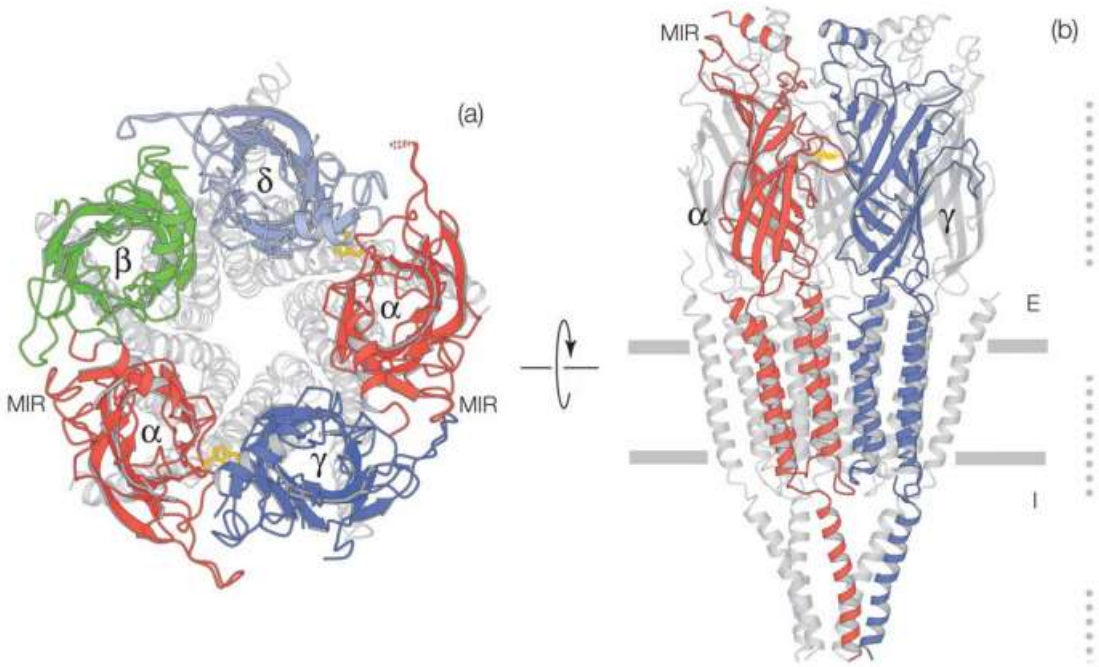


Figure 1.1.9. Ribbon diagram of a muscle type nAChR isolated from *Torpedo marmorata* postsynaptic membranes, based on images obtained by electron microscopy at 4Å resolution. Viewed from the synaptic cleft (left) and horizontally through the plasma membrane (right). The acetylcholine binding domain is highlighted in yellow at the interface of α and adjoining subunits. From Unwin (2005).

The channel is a dynamic structure and 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. Crystallography of the glial ACh binding protein (AChBP) of *Lymnaea*, a water-soluble homolog of the ACh binding domain, reveals that the quaternary ammonium group of the ACh molecule interacts with the Arg104, Val106 and Leu112 residues of the binding site (Brejc *et al*, 2001). The ACh binding

site of the nAChR is formed by the interface of the extracellular domains of the α-subunits and neighbouring subunit (Silman & Karlin, 1969; Karlin, 2002) and is composed of three loops contributed by the α subunit (loops A-C) and three from the complementary subunit (D-F) (Arias, 1997). 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. Electron microscopy 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). The α-subunits fall slightly out of symmetry with the non-α subunits when in the closed conformation. Activation of the channel by a ligand causes the inner 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 (Unwin, 2005).

The functional roles of nAChRs are broad and much of our understanding comes from disorders caused by nAChR dysfunction, or from knockout studies. M-nAChRs mediate muscle contraction (Langley, 1905) or electric organ discharge (Bennett *et al*, 1961). Several conditions arise from M-nAChR dysfunction, such as myasthenia gravis and congenital myasthenic syndrome. Myasthenia gravis is an autoimmune disease caused by the generation of autoantibodies to M-nAChRs and is treated with immunosuppressant drugs. Congenital myasthenic syndrome is caused by mutations in genes encoding nAChR subunits (Ohno *et al*, 1995), or the clustering protein RAPSN (Müller *et al*, 2003). Depending upon the mutation,

myasthenic syndrome can manifest in a fast-channel or slow-channel phenotype. Both conditions result in muscle weakness but can be effectively managed with acetylcholine esterase inhibitors or nAChR antagonists, respectively. The precise functions of N-nAChR subtypes are more challenging to elucidate because of the difficulties in determining stoichiometry of native receptors. Aberrations in N-nAChRs are involved in the pathophysiology of diseases including schizophrenia (Ripoll *et al*, 2004), depression (reviewed in Philip *et al*, 2010), epilepsy (Steinlein *et al*, 1995) and neurodegenerative conditions such as Parkinson's and Alzheimer's disease (Whitehouse *et al*, 1988). Studies on rats demonstrate that nicotine is able to improve working memory and this improvement is blocked by the nAChR antagonist mecamylamine (Levin *et al*, 1993). Selective agonists of both α7 and α4β2 similarly enhance memory in rats (Levin *et al*, 1999; Levin & Christopher, 2002) and primates (Buccafusco *et al*, 1995). nAChRs are therefore clearly involved in higher cognitive function and represent major drug targets for cognitive impairments. Nicotine has been shown to improve cognitive function in non-smokers as well as sufferers of ADHD, Alzheimer's and schizophrenia (Levin & Rezvani, 2002). The nAChR is also a major drug target for non-opioid and non-anti-inflammatory analgesics, anaesthetics, anti-depressants, anxiolytics (Tomizawa & Casida, 2003). Modulators of nAChRs are used clinically as muscle relaxants (antagonists of M-nAChR, such as pancuronium and agonists such as suxamethonium), treatments for nicotine addiction (varenicline, a partial agonist of α4β2), antitussives (dextromethorphan, an antagonist of N-nAChR) and treatments for dementia (galantamine, a positive allosteric modulator of nAChR). The α7

homomer is also known to play a role in inflammation regulation (Wang *et al*, 2003).

The size and complexity of the nAChR protein allows compounds to interact with the channel at several different sites. Allosteric modulators are known to bind to the central lumen of the pore (Giraudat *et al*, 1986; Charnet *et al*, 1990; Arias, 1998; Jozwiak *et al*, 2007; Tikhonov *et al*, 2004) and binding sites at the subunit interface of extracellular domains, as well as on both intra and inter-subunit sites of transmembrane domains have been proposed (reviewed in Chatzidaki & Millar, 2015). The most well-known nAChR antagonist, α-BgTX, binds irreversibly with the orthosteric site 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. Structural analogues are able to bind to alternative parts of the pore and display different potencies, voltage-dependencies and modes of action (Brier *et al*, 2003).

The nAChRs of vertebrates are much better studied than those of insects due in part to the difficulty in heterologously expressing functional insect receptors without creation of chimeric channels by co-expression of vertebrate β subunits (Tomizawa & Casida, 2001; Tomizawa & Casida, 2003). Much of our knowledge on non-vertebrate nAChRs is gleaned from α and β subunits cloned from *Drosophila*, *Myzus*, *Locusta*, *Schistocerca*, *Manduca* and the nematode worm *Caenorhabditis elegans*. Genome sequencing reveals that insect nAChR gene families are comparatively smaller than those of vertebrates or nematodes, consisting of 10-12 subunit genes (Jones & Sattelle, 2010). A transcriptome of *Locusta migratoria* neurons revealed genes encoding 9 α and 2 β subunits (Wang *et al*, 2015).

Drosophila melanogaster possess 10 genes encoding nAChR subunits, 7 α and 3 β (Sattelle *et al*, 2005) but the actual diversity is greatly expanded by alternative splicing and RNA editing (Lansdell & Millar, 2000; Grauso *et al*, 2002: Sattelle *et al*, 2005). No equivalent of the vertebrate M-nAChR has been found in insects, where fast excitatory neuromuscular transmission is mediated by glutamate receptors instead (Usherwood & Machili, 1966; Usherwood & Machili, 1968). The functional properties of insect β subunits remain enigmatic, even though genes encoding β subunits have been detected in diverse insect taxa, including: *Anopheles*, *Drosophila*, *Locusta, Schistocerca, Heliothis, Tribolium* and *Myzus*. Attempts to co-express these β subunits with insect α subunits have failed to form functional channels *in vitro*, in contrast to insect α/ vertebrate β chimeras (e.g. Dα3/chickβ2 – Shulz *et al*, 1998). Generally insect nAChR subunits show high amino acid sequence identity with equivalent subunits (greater than 60 % within insect, and around 40 % when compared with human) (Jones & Sattelle, 2010). Genome sequencing has also revealed the presence of insect nAChR subunits which show low sequence homology with known vertebrate or insect subunits. These consist of both α and non-α subunits and have notably truncated intracellular domains between the third and fourth transmembrane segment (Jones & Sattelle, 2010). The function and diversity of these subunits remains to be fully elucidated, but their low sequence homology may allow for the development of insecticidal compounds targeted towards particular species.

Despite the ubiquity of nAChRs, sufficient structural differentiation exists between vertebrate and insect receptors to make the insect nAChR a viable and widely exploited insecticide target, utilised by compounds such as the

neonicotinoids which bind with considerably higher affinity to insect nAChRs (Matsuda *et al*, 2005). The key structural feature responsible for this selective binding is an anionic nitro or cyano 'tip' that interacts with a cationic subsite unique to the ACh binding site of insect nAChRs (Tomizawa *et al*, 2000; Ohno *et al*, 2009; Talley *et al*, 2008). This attribute is reversed in nicotinoids such as desnitroimidacloprid, which show selectivity for mammalian nAChRs over insect (Tomizawa *et al*, 2000). Expression studies using *Xenopus* oocytes have shown that non-α subunits of the nAChR are responsible for the selective affinity of neonicotinoids to insect nAChRs (Lansdell & Millar, 2000).

Spinosad is a macrocyclic lactone insecticide which causes excitatory toxicity by modulating insect nAChRs (Salgado & Saar, 2004). The exact mode of action is not fully elaborated but the binding site is allosteric and is not shared with other insecticides (Salgado and Saar, 2004; Orr *et al*, 2009). Dα6 knockout *Drosophila melanogaster* show 1181 fold resistance to spinosad (Perry *et al*, 2007) and a point mutation has been identified in the α6 subunit of spinosad resistant population of the Western Flower Thrip (*Frankiniella occidentalis*). This results in the replacement of a glycine with a glutamic acid in the third transmembrane domain. When the analogous mutation is introduced to the human α7 nAChR, spinosad antagonism is abolished (Puinean *et al*, 2013).

Studies on the function of insect nAChRs are limited, but several experiments on the honey bee (*Apis mellifera*) reveal that injection of 0.5 nL nicotine into the antennal lobes increases sensitivity to sucrose (at concentrations >100 μM) and improves short-term olfactory memory (at concentrations >10 μM) (Thany & Gauthier, 2005). The injection of the nAChR antagonist, mecamylamine

into the brain of *A. mellifera* impedes antennal tactile memory for up to an hour whereas the injection of α-BgTX only affects longer term memory (>24 hr post learning), suggesting both α-BgTX sensitive and insensitive nAChRs are involved in memory formation and modulating gustation (Lozano *et al*, 1996; Dacher *et al*, 2005).

1.12 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 (Galvani & Aldini, 1792) 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 (e.g. Hodgkin & Huxley 1952) 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 of cells. 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 and the advent of electrical amplifiers which allow the simultaneous recording of membrane potential

and injection of current has made this possible. A number of techniques have been developed to measure the electrical activity of cells; two-electrode 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 concomitant DNA or RNA. This is then transcribed and the protein assembled. In the case of ion channels and receptors these are often translocated to the membrane, allowing functional characterisation.

The patch-clamp technique (Figure 1.1.10) was developed by Neher and Sakmann in the 1970s (Neher & Sakmann, 1976). Patch-clamp is a technique that requires a single electrode to measure membrane potential and simultaneously inject current. It is possible to record the electrical activity of the whole cell, or individual ion channels in the membrane. An electrode 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 (gigaohm) 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 seal of high resistance, but does not damage the membrane patch. 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 neurons isolated from small insects.

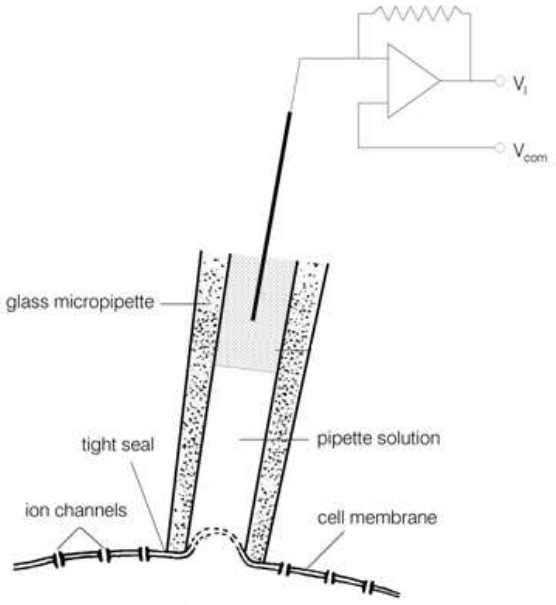


Figure 1.1.10 Simplified diagram showing the patch-clamp technique. From Mergler, 2010.

1.13 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 (Figure 1.1.11) is one such immortalised culture of human origin. This line was first derived by McAllister *et al* (1977), supposedly from a cerebellar medulloblastoma and consisting of undifferentiated preneuroglial cells.

It was found that TE671 cells have a doubling time of approximately 18 hours if kept at 37˚C in an atmosphere of 5 % CO_2 (Syapin *et al*, 1982). Five visibly differentiable cell types were recognised by Syapin *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 (Syapin *et al*, 1982). For this reason the ACh response of TE671 is thought to be mostly propagated by nicotinic receptors. This is further supported by the discovery that the TE671 nicotine response is identical to the ACh response (Grassi *et al*, 1993) and although M3 muscarinic receptors are present (Bencherif & Lukas, 1991), they are not detectable using electrophysiology (but are easily detectable with Ca^{2+} imaging). 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 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. However, Schoepfer *et al* (1987) demonstrated that the nAChR expressed by TE671 actually contained muscle type α-subunits. Further work revealed that the TE671 nAChR has subunit stoichiometry $(\alpha1)_2\beta1\gamma\delta$, the presence of γ subunits instead of ε showing that TE671 is an embryonic muscle cell line (Schoepfer *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. This explains the presence of muscle-type nAChRs and other phenotypic characteristics of TE671 (Stratton *et al*, 1989).

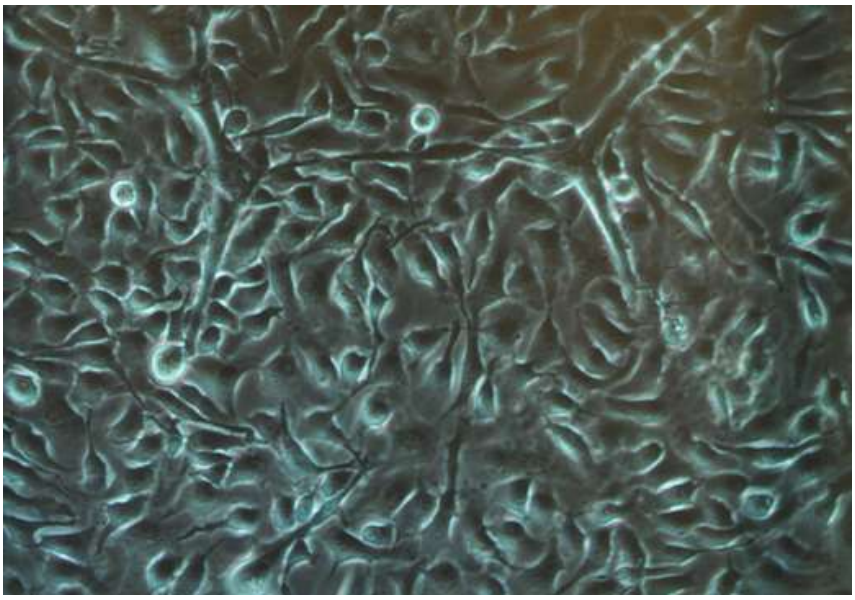


Figure 1.1.11 TE671 cells viewed at 200x magnification. A number of morphologically distinct cell types can be seen.

1.14 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 neuromuscular systems (Usherwood & Machili, 1966; Usherwood & Machili, 1968) but ACh is responsible for transmission in the CNS (Suter & Usherwood, 1985; Breer & Sattelle, 1987) and insect nervous

tissue is particularly rich in nAChRs (Matsuda *et al*, 2001). *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). A single α and non-α subunit have been identified in *S. gregaria*, namely αL1 (*Sg*α1) (Marshall *et al*, 1990) and *Sg*β1 (Jones *et al*, 2005). These subunits are orthologs of *Drosophila* Dα2 and Dβ1. Expression of Sgα1 in *Xenopus* oocytes generates a functional channel which is activated by nicotine and blocked by α-BgTX, d-tbc, mecamylamine, tetraethylammonium and bicuculline (Marshall *et al*, 1990). Attempts to co-express *Sg*α1 and *Sg*β1 result in channels with identical pharmacology to Sgα1 alone (Jones *et al*, 2005). Isolated *S. 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 10 μM (Suter & Usherwood, 1985). Treatment of neurons separately with atropine and d-tbc revealed that both nicotinic and muscarinic acetylcholine receptors are present (Suter & Usherwood, 1985). The conclusion 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, isolated from mushroom bodies (Figure 1.1.12) of 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 and 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 nAChRs (Hanke & Breer, 1986) and these provide a convenient model insect nAChRs for the purposes of electrophysiology.

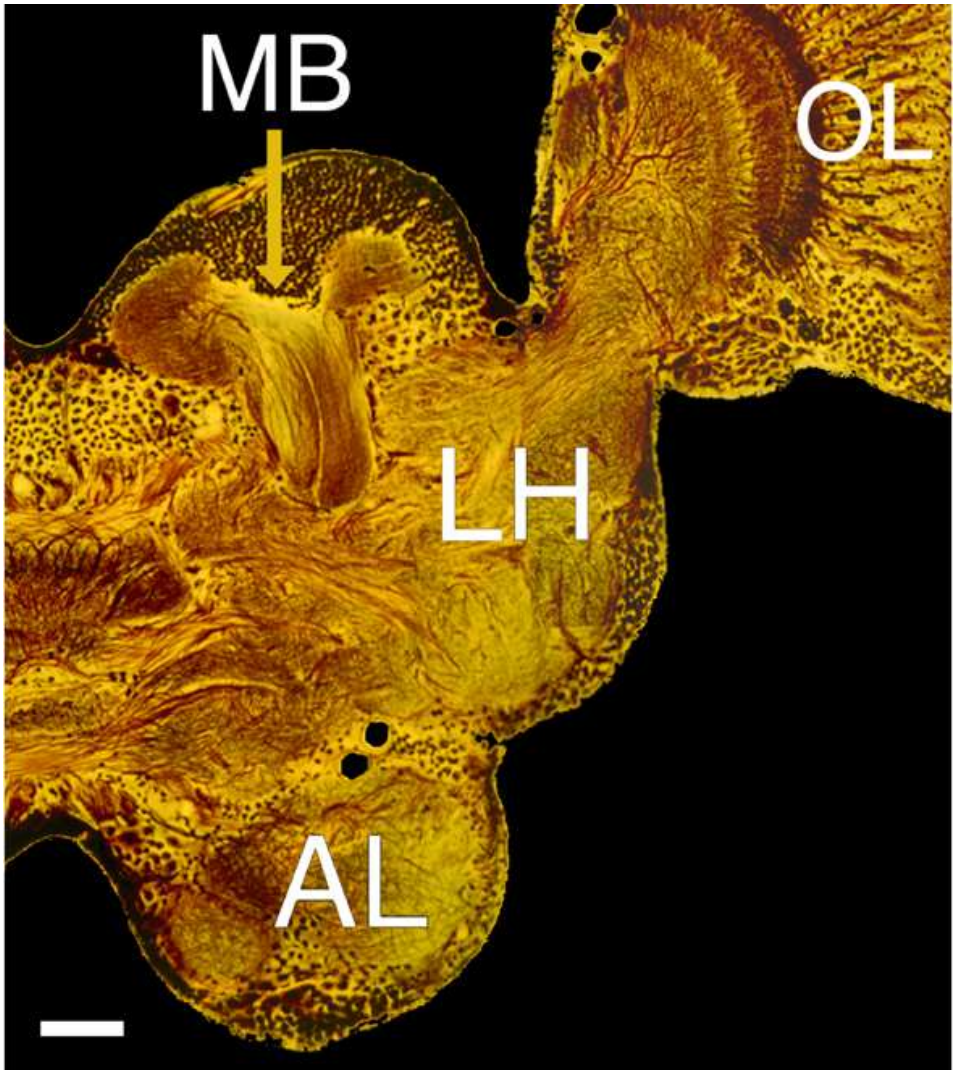


Figure 1.1.12. Transverse section of a Bodian stained locust (*Schistocerca americana*) brain. MB = mushroom bodies (containing Kenyon cells), OL = optic lobe, LH = lateral horn and AL = antennal lobe. Image from Perez-Orive *et al* (2002).

1.15 Aims

The aim of this project is to investigate the effects of ladybird alkaloid extracts and pure alkaloids on human muscle type nAChRs expressed by TE671 cells and locust (*Schistocerca gregaria*) neuronal nAChRs using patch-clamp electrophysiology. It has been repeatedly suggested that ladybirds produce alkaloids for the purpose of chemical defence. We wish to discover whether these alkaloids display channel modulating activity and may be the basis for ladybird toxicity and aposematism. The main predation pressure exerted upon ladybirds appears to come from other insects, notably other ladybirds. For this reason, we wish to compare potency and discover whether ladybird alkaloids display selectivity for insect over vertebrate nAChR, and could thus serve as lead structures for novel insecticides.

Seven species of commonly encountered British ladybird; the 2-spot (*Adalia bipunctata*), 10-spot (*Adalia decempunctata*), 7-spot (*Coccinella septempunctata*), 14-spot (*Propylea quatuordecimpunctata*), cream spot (*Calvia quatuordecimguttata*), pine (*Exochomus quadripustulatus*) and orange ladybird (*Halyzia sedecimguttata*) along with the invasive Asian harlequin ladybird (*Harmonia axyridis*) will be wild collected in the UK. Alkaloids will be extracted in methanol and purified by acid/base separation. Whole-cell patch-clamp will be used to compare the alkaloid extract of each of these species against human and locust nAChR. Concentration-inhibition plots will be constructed and IC_{50} values compared to examine the potency and selectivity of each extract.

The alkaloid extract of the most abundant species, the harlequin ladybird (Figure 1.1.13) will be subjected to detailed analysis of voltage-dependence and

mechanism of binding to establish mode of action. Alkaloid fractions will then be generated to determine which component/s is/are responsible for any activity observed.

Finally, adaline, an alkaloid produced by both 2-spot and 10-spot ladybirds and previously shown to display selective inhibition of locust nAChRs in preliminary experiments, will be subjected to both voltage-dependence and mechanism of action experiments. The effects on both human muscle-type and locust nAChRs will be compared in detail.

Fundamentally, establishing whether these compounds show activity at nAChRs will allow us insight into their functional significance, as well as providing a unique opportunity to potentially discover lead compounds for novel insecticides.



Figure 1.1.13 Invasive harlequin ladybird beetles (*Harmonia axyridis*). This species produces the alkaloid harmonine, the functional significance of which has been the subject of debate.

Chapter 2: Materials and Methods

2.1 Beetles

Adult beetles of eight ladybird species (*Adalia bipunctata*, *Adalia decempunctata*, *Calvia quatuordecimguttata*, *Coccinella septempunctata*, *Exochomus quadripustulatus*, *Halyzia sedecimguttata*, *Harmonia axyridis* and *Propylea quatuordecimpunctata*) were collected between September 2010 and September 2014 from the grounds of University Park (Nottingham) and Rothamsted Research (Harpenden). Both sexes were collected and these were stored in 25 mL polystyrene tubes at -20˚C until extraction. Live *Adalia bipunctata* beetles were purchased from Syngenta Bioline (product - Adalline B) and frozen at -20˚C upon delivery, until extraction.

2.2 Alkaloid Extraction & Purification

2.2.1 Solvent Extraction

Beetles were frozen in liquid nitrogen and crushed in a pestle and mortar (Figure 2.1.1). The resulting powder was washed with ~50 mL/100 beetles in reagent grade methanol (all reagents purchased from Sigma-Aldrich, UK unless otherwise stated) in a conical flask. This was placed on a magnetic stirrer and left for 24 hrs at room temperature. The methanol was decanted into a round-bottom flask and evaporated using a Rotavapor evaporator (BUCHI, Switzerland). This process was repeated with fresh methanol and the precipitate from both extractions combined.

2.2.2 Acid-Base Separations

To isolate alkaloids from other methanol soluble organic compounds, an acid-base separation was used (Al Abassi *et al*, 2001). The dried methanol extract

was reconstituted with 50 mL 1 M hydrochloric acid (HCl) and decanted into a glass separation funnel. The flask was then washed with HPLC grade diethyl ether (Fisher Scientific UK, Ltd) and added to the separation funnel. The contents were mixed and left to separate (Figure 2.1.1). The two layers were transferred into separate flasks and the top, organic layer was discarded, the lower aqueous layer was returned to the separating funnel. The flask was once again washed with ether and the contents returned to the funnel. This process was repeated three times. The resulting aqueous solution was adjusted to ~pH 10-12 using 2 M sodium hydroxide (NaOH - Fisher Scientific UK, Ltd) and returned to the separating funnel. The flask was washed with dichloromethane (DCM – Fisher Scientific UK, Ltd) and then added to the funnel. The contents were mixed and left to separate. The bottom organic layer was retained in a conical flask and the top, aqueous layer returned to the funnel and washed again with DCM. The process was repeated three times. The final wash was made using saturated NaCl (Fisher Scientific UK, Ltd) solution. Magnesium sulphate powder ($MgSO_4$ – BDH Chemicals) was added to the organic layer to remove any water from solution. The remaining liquid was then passed through a Whatman filter paper (Grade 4). The DCM was evaporated and the dry solid 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 alkaloid. The remaining extract was transferred to a clean glass vial, dried under a stream of nitrogen and weighed (Figure 2.1.1). The resulting alkaloid was aliquoted into glass ampoules that were sealed under nitrogen and stored at 4˚C.

2.2.3 Fractionation of Alkaloid Extracts

In order to learn more about the activity and selectivity of ladybird alkaloids, it was necessary to purify individual compounds from the mixtures extracted from beetles and revealed by thin-layer chromatography. Various separation techniques were tested in order to find the most effective means of achieving this.

2.2.4 High-Performance Liquid Chromatography (HPLC)

Dried extracts were dissolved in 1:1 acetonitrile:H_2O at concentrations ranging from 1-33 mg/mL (*H. axyridis*) and then subjected to reverse phased HPLC (Shimadzu SCL-10AVP, Shimadzu Instruments, Japan). The stationary phase (250x4.6mm, ACE HPLC Columns) was packed with C18 pellets and the injection volume was 10 µL. The organic solvents used were either acetonitrile (CH_3CN) or methanol (CH_3OH) and the aqueous solvent was HPLC grade water with 0.1 % formic acid. The concentration of organic solvent increased from 5 % to 100 % over fifty minutes, remained at 100 % for ten minutes before returning to 5 % for a further five minutes. The UV absorbance was measured as compounds eluted off the column at 215, 230, 254, 270, 280 and 360 nM.

2.2.5 Liquid Chromatography

A 30 cm glass column was packed with 2 cm neutral aluminium oxide, activity grade I (Woelm Pharmaceuticals). The column was washed with DCM before alkaloid extract dissolved in DCM was added to the top. The column was first washed with DCM and ten 1 mL fractions were collected in clean, glass vials and dried under nitrogen. The running solvent was then replaced with 4:1 DCM:methanol for the following ten fractions before being replaced again with

methanol with 1 % ammonium hydroxide. 1 µL of each fraction was blotted onto an aluminium backed silica thin-layer chromatography plate (Sigma-Aldrich, UK) and allowed to run in methanol with 1 % ammonium hydroxide. Plates were then stained with Dragendorff's reagent to reveal the alkaloid composition of fractions.

2.2.6 Thin Layer Chromatography

Having used thin-layer chromatography to visualise fractions generated by liquid chromatography, it was deemed an effective method to separate ladybird alkaloids. *H. axyridis* alkaloid extract was dissolved in methanol to a concentration of 5 mg/mL and 1 mL was loaded along a 20 cm^2 silica gel glass plate (Fluka) in a straight line, 2 cm from one edge, using a glass capillary tube. The plate was transferred to a 3000 mL Pyrex beaker with a small volume of methanol with 1 % ammonium hydroxide (below the loading level of the plate), covered and allowed to run for 4 cm. Two fractions were clearly visible without staining, and Dragendorff's reagent confirmed that these fractions were discrete, one mobile and one that remained at the loading level. The silica containing the two fractions was separately scraped off the plate using a flat measuring spatula. The silica was collected in a glass vial and extracted in methanol. The supernatant was removed to a clean glass vial and dried under nitrogen.



Figure 2.1.1 (A) Whole orange ladybirds (*Halyzia sedecimguttata*) were frozen with liquid nitrogen, (B) crushed in a pestle and mortar and extracted in methanol. The methanol extract (D) was dried and the alkaloids purified by acid-base separation (C). Dried alkaloid extracts from five species of ladybird are shown in image E.

2.3 Cell Culture

2.3.1 TE671 Human Rhabdomyosarcoma Cells

Cells were cultured in a 4.5 g/L glucose Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10 % foetal calf serum (FCS), 2 mM glutamine (Gln), 10 IU/mL penicillin and 20 µg/mL streptomycin (pen/strep). Cultures were passaged once per week and plated in 35 mm Petri dishes over heat-sterilised sections of

glass coverslip (5x20 mm) in 2 mL of DMEM. These were kept in a 36.5˚C incubator in a 5 % $C0_2$ atmosphere. After 20 passages, the culture was re-derived from stock stored in liquid nitrogen. For recordings, confluent coverslips were transferred to a perfusion chamber (Figure 2.1.7) with mammalian ringer (135 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 10 mM D-glucose adjusted to pH 7.4 with NaOH) flowing at 5 mL/min.

2.3.2 Primary Locust Neurons

Gregarious adult desert locusts (*Schistocerca gregaria*) were purchased from www.livefoods.co.uk and kept in locust breeding cages measuring 50x40x32 cm (Figure 2.1.2). These were maintained at an ambient temperature of 26-28˚C with a 40 W 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.*). Two 6^{th} instar locusts were used to harvest neurons to cover a 35 mm Petri dish 0-8 days after ecdysis, before the cuticle had hardened. 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 (135 mM NaCl, 25 mM KCl, 0.4 mM $NaHCO_3$, 0.5 mM glucose, 5 mM HEPES adjusted to pH 7.2 with NaOH) over ice until dissection. The head was pinned through the mandibles, facing upwards (Figure 2.1.3) with 25G hypodermic needles (Microlance, BD UK) into a 35 mm Petri dish with a 4 mm layer of Sylgard® (Dow Corning, USA). An incision was made from each eye to the frontal ridge using Vanna's micro scissors (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 (Figures 2.1.4 & 2.1.5). The optic nerves were cut and the brain removed using forceps. Air sacs were discarded and the brain placed in cooled Rinaldini's saline (Figure 2.1.5). When brains from both heads 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 a 1.5 mL Eppendorf tube with 200 µL of Rinaldini's saline containing 2 mg/mL collagenase and 0.5 mg/mL dispase (Boehringer Mannheim UK Ltd). This was then incubated for 15 minutes at 36.5˚C. Following enzyme treatment, the neurons were centrifuged at 800g for 1 minute at room temperature, the supernatant was removed and replaced with 200 µL of locust culture medium (5:4 DMEM[10% FCS, 2 mM Gln] : Schneider's insect medium, with 10 IU/mL penicillin and 20 µg/ml streptomycin). The mushroom bodies were then gently triturated through a 200 µL Gilson pipette tip ten times before being distributed over heat-sterilised, poly-lysine coated (see below) glass coverslips (5x20 mm) which had been placed into 35 mL Petri dishes containing 2 mL of culture medium. Dishes were incubated at 36.5˚C in a 5 % CO_2 atmosphere. Locust neuron preparations yielded up to ~200-300 neurons/cm^2 and remained viable for up to two days but quantity, quality and adherence of cells varied considerably. To make recordings, coverslips were transferred to a patch-clamp perfusion chamber and constantly washed with locust Ringer (180 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES made up to pH 7.2 with NaOH) at a rate of 5 mL/min.



Figure 2.1.2 Locust breeding cage in which locusts (*Schistocerca gregaria*) were kept. Heat was provided by a 40 W incandescent bulb and fresh food was added daily.



Figure 2.1.3 Locust head pinned through mandibles. Front of head capsule has been removed to expose brain. Scale bar represents 5 mm.

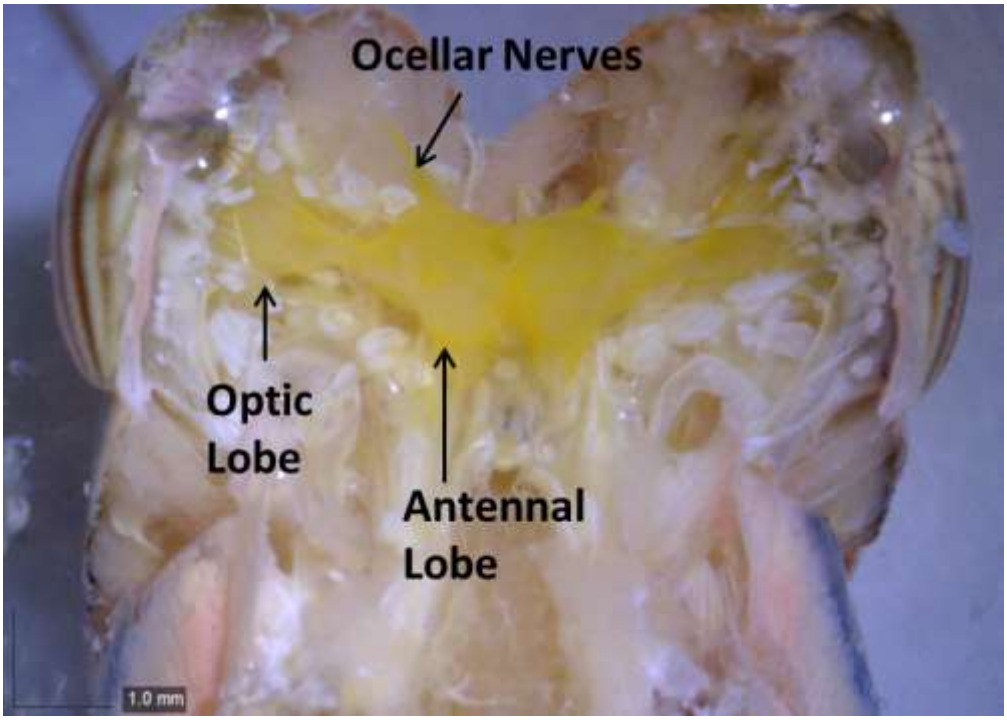


Figure 2.1.4 Locust brain in situ, optic nerves can be seen connecting to the eyes on either side of the head, antennal nerves have been severed but antennal lobes are visible below optic nerves.



Figure 2.1.5 A locust brain after removal from head capsule. The mushroom bodies are located at the top of the picture, above the optic nerves.

2.3.3 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 using a drug application device. Glass coverslips were cut into 5x20 mm strips using a diamond tipped glass cutter, heat sterilised at >100°C in a lab oven and arranged individually on the bottom of 35 mm Petri dishes. The first treatment was poly-l-lysine (PLL), a homopolymer of L-lysine produced commercially by fermenting *Streptomyces albulus*. The positively charged amino groups of poly-L-lysine promote cell adherence by interacting with negatively charged portions of cell membrane. 2 mL of 0.01 % poly-l-lysine solution was placed in each dish with a Pasteur pipette, covering the sections of glass. This was left at room temperature for two hours. The liquid was then removed and dishes left to dry in a sterile cabinet. The second compound tested was poly-D-lysine hydrobromide (PDL). PDL is an artificial analogue of PLL that is thought be to be more resistant to digestion by enzymes and therefore possibly more effective. Dry solid PDL was dissolved in deionised water to a concentration of 0.01 %, and coverslips were coated in the same way as with PLL. In order to test the efficacy of the two treatments, whole-cell currents in response to 100 µM ACh application were recorded (as described below). Both treatments proved equally effective at adhering cells without affecting the ACh induced currents. PLL coated coverslips were used for the majority of subsequent recordings.

2.4 Electrophysiology

2.4.1 Whole-cell Patch Clamp

Borosilicate glass patch-pipettes (World Precision Instruments, USA) were pulled to ~5-7 MΩ using a P-97 Flaming/Brown micropipette puller (Sutter Instrument Co., USA) and filled with a caesium pipette solution (140 mM CsCl, 1 mM $MgCl_2$, 11 mM EGTA and 5 mM HEPES, pH 7.2). Whole-cell currents were monitored using an Axopatch 200A (Axon instruments, USA) patch-clamp amplifier and recorded to the hard disk of a Dell computer using WinWCP V4.5.7 software (Dr John Dempster, University of Strathclyde). Compounds were applied to cells using a DAD-12 Superfusion system (Adams and List Associates, USA) fitted with a 100 µm polyamide coated quartz output tube (Figures 2.1.6 & 2.1.7) with a solution exchange time of 30-100 ms (Boehm & Betz, 1997; Karlsson *et al*, 2011). 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 (Figure 2.1.8). Both the amplifier and perfusion system were controlled by WinWCP. A wash-off of either mammalian or locust Ringer was constantly flowing from the tip of the perfusion manifold at ~3 mL/hr. The drug application device was pressurised with compressed nitrogen and ACh and test compounds were applied at 200 mm/Hg. All recordings were taken at room temperature (22˚C-25˚C). Alkaloids were stored in glass ampoules under nitrogen at 4°C and dissolved in Ringer solutions prior to experiments.

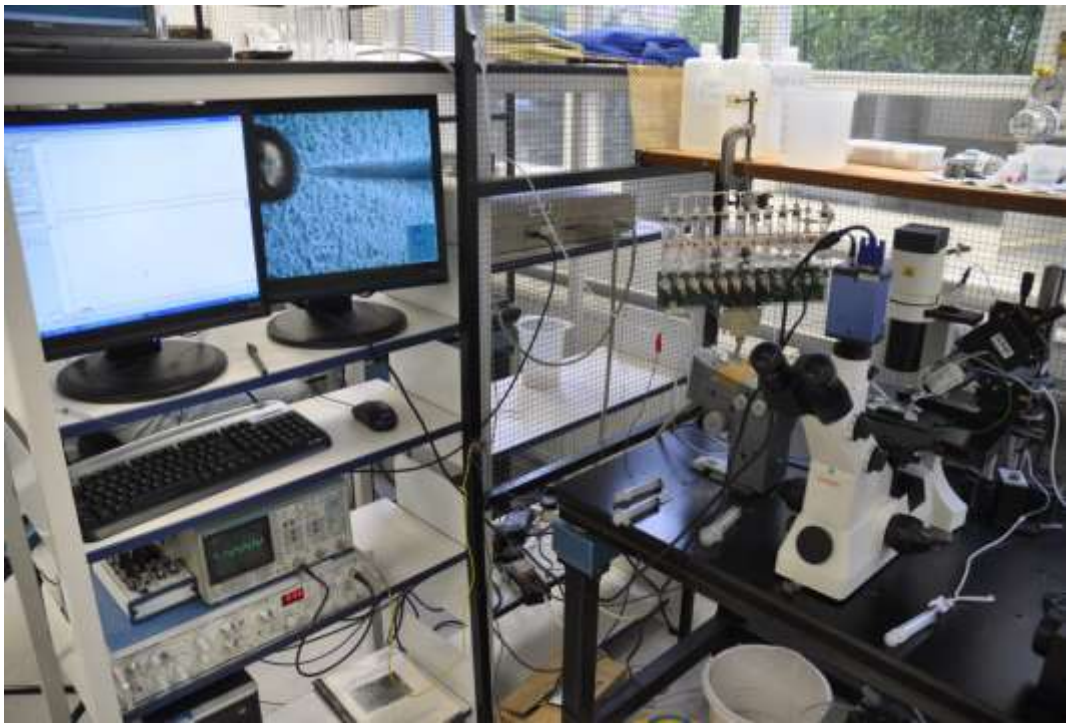


Figure 2.1.6 Whole-cell patch-clamp rig with single cell perfusion system.

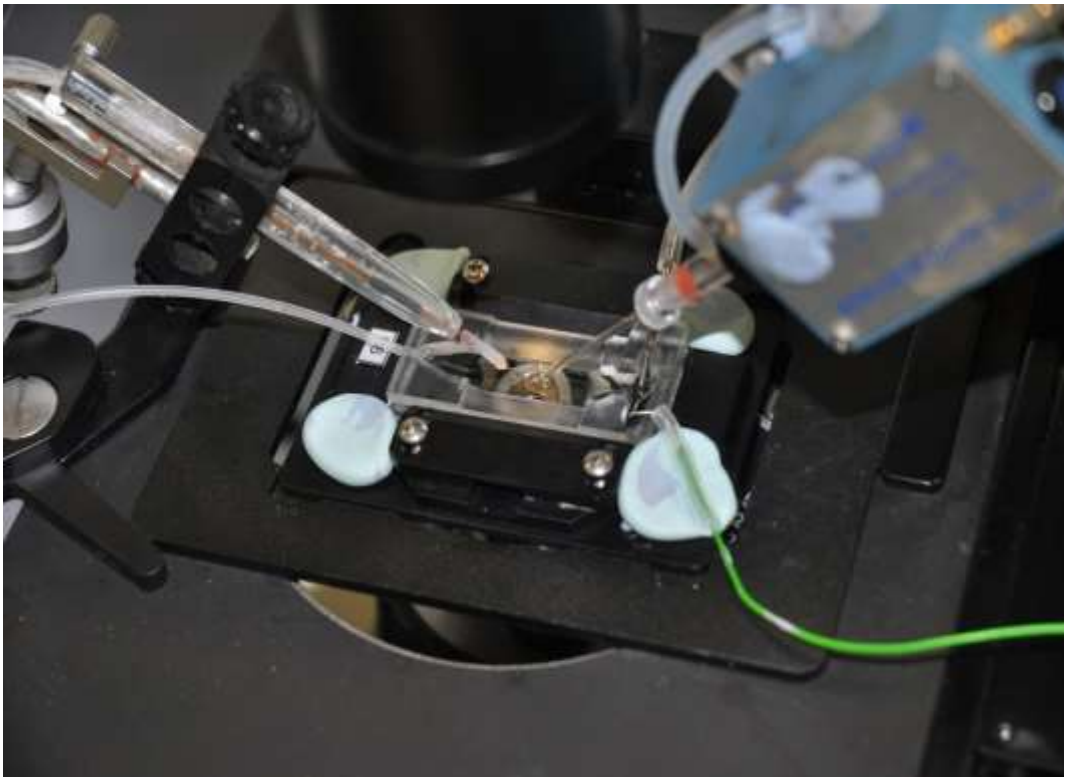


Figure 2.1.7 Perfusion chamber with micro manifold (left) and patch-clamp head stage with patch pipette electrode (right). Bath electrode can be seen leaving perfusion chamber to the right. Cover-slips with cells were placed in this set-up to make whole cell recording.



Figure 2.1.8 Locust neuron clamped (centre and right) with 100 µm manifold tip (left). Compounds were applied to cells at 200 mm/Hg. 200x magnification.

2.4.2 Current Measurement

The whole-cell nAChR currents generated in response to ACh exposure consisted of a rapid onset peak followed by desensitisation before ringer wash-off caused return to baseline current (Figure 2.1.10). Various parameters of this current trace can be measured to determine response size and toxin activity. Depending upon the mode of action, different parameters are more meaningful. We took three major components of the current response into consideration, these were; peak current, 1 s current ("late current" Brier *et al*, 2003) and net-charge movement (area under graph/AUG) (Figure 2.1.10). Peak current was the maximum current reading recorded, 1 s current was the current at 1s after onset of the ACh response, both measured in pA. Net-charge movement is the total flux of cations across the membrane during the ACh response, measured in pA.ms (fC).

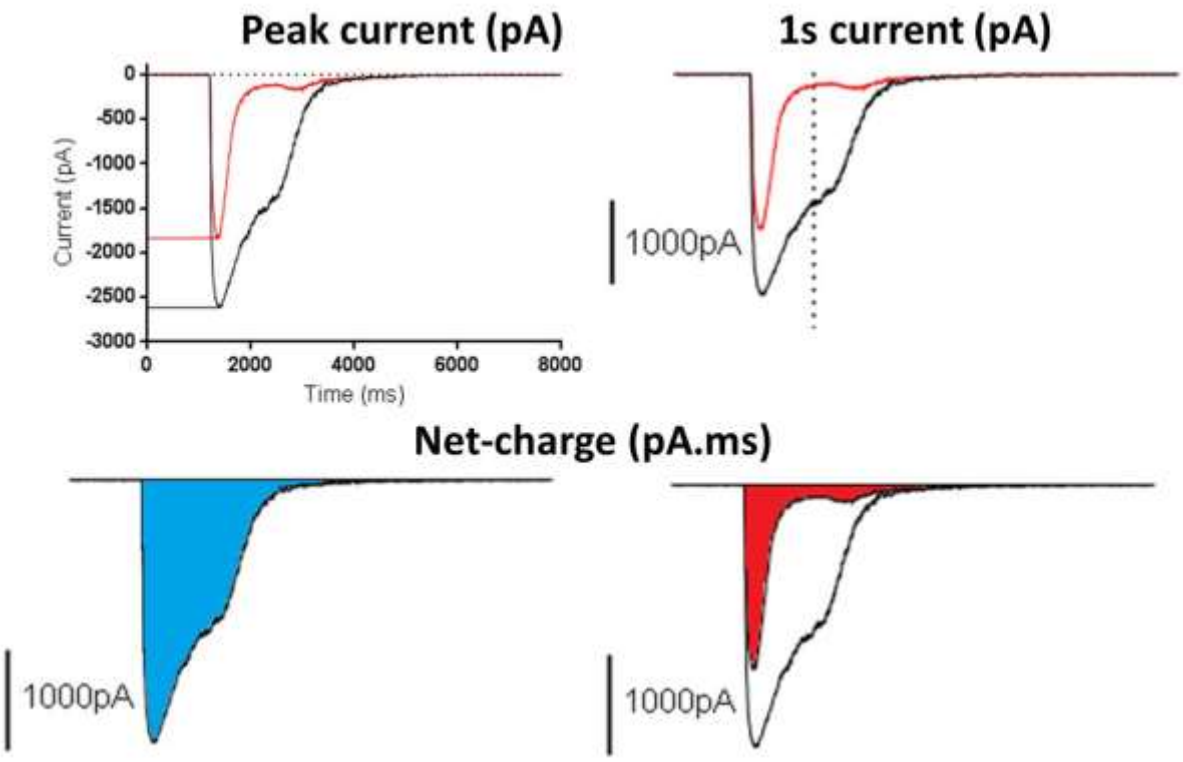


Figure 2.1.10 Three different aspects of the current trace were measured during analysis. These were the Peak (top left), 1s current, dotted line indicates 1s after current onset (top right) and the net-charge movement (bottom). A control response to ACh is shown in black (coloured blue for net-charge movement) and a trace showing the effect of co-applying a nAChR antagonist with ACh is shown in red.

2.5 Pharmacological Characterisation

It was essential to verify that the ACh responses displayed by both the human and insect model were nicotinic and free from muscarinic interference. This was achieved by characterising the responses to the nAChR selective agonist, nicotine, antagonist d-tubocurarine (d-tbc) and selective muscarinic antagonist, atropine. These were applied to ACh responsive cells individually (nicotine) or co-applied (d-tbc and atropine) with ACh. To verify our models, we also wished to examine the activity and selectivity of imidacloprid, a nAChR targeting neonicotinoid insecticide. Imidacloprid acts as a partial agonist of insect nAChR. It is known to be highly effective and selective, both in the field and laboratory. In order

to quantify activity and selectivity, increasing concentrations of imidacloprid were applied to both TE671 cells and locust neurons at V_H = -75 mV, and the inward currents were normalised against an ACh control response, applied prior to imidacloprid. Imidacloprid acts as a partial agonist, it was therefore co-applied with ACh to measure inhibition of ACh induced currents. The results were again normalised against an ACh control and concentration inhibition curves plotted.

2.5.1 EC_{50}

The whole-cell nAChR response of cells to ACh is concentration dependent, but exposure to high concentrations of ACh leads to receptor desensitisation and rundown whereas low concentrations fail to elicit receptor activation. The subunit composition of nAChRs determines sensitivity to ACh. For this reason it was important to examine the ACh sensitivity of both TE671 and locust nAChRs by calculating the EC_{50}, so that appropriate ACh concentrations could be used in experiments. This involved clamping cells at V_H = -75 mV, applying increasing concentrations of ACh and then measuring the response size. Currents were normalised against those for the concentration that generated the largest current response. These values were then plotted against log ACh concentration and fitted with a four-parameter logistic equation (4PL) (see below) with variable slope using Graphpad Prism 6. The EC_{50} value was then derived from this curve.

$$Y = 100\left(1 + 10^{(LogEC50 - x) \times Hillslope}\right)$$

2.6 Determining activity and selectivity of ladybird alkaloids

2.6.1 IC_{50}

IC_{50} or half-maximal inhibitory concentration is a measure of toxin or drug potency. In this study, it refers to the concentration of a compound that inhibits the maximal nAChR response by 50 %. To calculate IC_{50} values, peak and late current as well as net-charge movement were measured using WinWCP and exported to GraphPad Prism 6 (GraphPad Software, USA). All values were normalised against an ACh control applied either before or after alkaloid application (whichever was largest) to generate % control response values. The mean and standard error of the normalised current measurements were plotted against antagonist concentration to generate concentration-inhibition plots. A regression curve was fitted to these points using the four-parameter logistic (4PL) (equation shown below) and the IC_{50} value taken from this.

$$Y = 100\left(1 + 10^{(LogIC50 - x) \times Hillslope}\right)$$

To evaluate the differences in potency between TE671 cells and locust neurons, sum of squares F-test comparisons were conducted using Graphpad Prism 6.

2.6.2 Voltage-Dependence

In order to predict the mode of action and binding site it was necessary to examine how the holding potential of a cell affected the inhibitory activity of ladybird alkaloids. To test this, we compiled concentration inhibition plots at holding potentials ranging from +50 to -120 mV in TE671 cells and -50 to -120 mV in locust neurons. Locust neuron nAChR (like other neuronal-nAChR) display inward rectification and therefore could not conduct current at positive holding potentials.

The IC_{50} values were then plotted against holding potential and a line fitted using the Woodhull equation (Woodhull, 1973) to calculate δ value, corresponding to the fraction of membrane electric field entered by the molecule.

$$IC_{50}\ (V) = IC_{50}(0)\exp(\frac{z\delta VF}{RT})$$

Where V = command voltage, δ = fraction of the membrane field sensed by the blocker as it binds, R = the gas constant, T = the absolute temperature, F = Faraday's number and z = the valence of the blocker.

2.6.3 Competition Assay

The nAChRs of TE671 and locust neurons displayed different sensitivity to ACh and therefore it was necessary to use different concentrations of ACh in experiments. In order to compare activity of ladybird alkaloids towards the two models, we first needed to establish whether ladybird alkaloids displayed competitive or non-competitive activity. If the inhibitory activity was competitive, we would expect ACh concentration to affect the IC_{50}. We conducted a competition assay, whereby increasing concentrations of ACh are applied to a cell clamped at V_H = -75mV and an EC_{50} plotted, as described previously. The same concentrations of ACh were then co-applied to the cell with a single inhibitory concentration of alkaloid. The peak, plateau and net-charge values were then normalised against the corresponding values for ACh that generated the largest nAChR response, to construct a separate EC_{50} curve. The action of competitive inhibitors is overcome by increasing agonist concentrations, causing the EC_{50} curve to shift to the right but ultimately return to 100 % of control. Non-competitive inhibitors bind at a separate

site and therefore cannot be displaced by high agonist concentrations, causing the EC_{50} curve to plateau before response size reaches that of the control.

2.6.4 Activation Experiments

To determine whether ladybird alkaloids and alkaloid extracts caused any ion-channel activation in the absence of agonist, they were applied individually to both TE671 and locust neurons clamped at V_H = -75 mV. ACh was applied to cells for 1 s to establish that the cell exhibited a nAChR response, followed by a Ringer control to test for flow artefacts and then increasing concentrations of ladybird alkaloid and alkaloid extracts were applied. Recordings were reviewed in WinWCP and any currents generated were compared to a Ringer control. If the response size was greater than the control, it was considered to have caused receptor activation.

Chapter 3: Characterising the Nicotinic Acetylcholine Receptor Responses of Locust Neurons and TE671 Cells

Introduction

In order to use locust neurons and TE671 as models to compare the nAChR activity of ladybird alkaloids, it was first necessary to characterise and compare the nAChR responses of both. TE671 cells have been widely used as a model of muscle-type nAChRs and therefore, the response has been well characterised (Shao *et al*, 1998; Brier *et al*, 2003; Zalat *et al*, 2005; Barthelme *et al*, 2013; Procopiou *et al*, 2014). The locust neuron preparation used in this study is novel and therefore thorough characterisation was essential. Before conducting experiments, it was first necessary to establish the ACh sensitivity of models, so that appropriate ACh concentrations could be selected for experiments. The responses of both models to increasing concentrations of ACh were compared by constructing concentration-response curves to calculate EC_{50} values. A pharmacological characterisation was then conducted using the selective antagonists of nAChRs and mAChRs, d-tbc and atropine, and selective nAChR agonist, nicotine to ensure that the ACh responses observed were the result of nicotinic rather than muscarinic AChR activation. The excitatory and inhibitory activity of imidacloprid, a neonicotinoid insecticide that acts as a partial agonist of insect nAChRs was used to validate the models as appropriate for identifying selective nAChR toxins. Finally, the ease with which both models formed high-resistance seals, responded to ACh and withstood repeated perfusion to give stable ACh responses were compared.

3.1 Acetylcholine Induced Inward Currents

3.1.1 ACh EC_{50}

Different isoforms of nAChR display differential sensitivity to the natural neurotransmitter ACh, as a result of subunit composition. Prior to conducting inhibition experiments, it was necessary to establish the ACh sensitivity of both locust and TE671 nAChRs to ensure that the ACh concentration generated comparable whole-cell currents and did not cause excessive receptor rundown. This was accomplished by constructing EC_{50} plots, demonstrating the effect of increasing ACh concentration on magnitude of current response. Cells were subjected to whole-cell patch-clamp at V_H = -75 mV and increasing concentrations of ACh were applied for one second at 60 s intervals. The response sizes were measured and normalised against the concentration which generated the largest inward current (Figure 3.1.1). TE671 cells showed greater sensitivity to ACh than locust neurons, with EC_{50} values of 1.78 (95% CL = 1.48–2.15) to 11.6 µM (95% CL = 9.13–14.7) (peak and AUG) whereas locust neuron EC_{50} ranged from 20.26 (95% CL = 10.65–28.24) to 17.34 µM (95% CL = 16.2 – 25.35). Figure 3.1.2 shows representative traces of a locust neuron exposed to increasing concentrations of ACh. Based on these data it was decided to use ACh concentrations of 10 and 100 µM for TE671 and locust neurons respectively. This represented a concentration that generated a near-maximal but not rapidly desensitising inward current.

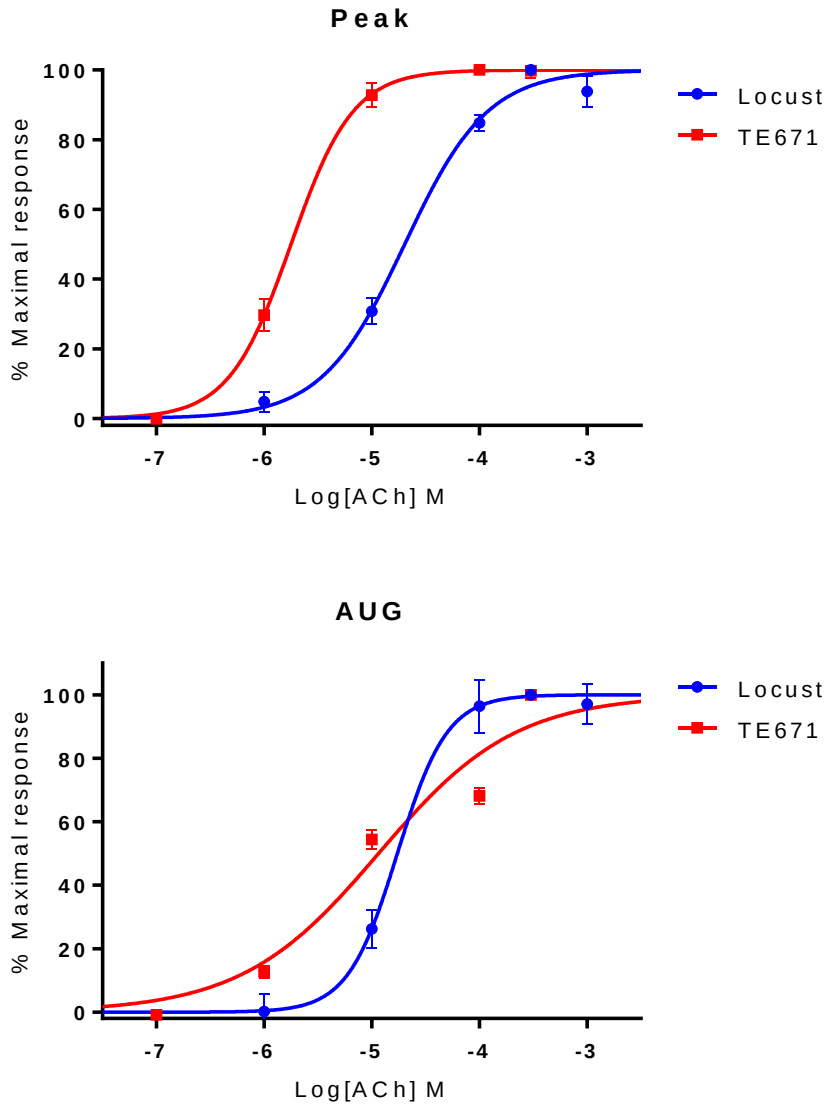


Figure 3.1.1. EC_{50} curves showing the data for peak and area under graph (net-charge) measurements for locust neurons (blue) and TE671 (red) at V_H= -75 mV. The calculated EC_{50} values were 20.26 µM and 1.8 µM for peak and 17.34 µM and 11.6 µM for AUG, locust and TE671 respectively. N = 11 for locust, N = 19 for TE671. Error bars indicate ±SEM.

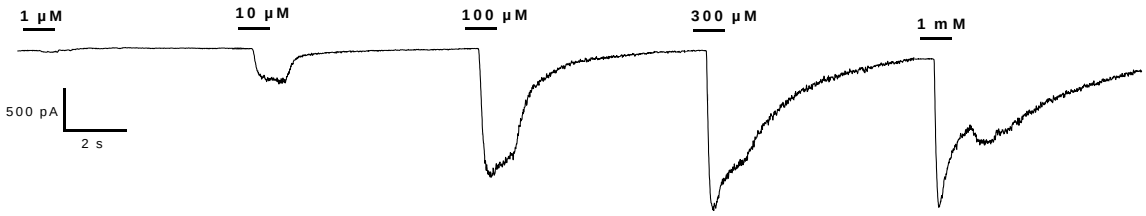


Figure 3.1.2. Representative traces showing standard response of a locust neuron to increasing concentrations of ACh. V_H = -75 mV. Horizontal bars indicate 1 s application period.

3.1.2 Repeated ACh Exposure

Many of the experiments required repeated applications of ACh and compounds to cells. It was therefore necessary to determine whether the ACh responses of both models were stable and to establish the minimum recovery time needed between applications. Six applications of ACh were made to TE671 cells at 10, 30 and 60 second intervals. The peak, AUG and 1 s currents were measured and normalised against the largest response and mean ±SEM plotted in the column chart shown in Figure 3.1.3. A linear-regression was fitted to the points (not shown) and deviation from zero calculated to determine whether the response was stable. The results for 10 and 30 seconds show that the response to ACh diminishes sequentially and the linear-regression differs significantly from zero (10 s $p = 0.0039$, 0.0064 and 0.0061 [peak, AUG and 1 s], 30 s $p = 0.0062$, 0.0182 and 0.0057). Applications at 60 s resulted in stable ACh responses and the linear regression did not differ significantly from zero in any of the current measurements. This was repeated with locust neurons (Figure 3.1.4) with a 60 s time interval and the responses were stable and the linear-regressions did not differ from zero. Figure 3.1.3 shows that at all three time intervals, the first response to ACh is larger than subsequent responses. In all later experiments, at least two applications of ACh were made to cells in order to establish a stable response, prior to application of test compounds, the second served as the control response.

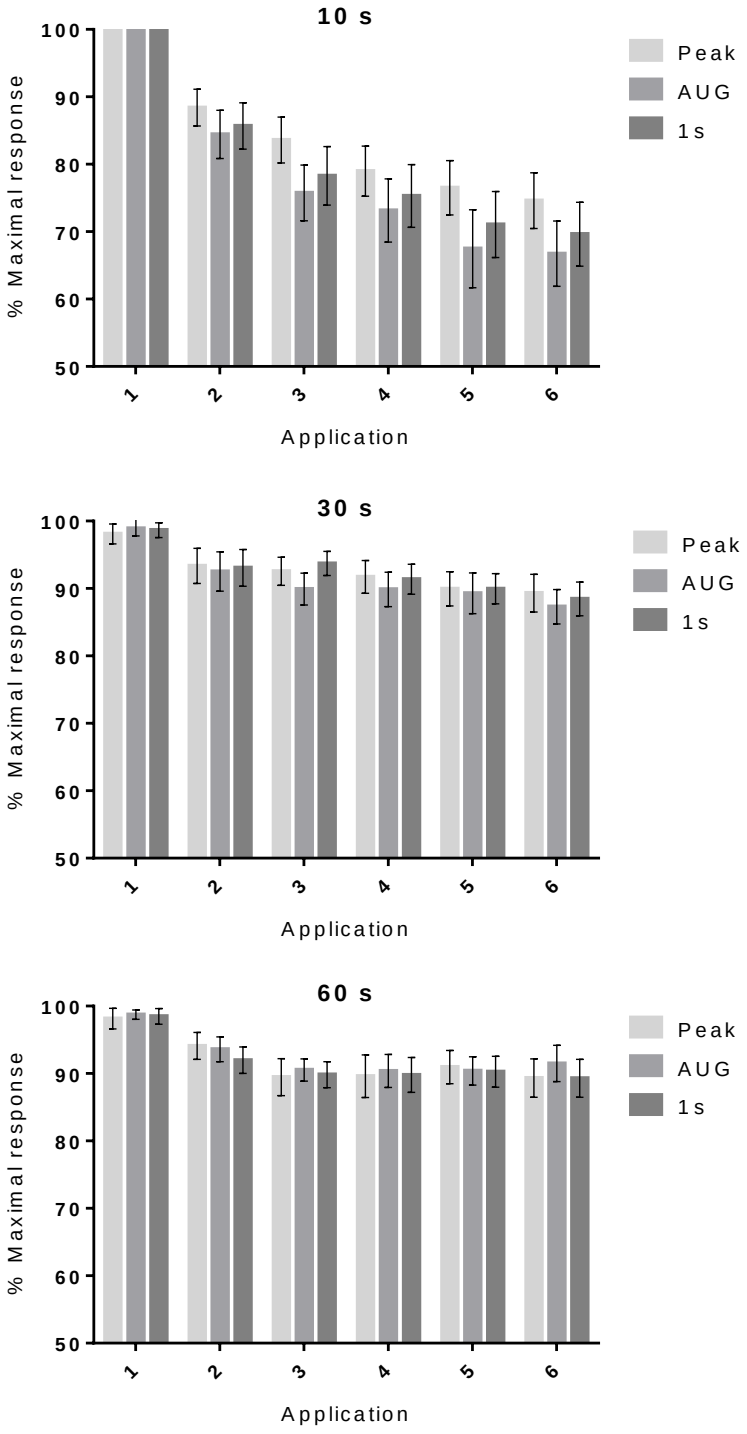


Figure 3.1.3. 10 µM ACh was repeatedly applied to TE671 cells at intervals of 10 s, 30 s and 60 s and the peak, AUG and 1 s current measured and normalised against the largest response. A linear regression was fitted to the points (not shown) and deviation from 0 determined. Applications at 10 and 30 second intervals resulted in decreasing current responses and linear regressions that differed significantly from zero (10 s p = 0.0039, 0.0064 and 0.0061 [peak, AUG and 1 s], 30 s p = 0.0062, 0.0182 and 0.0057). Applications at 60 s reduced slightly after the first application but then reached a plateau. The slope of the lines does not differ significantly from zero. N=11 and error bars indicate ±SEM.

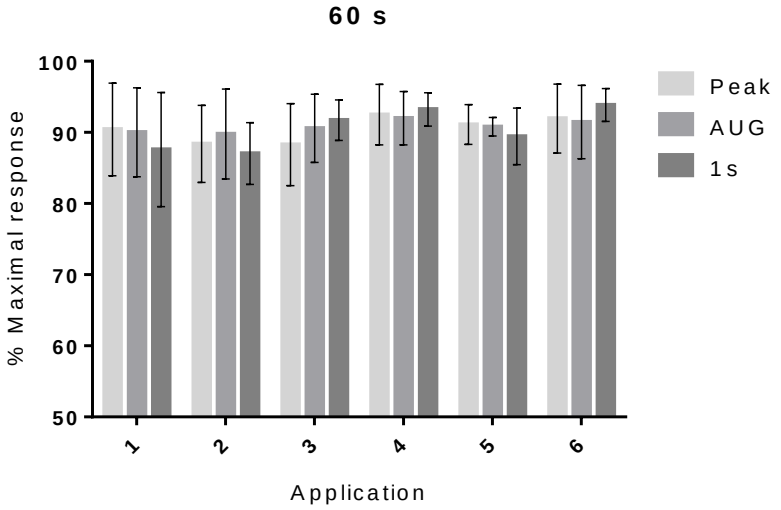


Figure 3.1.4. 100 µM ACh was applied repeatedly to locust neurons at 60 s intervals and the peak, AUG and 1 s current measured and normalised against the largest response. A linear regression was fitted to the points (not shown) and deviation from zero determined. The slopes of the lines do not differ significantly from zero. N = 4 and error bars indicate ±SEM.

3.2 Pharmacological Characterisation

Prior to using the models to compare the activity of ladybird alkaloids, it was essential to verify that the response to ACh was the result of nicotinic rather than muscarinic AChR activation. Responses to a selection of selective nicotinic and muscarinic ligands were recorded in cells that had been voltage-clamped at V_H = -75 mV. Both TE671 and locust neurons were responsive to both ACh and nicotine (Figure 3.2.1). ACh responses of both TE671 and locust neurons were blocked by d-tbc (Figures 3.2.2 & 3.2.3) with IC_{50}s shown in Table 3.2.1. This ACh response was not blocked by 10 µM atropine (Figure 3.2.1). This is consistent with the action of nAChR. High concentrations of ACh (>300 µM) generated slow onset (> 2 second after ACh application), oscillating responses in both models, consistent with muscarinic AChR activation, but these were not seen at the ACh concentrations used in later experiments.

	Peak		AUG		1 s	
	TE671	**Locust**	**TE671**	**Locust**	**TE671**	**Locust**
IC_{50} (µM)	2.13	15.8	1.23	5.05	1.02	2.73
95% CL	1.48-3.07	11.9-21.5	0.84-1.8	3.15-8.11	0.78-1.34	1.75-4.27
P	<0.0001		<0.0001		0.0002	

Table 3.2.1. IC_{50} values, 95 % confidence limits and sum of squares F-test P values for the inhibition of nAChR responses in TE671 cells and locust neurons by d-tbc.

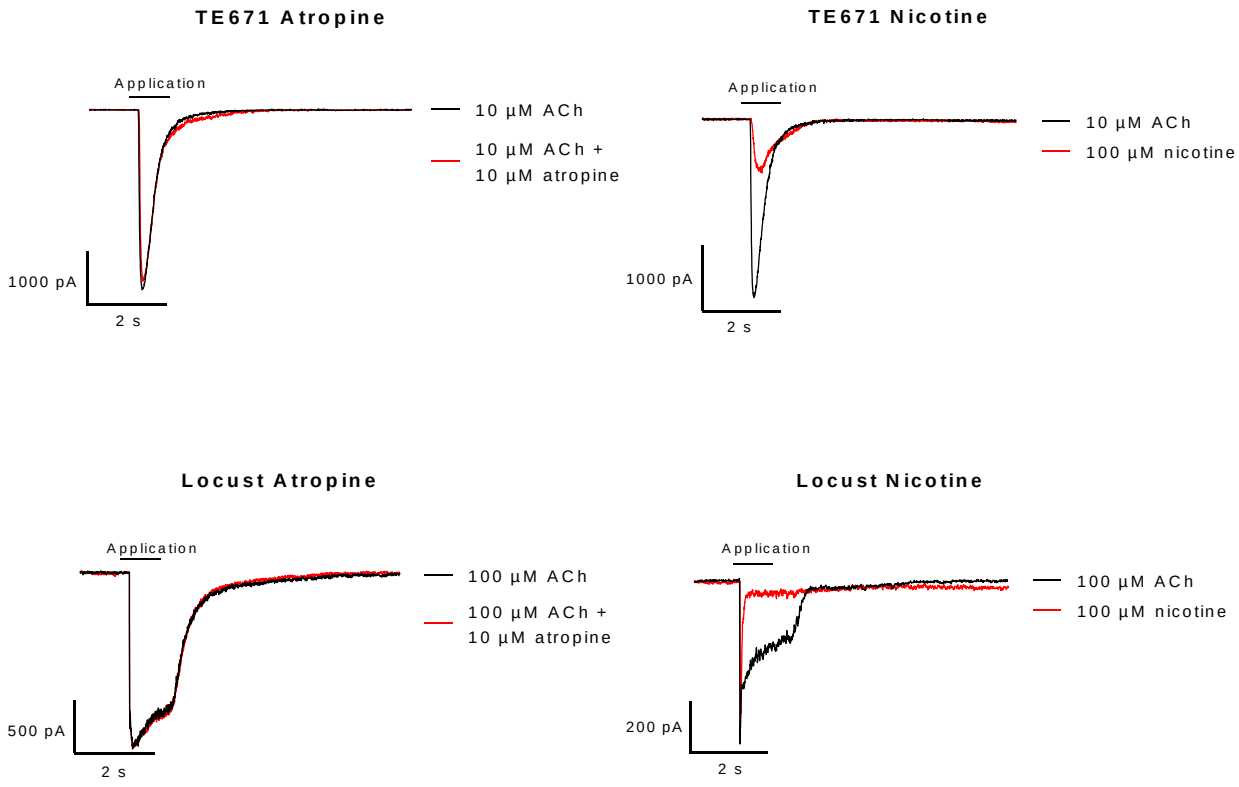


Figure 3.2.1 Representative traces showing the responses of TE671 cells (above) and locust neurons (below) to 10 µM atropine (left) and 100 µM nicotine. A control response to ACh is shown in black and the response to nicotine or co-application of atropine with ACh is shown in red. Applications were made for one second and V_H = -75 mV.

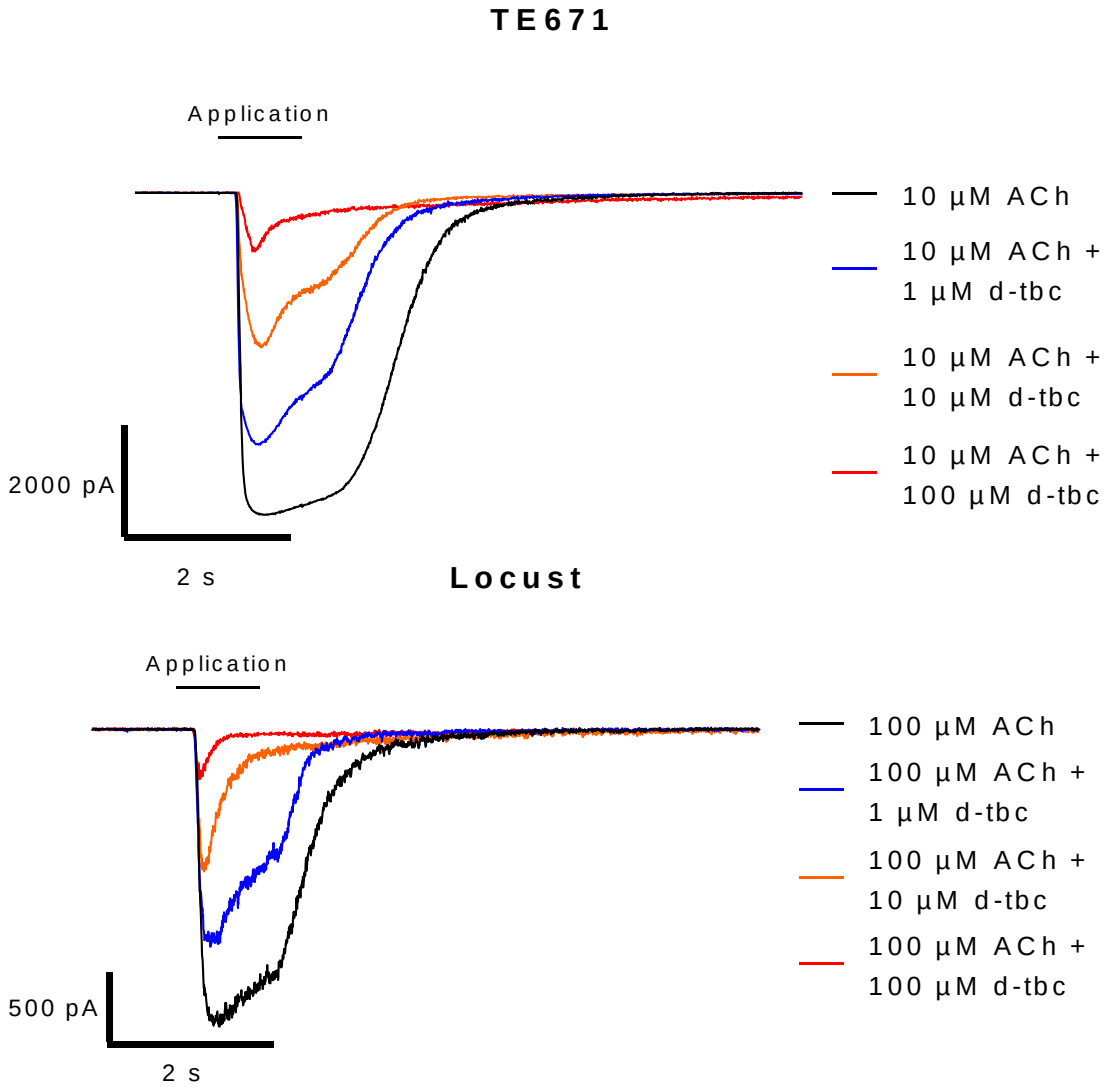


Figure 3.2.2 Representative traces showing the effect of Increasing concentrations of d-tbc when co-applied with ACh to TE671 cells (above) and locust neurons (below) at V_H = -75 mV.

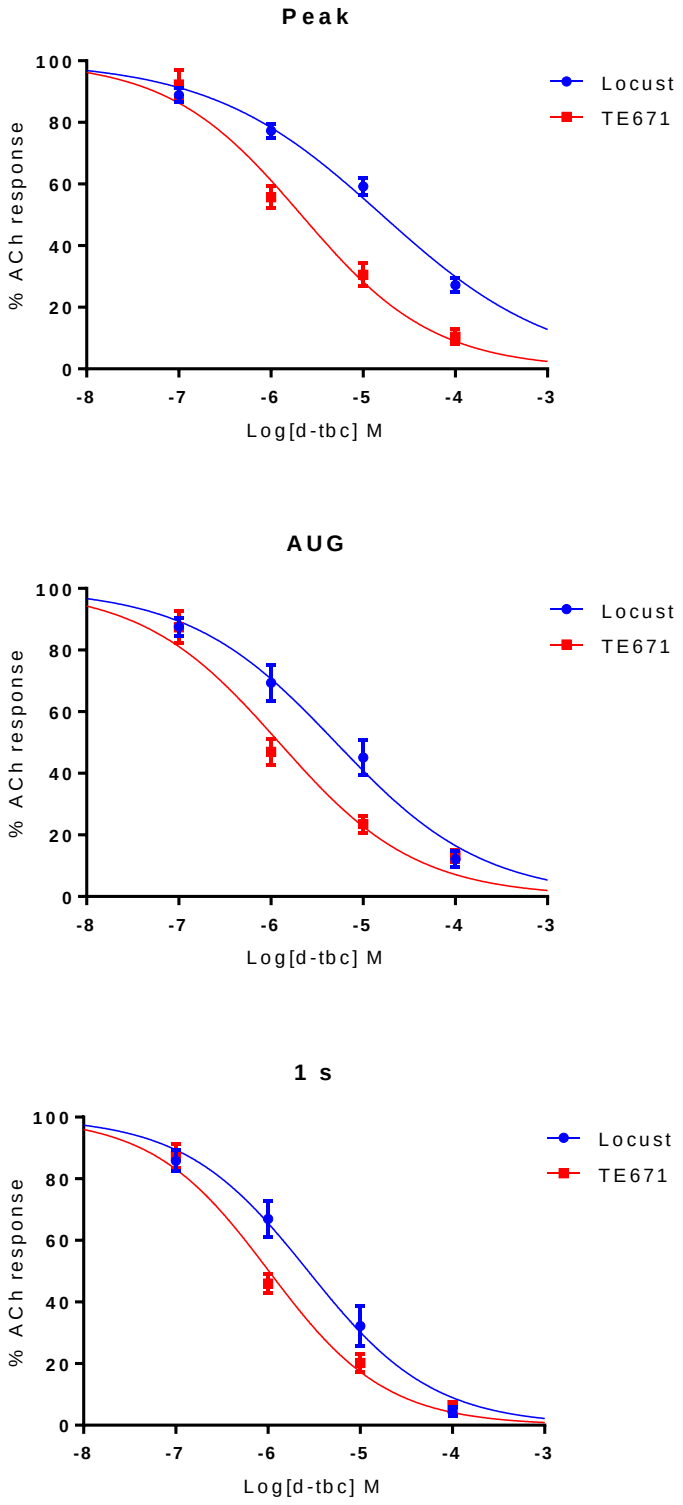


Figure 3.2.3. IC_{50} curves for d-tbc against locust neuron (blue) and TE671 (red) nAChR. Increasing concentrations of d-tbc were co-applied with 100 µM (locust) or 10 µM (TE671) ACh and peak, net-charge and 1 s current readings were normalised against control at V_H = -75 mV. The IC_{50} values for peak, net-charge and 1 s current were; 2.13 µM, 1.23 µM and 1.02 µM respectively for TE671 cells and 15.97 µM, 5.1 µM and 2.73 µM respectively for locust neurons. N = 11 (locust) 18 (TE671) error bars show ±SEM.

3.3 Imidacloprid Sensitivity

3.3.1 Activation of nAChRs with Imidacloprid

Imidacloprid is a neonicotinoid insecticide that acts as an insect selective partial agonist of nAChRs. Imidacloprid mediated activation of human muscle-type and locust neuronal nAChRs was compared. Increasing concentrations were applied to cells and the peak response and net-charge movement were normalised against an ACh control (Figure 3.3.1 & 3.3.2). The inhibitory activity of imidacloprid was then examined by co-applying increasing concentrations with ACh. Responses were measured and normalised against a control response (Figure 3.3.3). A final application of ACh after all imidacloprid applications revealed that the peak ACh response of TE671 cells returned to 81.7 % of the initial control response (N = 45), whereas that of locust neurons returned to 22.3 % (N = 14). The AUG returned to 77.9 % for TE671 (N = 13) and 22.6 % for locust (N = 14). It was not possible to estimate an EC_{50} for activation of human muscle-type nAChR because the activity was so minimal. The EC_{50} values calculated for locust neurons were 2.25 µM (95% CL = 1.09–4.65 µM) for peak and 0.6 µM (95% CL = 0.13–2.77 µM) for AUG.

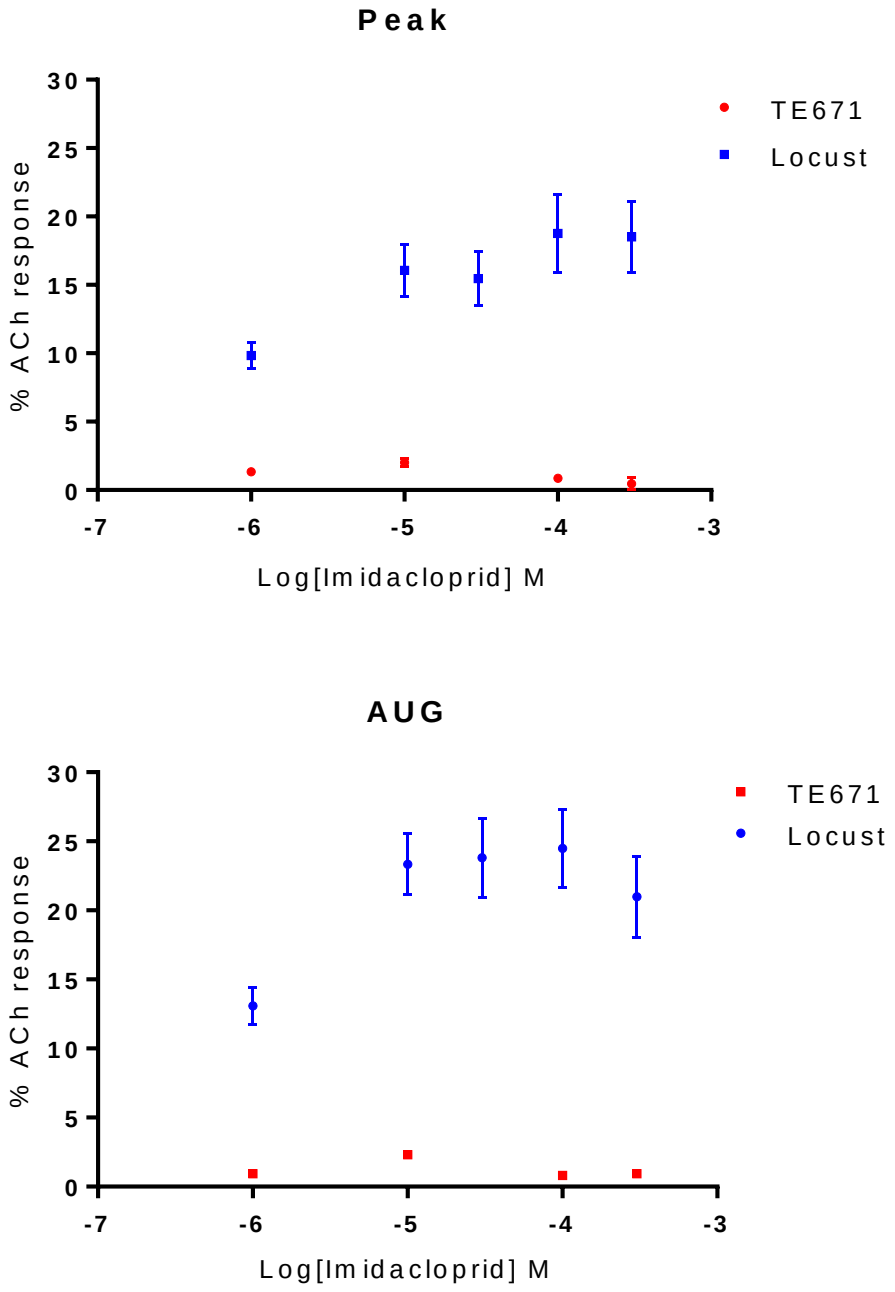


Figure 3.3.1. Activation of human muscle-type nAChR (red) and locust neuron nAChR (blue) by increasing concentrations of imidacloprid. Cells underwent whole-cell patch-clamp at V_H = -75 mV and compounds were applied for one second. Peak currents (A) and net-charge movement (B) were normalised against a control of 10 µM (TE671) or 100 µM (locust) ACh. Locust neurons were considerably more sensitive to imidacloprid with maximum responses up to 24.5 % of control as opposed to just 2.3 % for TE671 (AUG). N = 9-31 for TE671 and 14 for locust (peak); N = 13 for TE671 and 14 for locust neurons (AUG) and error bars indicate ± SEM.

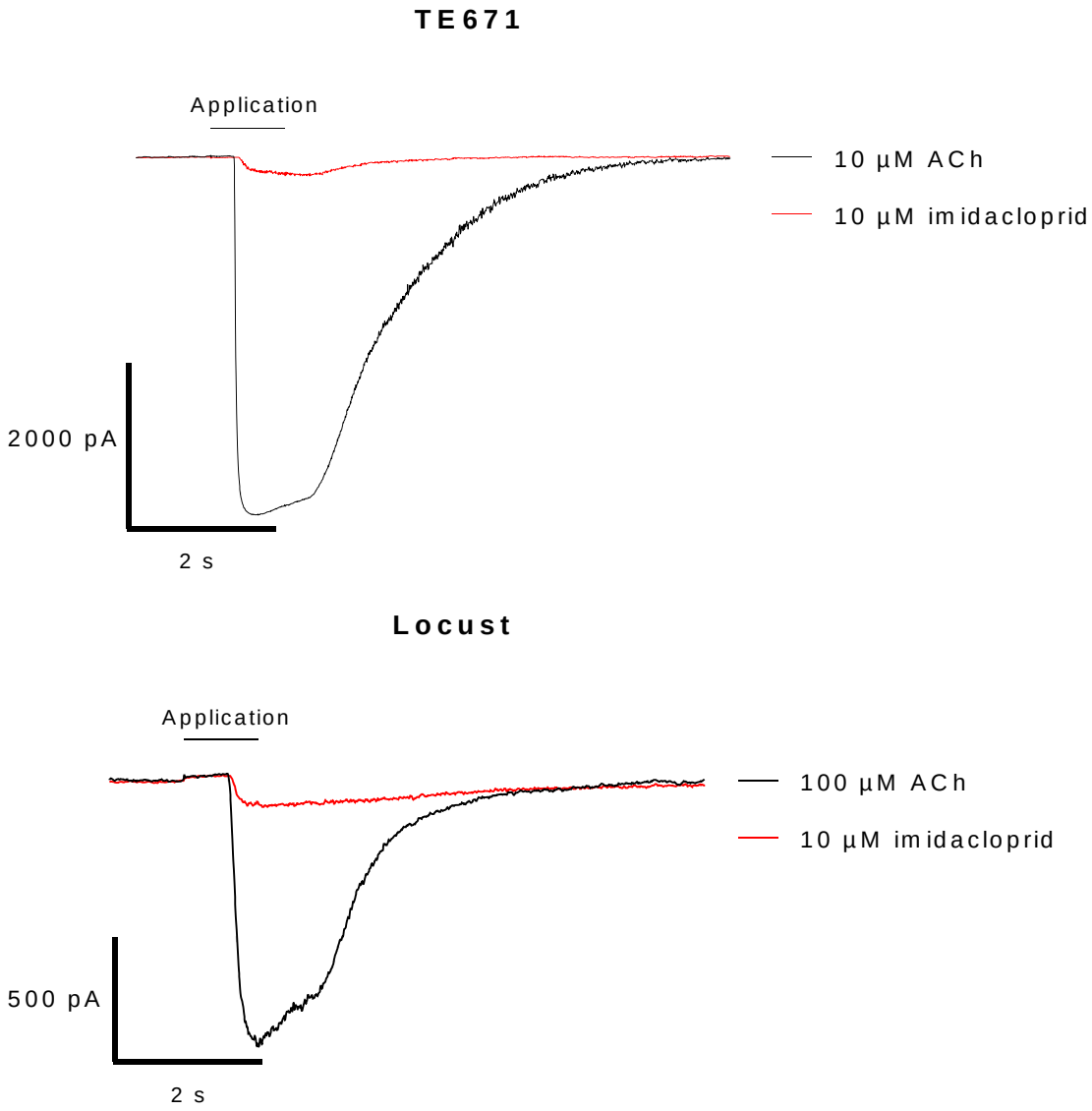


Figure 3.3.2. Representative traces showing the response of TE671 cells (above) and locust neurons (below) to a one second control response of ACh (black) and 10 µM imidacloprid (red) when patch-clamped at V_H = -75 mV. A small inward current is generated in both instances. This washes off rapidly in the case of TE671 but not in locust neurons.

3.3.2 Antagonism of ACh induced currents by co-application with imidacloprid

Co-application of increasing concentrations of imidacloprid with ACh resulted in diminishing peak and 1 s currents in both TE671 and locust neurons (Figure 3.3.3 & 3.3.4). Imidacloprid had no effect on locust neuron ACh response

size at 10 nM and caused slight potentiation at 30 nM before causing concentration dependent inhibition at concentrations in excess of 100 nM. The antagonistic activity of imidacloprid began to plateau at concentrations between 10-100 µM and 1 mM for locust neuron and TE671, respectively. For this reason, the curve that was fitted to the inhibition plots was not constrained to 0. The peak IC_{50} value calculated for locust neurons was 119 fold lower than that for TE671 and that for AUG IC_{50} was 389 fold lower in locust neurons (Table 3.3.1) In both comparisons, locust neurons were two orders of magnitude more sensitive to imidacloprid, and this was strongly significant by sum of squares F-test ($P < 0.0001$).

To confirm that imidacloprid activity was competitive with ACh, a concentration-response plot in the presence and absence of imidacloprid was conducted on TE671. The protocol for constructing an EC_{50} plot was followed, as described previously on TE671 cells. This was then repeated on the same cell but with the co-application of a fixed 300 µM imidacloprid concentration. The peak current was measured and normalised against the control response which generated the largest response. The mean ±SEM are shown in Figure 3.3.5. The EC_{50} for ACh was shifted from 2.03 (95% CL = 1.44-2.85) to 7.89 µM (95 % CL = 3.83-16.24) by 300 µM imidacloprid, suggestive of competitive antagonism. When co-applied with imidacloprid, the highest ACh concentration only generated a peak response 81.53 % that of the ACh alone.

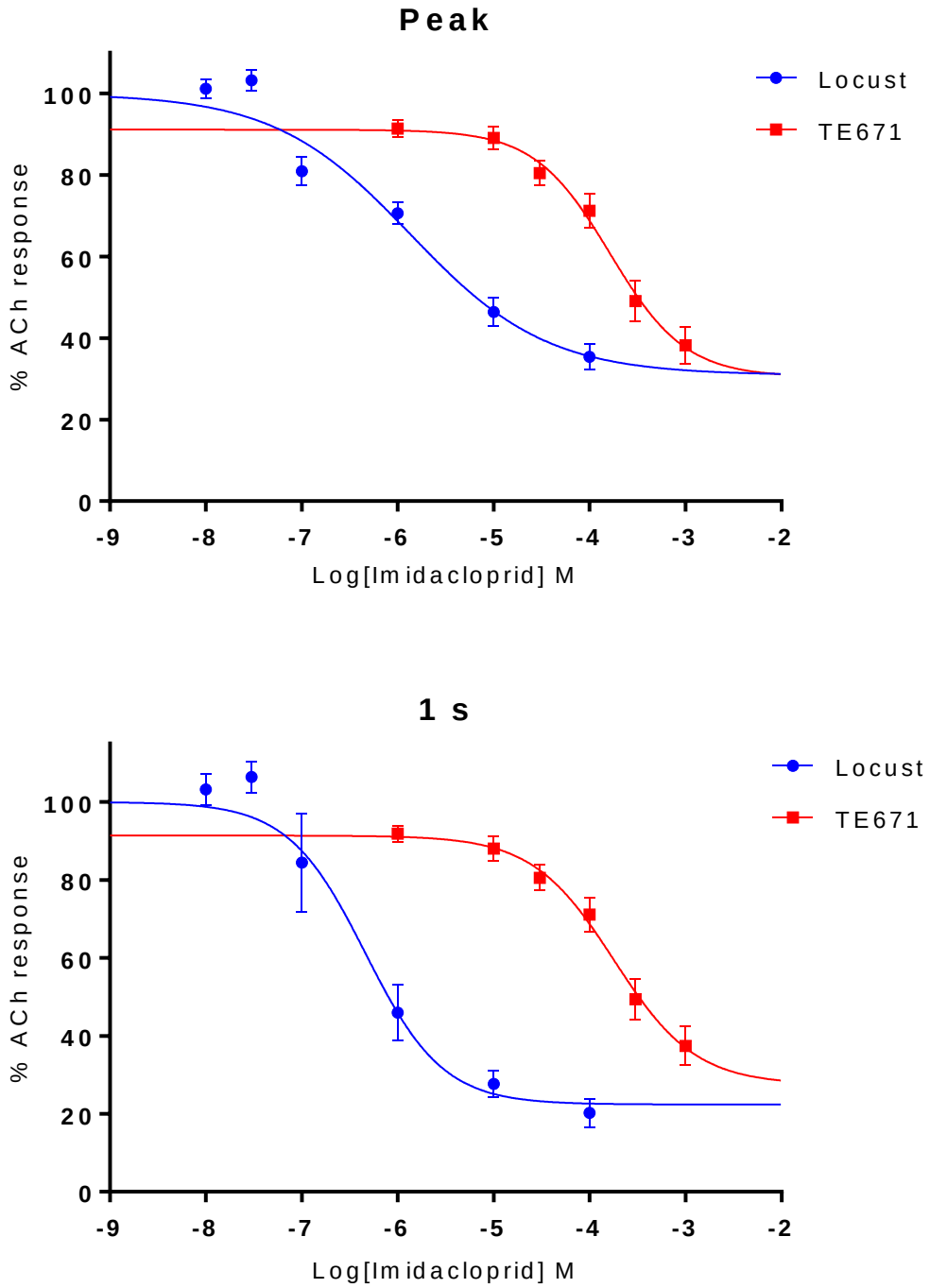


Figure 3.3.3. Concentration inhibition plots comparing the antagonist activity of imidacloprid on the response to 10 and 100 µM ACh on TE671 cells (red) and locust neurons (blue) respectively. Increasing concentrations of imidacloprid were co-applied with ACh and the peak (top) and 1 s currents measured and normalised against a control. The mean ±SEM are shown and a non-linear regression fitted with a 4PL equation constrained to max = <100 and min =>0. IC_{50} values were derived (see table 3.3.1). Sum of squares F-test revealed that in all cases, locust neurons were significantly more sensitive to imidacloprid.

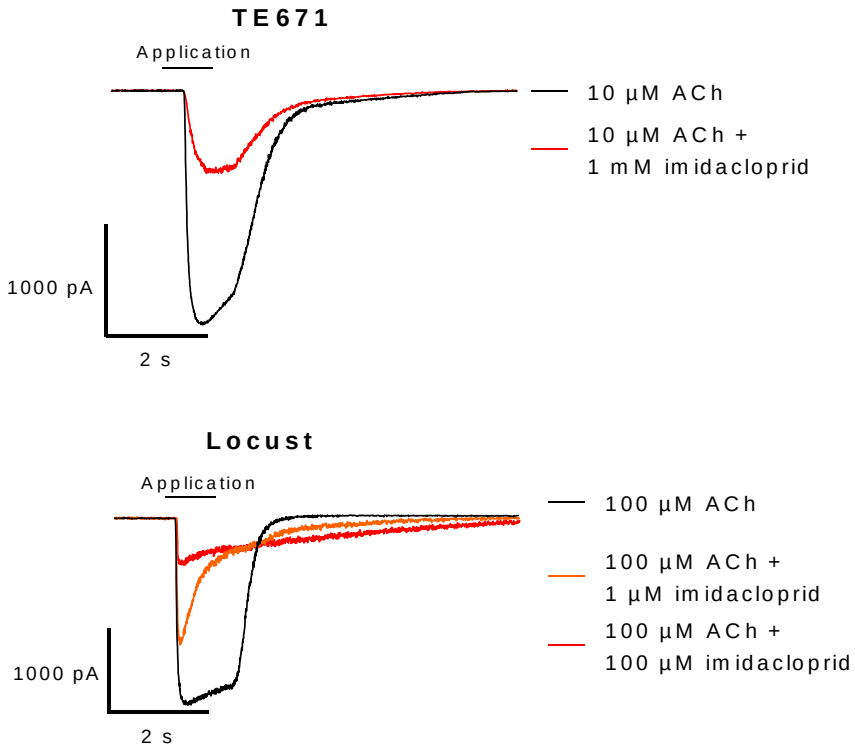


Figure 3.3.4. Representative traces showing the inhibition of nAChR responses caused by imidacloprid on TE671 cells (above) and locust neurons (below). Cells were patch-clamped in the whole-cell conformation at V_H = -75 mV. ACh (black) and increasing concentrations of imidacloprid (red and orange) were co-applied over cells for one second and the inward currents measured.

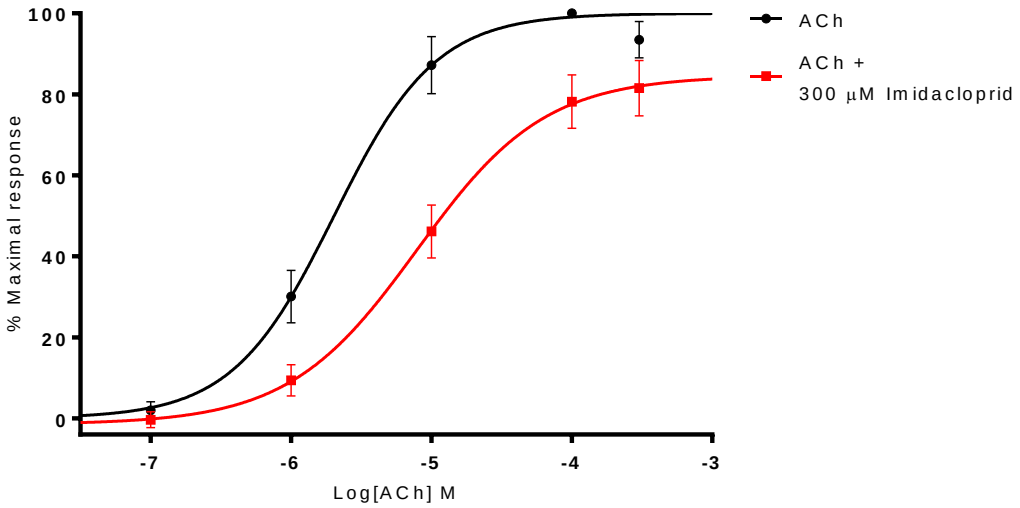


Figure 3.3.5. Concentration response curves showing the effect of imidacloprid on the response of TE671 cells to increasing ACh concentration. Increasing concentrations of ACh were applied to TE671 cells to establish an EC_{50} curve (black). This was then repeated with the co-application of 300 µM imidacloprid. Peak currents were normalised against the ACh response that generated the largest response and mean ±SEM plotted (red). The results show that imidacloprid shifts the EC_{50} curve to the right but also inhibits the maximal peak response The EC_{50} values were 2.03 µM for ACh shifted to 7.9 µM for ACh with imidacloprid. N = 10.

	Peak		1 s	
	TE671	**Locust**	**TE671**	**Locust**
IC_{50} (μM)	162	1.36	179	0.46
95% CL	70.6-374	0.57-3.2	63.2-507	0.17-1.25
P	<0.0001		<0.0001	

Table 3.3.1 IC_{50} values, 95 % confidence limits and the sum of squares F-test p values calculated for the imidacloprid inhibition of nAChR responses of TE671 cells and locust neurons at V_H = -75 mV.

3.4 Patch-Clamp of Locust Neurons and TE671 Cells

The quality and quantity of patch-clamp data generated is largely dependent upon the ease with which high-resistance seals can be formed and maintained between electrode and cell. Having optimised the protocols used to prepare cells and conduct experiments, the outcome of patch-clamp attempts was recorded for 323 TE671 cells and 361 locust neurons over 33 and 24 days of experiments, respectively. The number of attempts that resulted in: high resistance (gigaohm) seal formation, an inward current in response to ACh exposure and >6 stable responses to ACh were recorded. The results are shown in Figure 3.4.1, both as a proportion of cells that had met pre-determined requirements (i.e. it is only possible to know if a cell was ACh responsive if a high-resistance seal had been formed), and of the total number of attempts. Multiple T-tests revealed that there were no significant differences between TE671 or locust neurons in terms of the success with which high-resistance seals were formed and whole-cell currents recorded. An average of 57 % of attempts to record from TE671 cells resulted in a stable ACh response which could be used for experiments. This varied from 0-100 % between testing days. An average of 46 % of attempts to record from locust neurons resulted in a stable ACh response. This varied from 10-73 % between testing days. The main cause of seal-loss or unstable ACh response was pressure

from the perfusion system disrupting the seal, or causing the cell to detach from the glass cover-slip.

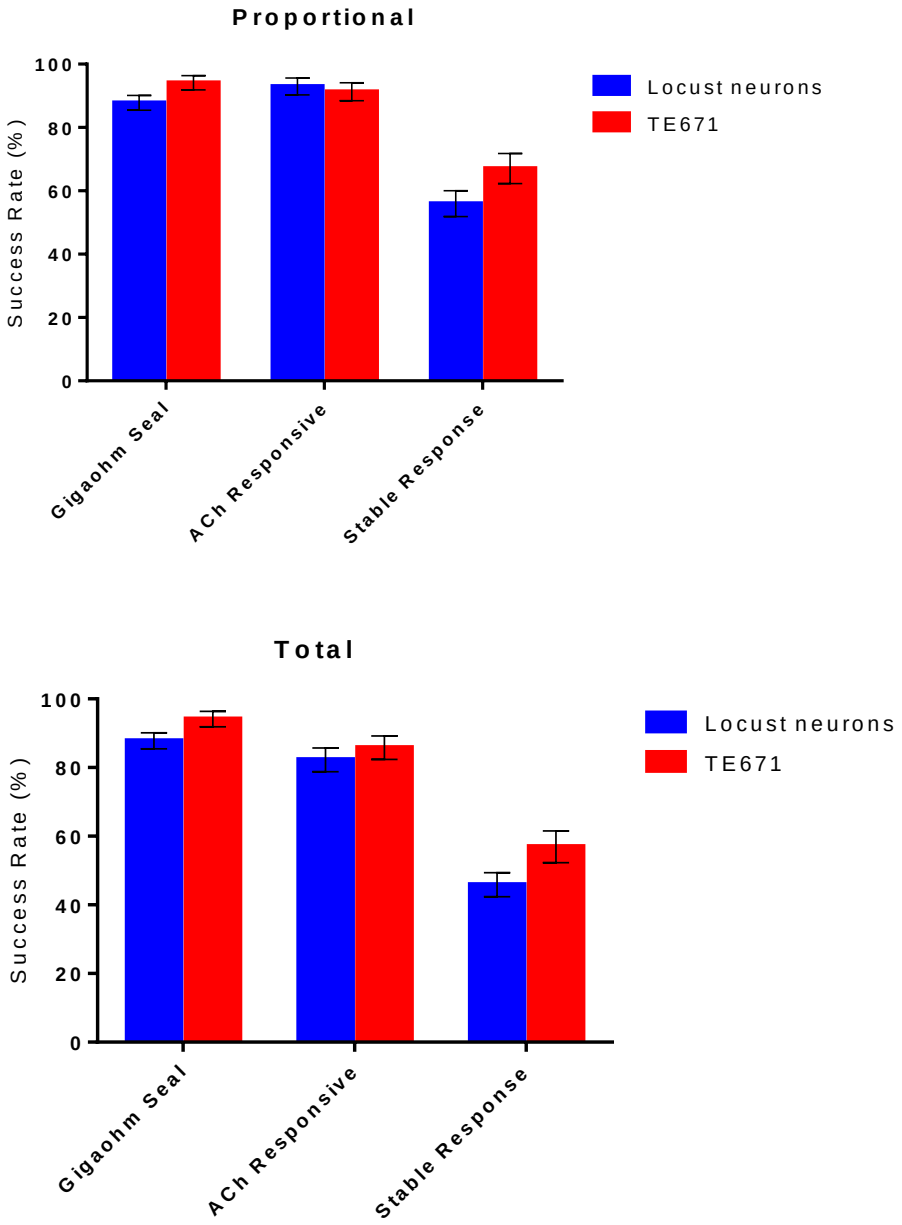


Figure 3.4.1. Column charts showing the whole-cell patch-clamp success rate of locust neurons (blue) and TE671 cells (red). The top chart shows the results as a proportion of cells that had met the prerequisite conditions (e.g. it is only possible to know if a cell displayed a stable ACh response if a gigaohm seal had been formed). The bottom chart shows the success rate as a percentage of the total number of patch-clamp attempts. It can be seen that TE671 cells are marginally more likely to form high-resistance seals and display a stable ACh response, but multiple T-tests revealed that these differences were not significant. N = 361 (locust) and 323 (TE671) error bars show ±SEM.

3.5 Discussion

3.5.1 Pharmacology of the ACh Response of TE671 cells and Locust Neurons

Prior to investigating the activity of ladybird alkaloids it was essential to develop protocols to make recordings of both vertebrate and insect nAChR currents using whole-cell patch-clamp. The human rhabdomyosarcoma line, TE671 which is known to express embryonic muscle-type nAChRs with stoichiometry $(\alpha1)_2\beta1\gamma\delta$ (Schoepfer *et al*, 1987; Luther *et al*, 1989; Yamamoto *et al*, 1991) was selected. This cell line has been widely used as a source of nAChRs for electrophysiological and pharmacological experiments (Shao *et al*, 1998; Brier *et al*, 2003; Zalat *et al*, 2005; Barthelme *et al*, 2013; Procopiou *et al*, 2014) and has shown sensitivity to azaphenalene ladybird alkaloids (Leong *et al*, 2015). A technique for isolating locust neurons was developed, based on Cayre *et al*, (1998 & 1999) which yielded a consistent source of cells that were amenable to patch-clamp and expressed locust neuronal nAChRs. Patch-clamp of native receptors was deemed preferable to heterologous expression of locust nAChRs because of the inherent difficulty of expressing insect receptors (Tomizawa & Casida, 2001; Tomizawa & Casida, 2003; Jones & Sattelle, 2010) and the considerably smaller quantity of alkaloid required for patch-clamp experiments over voltage-clamp of *Xenopus* oocytes. The relatively crude nature of the locust neuron preparation meant that nAChR subunit composition could not be verified and may not have been homogenous, but the cells used in this study displayed consistent nicotinic pharmacology.

The sensitivity of both cell types to ACh was compared by constructing EC_{50} plots of both peak current and net-charge movement (Figures 3.1.1 & 3.1.2). The

EC_{50} values for both parameters in both cell types were in the low µM range but TE671 were considerably more sensitive than locust neurons, with values for peak current of 1.78 and 20.26 µM respectively. The comparatively low sensitivity to ACh displayed by locust neurons has been noted previously (Suter & Usherwood, 1985). At high ACh concentrations (> 100 µM) TE671 cells occasionally displayed late onset, oscillating responses that were blocked by atropine, these are apparent in the EC_{50} curve for net-charge movement (which was conducted without atropine) where response size continues to increase rather than plateau (Figure 3.1.1). Concentrations of 10 and 100 µM ACh were used in further experiments for TE671 and locust neurons respectively. These represented concentrations that generated near maximal peak currents, but did not cause rapid desensitisation. Repeated applications of these concentrations at 60 s intervals resulted in inward currents that were consistent in magnitude after the first application (Figures 3.1.3 & 3.1.4), not causing receptor rundown as seen with 10 and 30 s wash-off times. For further experiments, at least two applications of ACh were made prior to test compounds and the second was used as the control response.

Pharmacological characterisation with the selective antagonists of nAChRs and muscarinic AChRs, d-tbc and atropine, as well as the selective nAChR agonist, nicotine revealed that both cell types displayed typical nAChR pharmacology. The application of 100 µM nicotine resulted in rapidly desensitising inward currents (Figure 3.2.1). ACh currents were antagonised by the nAChR antagonist d-tbc (Figure 3.2.2 & 3.2.3) but not by the mAChR antagonist atropine (Figure 3.2.1). An α subunit cloned from a desert locust embryo cDNA library (αL1) and subsequently

expressed in *Xenopus* oocytes, showed high sensitivity to d-tbc (Marshall *et al*, 1990). 1 µM d-tbc abolished the nicotine induced current in these homomeric nAChRs, in contrast to the nAChRs expressed by the locust neurons used in this study which were strongly antagonised but still responsive to ACh when co-applied with 100 µM d-tbc and only weakly antagonised by 1 µM d-tbc (Figure 3.2.2) with a peak IC_{50} of 15.97 µM. It may be that ACh has a higher affinity and is therefore better able to compete with d-tbc for binding sites than nicotine. Alternatively homomeric αL1 may be more sensitive to d-tbc antagonism than the native nAChRs present in the neuron preparation used herein. αL1 displayed low sensitivity to nicotine compared with chick α7 when expressed in *Xenopus* oocytes, with EC_{50}s of 830 and 24 µM, respectively (Amar *et al*, 1995). In a similar patch-clamp study of neurons isolated from the thoracic ganglia of the migratory locust (*Locusta migratoria*) low sensitivity to d-tbc was also noted, with an IC_{50} of 8.5 µM (Benson, 1992). Contributions to the d-tbc binding site from the secondary subunit are known to affect d-tbc binding affinity in vertebrate M-nAChRs (Bren & Sine, 1997). An experiment to determine the inhibitory activity of d-tbc towards nicotine induced currents would shed light upon the differences in d-tbc sensitivity of the nAChRs present in the preparation used in this study and homomeric αL1.

3.5.2 Imidacloprid Acts as an Insect Selective Partial Agonist of nAChR

Imidacloprid is the most widely used neonicotinoid insecticide on the market (Jeschke *et al*, 2010). It acts as a partial agonist of nAChRs with selectivity for insect over vertebrate receptors (Matsuda *et al*, 2005; Ihara *et al*, 2006). Both the excitatory and inhibitory activities of imidacloprid were compared between

human and locust nAChR models to verify them as an appropriate method for detecting insect selective activity (Figures 3.3.1 & 3.3.3). It is also valuable to establish the effect that imidacloprid has on human muscle-type nAChRs because exposure to high concentrations is known to be acutely toxic, causing neurological, cardiovascular and respiratory abnormalities with dyspnea/apnea, coma (Glasgow Coma Scale <8), tachycardia, hypotension and nausea/vomiting reported in over 40 % of severe cases (Lin *et al*, 2013). This is thought to be mediated by activity at α4β2 nAChR, expression of which is significantly upregulated in response to chronic exposure to imidacloprid and metabolites (Tomizawa & Casida, 2000; Tomizawa & Casida, 2002), but studies of human toxicity are limited (Li *et al*, 2011). M-nAChRs are not thought to be clinically relevant in neonicotinoid poisoning (Tomizawa, 2004) but this question has received little research attention.

Application of imidacloprid to ACh responsive locust neurons resulted in moderate inward currents with maximum responses of 18.8 % of peak and 24.5 % of AUG of 100 µM ACh response. This was in stark contrast to the response of TE671 cells which displayed very small inward currents (maximum of 2 and 2.3 % for peak and AUG respectively). This is indicative of the highly selective nature of imidacloprid. The disparity in magnitude between the currents generated by ACh and imidacloprid in locust neurons can be explained by the fact that neonicotinoids activate a sub-conductance rather than the full conductance state of ACh activation and are thus considered partial agonists of insect nAChRs (Nagata *et al*, 1997; Matsuda *et al*, 2005). Electrophysiological studies of neonicotinoids indicate that cyclic gaunidine containing neonicotinoids such as imidacloprid generate smaller

whole-cell currents than neonicotinoids possessing an acyclic moiety (Ihara *et al*, 2006). When applied to honeybee neurones, imidacloprid activated maximal currents corresponding to 43 % of the response to 300 µM ACh in antennal lobe neurons (Barbara, 2008) and 36 % of the 200 µM ACh response in Kenyon cells (Déglise *et al*, 2002). Application of 100 µM imidacloprid to neurons isolated from the terminal abdominal ganglia of the American cockroach (*Periplaneta americana*) resulted in currents 11 % of the 100 µM ACh response (Ihara *et al*, 2006). It was not possible to calculate an accurate EC_{50} value for activation of human M-nAChRs because the activity was so weak, but those calculated for locust neurons were 2.25 (95% CL = 1.09–4.65 µM) and 0.6 µM (95% CL = 0.13–2.77 µM) for peak and AUG respectively. These values fall in line with published values for patch-clamp assays of insect and human cells with peak EC_{50} value calculated for honeybee antennal lobe neurones was 3.4 µM (Barbara *et al*, 2008), the EC_{50} for honeybee Kenyon cells was 25.1 µM (Déglise *et al*, 2002) and imidacloprid (300 µM) caused peak currents of 0.6 % of the 1 mM ACh response in human α4β2 expressed by HEK293 cells (Li *et al*, 2011).

Co-application of increasing concentrations of imidacloprid with ACh caused concentration dependent inhibition of nAChR currents (Figures 3.3.3 & 3.3.4). As with activation, inhibitory activity was considerably greater towards locust nAChRs than human muscle-type. The IC_{50} values calculated for locust nAChRs were 1.36 (95% CL = 0.57–3.2 µM and 0.46 µM (95% CL = 0.17–1.25 µM) for peak and late current whereas those for TE671 cells were 162 (95% CL = 70.6–374 µM) and 179 µM (95% CL = 63.2–507 µM). This equates to 119–389 fold selectivity for insect over

human nAChRs and this was significant in both current parameters (P = <0.0001). Co-application of 300 µM imidacloprid with 10 µM ACh to TE671 cells generated inward currents 49.1 % of control (Figure 3.3.3), this inhibition appears to be lower than that of human α4β2 which displayed responses 5-29 % of the EC_{50} ACh response when co-applied with 300 µM imidacloprid (Li *et al*, 2011). This further suggests that the activity of imidacloprid is greater at N-nAChR than M-nAChR and the clinical pathyophysiology of human imidacloprid poisoning is not predominantly influenced by activity at M-nAChR (Tomizawa & Casida, 2000; Tomizawa & Casida, 2002). Net-charge inhibition was omitted because the dual activity of imidacloprid made this parameter redundant. Co-application of 10 µM imidacloprid with 100 µM ACh to locust neurons generated responses 46.4 % of the ACh response. This is similar to the level of antagonism shown towards honeybee Kenyon cells, which had response sizes 36 % of the ACh control (Déglise *et al*, 2002). When co-applied with ACh, imdacloprid did not abolish inward currents at the highest concentration used in these experiments (Figure 3.3.3), 100 µM for locust neurons and 1 mM for TE671 cells. This may be because ACh is still competing with imidacloprid for binding sites. If this is the case, using higher concentrations of imidacloprid would have further reduced the response size. Alternatively there may be some insurmountable ACh binding that imidacloprid is unable to overcome.

To confirm that the antagonism caused by imidacloprid is competitive with ACh in vertebrate M-nAChRs, an ACh concentration-response curve was constructed in the presence and absence of imidacloprid. The concentration-response curve shown in Figure 3.3.5 clearly shows that increasing concentrations

are able to overcome the inhibitory activity of imidacloprid against human muscle-type nAChR. The EC_{50} value is right shifted from 2.03 to 7.9 µM by 300 µM imidacloprid but the response plateaus at 81.5 % of ACh alone. This could be caused by imidacloprid acting at a separate, non-competitive site but may also be a result of receptor desensitisation or insufficient wash-off between applications. Previous experiments into the agonist activity of imidacloprid in TE671 cells revealed that the inhibitory action of imidacloprid is not quickly reversible, with a final control application of ACh causing a peak response of just 81.7 % of the initial control response. To determine whether there is a second, non-competitive binding site for imidacloprid a radioligand binding assay could be employed with radioligands such as [3H]-TCP which occupies a luminal binding site (Leong *et al*, 2015).

The fact that imidacloprid acts as a competitive antagonist and that the concentration of ACh used for experiments on locust neurons was ten-fold higher than that used on TE671 cells may have skewed the results. It would be expected that applying a higher concentration of ACh would reduce the probability of imidacloprid reaching the orthosteric site. The fact that antagonist activity of imidacloprid was significantly greater towards locust neuron ACh responses despite the disparity in ACh concentration, highlights the considerably higher affinity that this compound displays for locust N-nAChRs over the human M-nAChRs of TE671 cells.

3.5.3 Patch-Clamp of TE671 and Locust Neurons

Once a protocol for dissecting, enzyme treating and coating coverslips had been developed and optimised for the whole-cell patch-clamp of locust neurons, the success with which it was possible to record whole-cell currents from both cell

types was compared. The proportion of cells that it was possible to form gigaohm seals with, the proportion that showed inward currents to ACh and the proportion that gave consistent responses to ACh were compared in Figure 3.4.1. The results show that TE671 were marginally more likely to yield consistent ACh responses that could be used in experiments than locust neurons (57 % as opposed to 46 %) but this was not statistically significant. Both cell types showed high levels of variability in terms of how well they tolerated patch-clamp. This could have been a result of variations in passage number or time since plating of TE671 cells. With locust neurons, every attempt was made to keep poly-L-lysine coating of coverslips and enzyme treatment consistent, but the practicalities of storing coated cover-slips and enzyme solutions led to some variability. This may explain the unpredictability of patch-clamp success with locust neurons. Generally both cell types were quite straightforward to patch-clamp and yielded a consistent source of both human muscle-type and locust neuronal nAChR.

Chapter 4: Screening Ladybird Alkaloid Extracts for Nicotinic Acetylcholine Receptor Activity

Introduction

Recently published data (Leong *et al*, 2015) suggest that the nAChR may be a target for alkaloids produced by toxic ladybird beetles. To date, only a very small number of ladybird alkaloids have been screened for nAChR activity. We wished to determine whether other ladybird alkaloids show activity at nAChRs and whether any show higher potency for locust over human nAChRs, indicating possible selectivity evolved to target insect predators. Alkaloid extracts were prepared from seven UK native ladybirds and the invasive harlequin. Concentration-inhibition plots were used to compare the nAChR inhibitory activity (IC_{50}) to both human muscle-type and locust neuronal nAChRs. Alkaloid extracts were then applied to cells in the absence of ACh to screen for agonist activity.

4.1 Alkaloid Extracts

Eight species of ladybird known to produce alkaloids (Table 4.1.1) were collected in sufficient numbers to allow alkaloid extraction and purification. These were: 2-spot (*Adalia bipunctata*), 10-spot (*Adalia decempunctata*), cream spot (*Calvia quatuordecimguttata*), 7-spot (*Coccinella septempunctata*), pine (*Exochomus quadripustulatus*), orange (*Halyzia sedecimguttata*), harlequin (*Harmonia axyridis*) and 14-spot ladybird (*Propylea quatuordecimpunctata*). The numbers of 2-spot ladybirds were supplemented with captive produced beetles purchased from Syngenta Bioline (product Adalline b). Organic extracts were prepared by spinning crushed beetles in methanol before using acid-base separation to isolate alkaloids. Thin layer chromatograms, stained with

Dragendorff's reagent revealed that all extracts contained alkaloid and that composition varied between species (Figure 4.1.1). This was consistent with previous studies that have identified novel alkaloids from each of the species used (Table 4.1.1). Of the species used in this study, three are polymorphic. Melanic and non-melanic individuals of the 2-spot and harlequin ladybird and spotted, chequered and dark forms (Honěk et al, 2005) of the 10-spot ladybird were collected together and combined for extractions.

Mean body mass varied considerably between the species examined in this study, but alkaloid content, measured as μg alkaloid extract/beetle remained relatively constant with a range of 9.1-90.5 μg/beetle but with most species clustered between 35-60 μg/beetle (Figure 4.1.2). Species with lower body mass generally had higher relative alkaloid content but similar total alkaloid content to larger species. The exception to this trend was the cream spot ladybird (*C. quatuordecimguttata*) which displayed both a low body mass and alkaloid content.

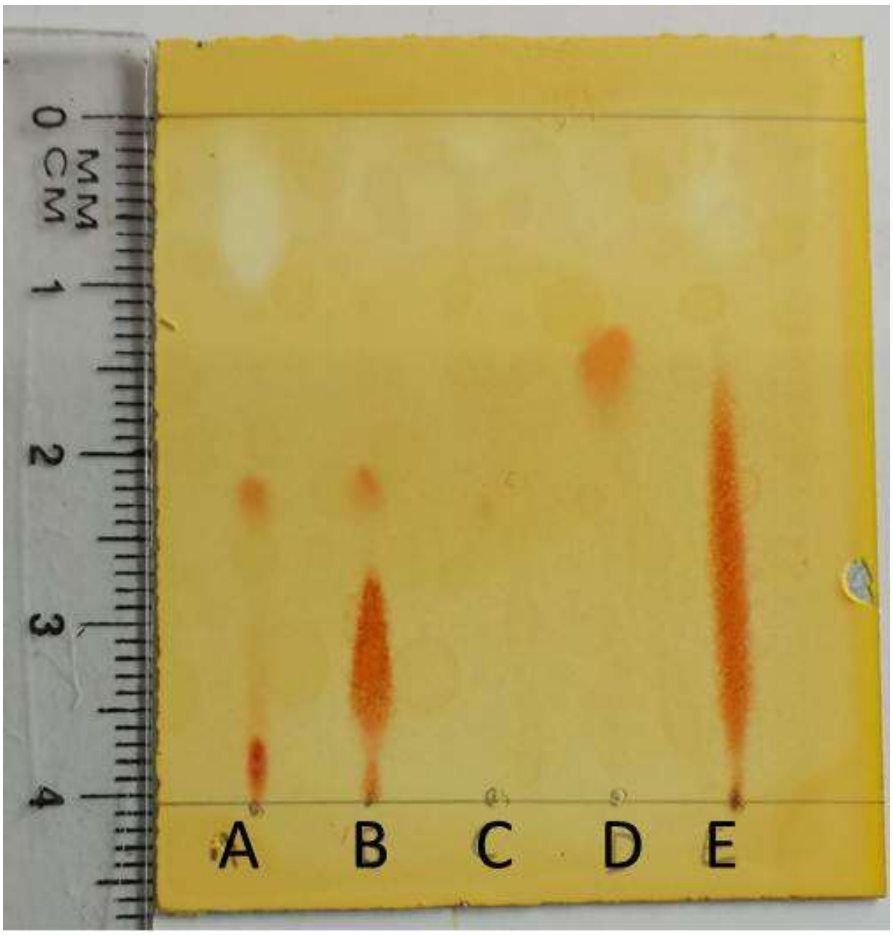


Figure 4.1.1 A qualitative silica TLC plate was spotted with 1 µL DCM containing 10 mg/mL alkaloid extract from the following five species of ladybird: A *Propylea quatuordecimpunctata*, B *Exochomus quadripustulatus*, C *Calvia quatuordecimguttata* (note, an alkaloid spot is visible around 2 cm but does not show up well in this figure), D *Adalia decempunctata* and E *Halyzia sedecimguttata*. The plate was allowed to run in methanol with 1 % ammonium hydroxide and then stained with Dragendorff's reagent.

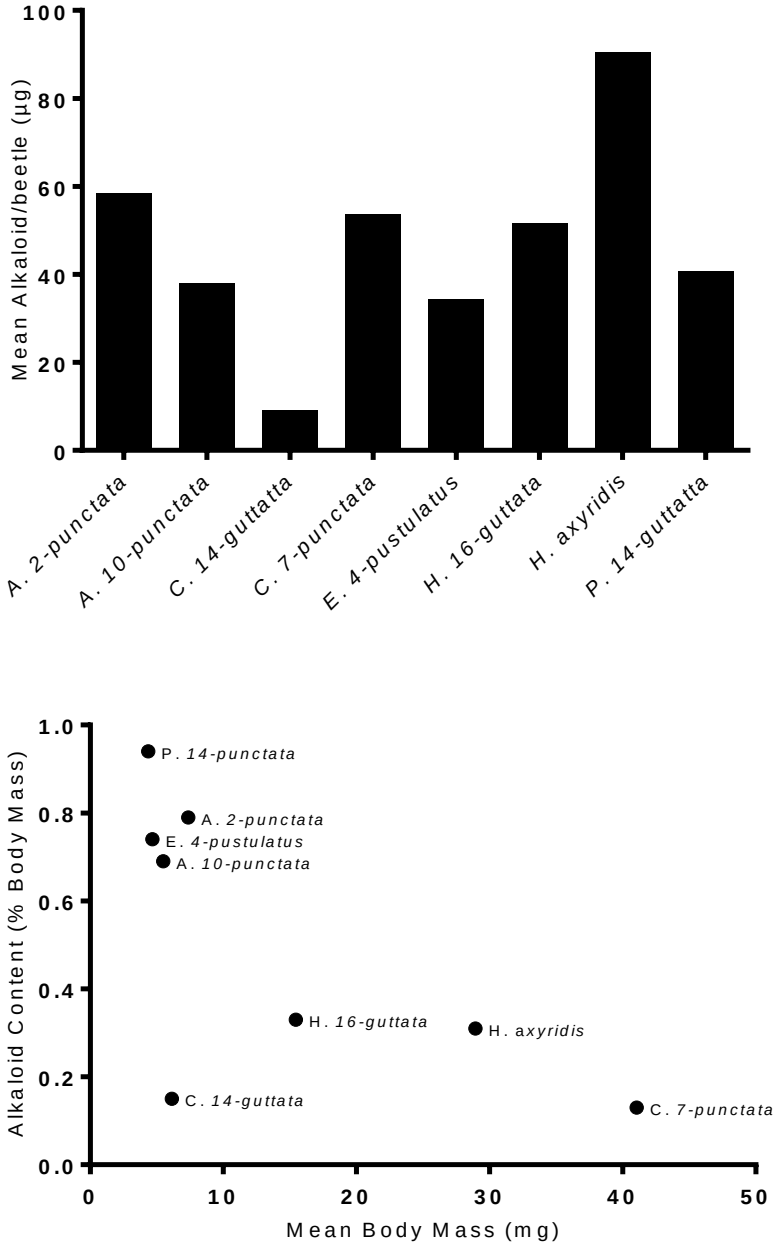


Figure 4.1.2 (Above) Column chart showing the concentration in µg/beetle of alkaloid extracted from eight species of ladybird. Whole beetles were frozen in liquid nitrogen, crushed and extracted in methanol for 48 hrs at room temperature. The resulting organic extract underwent acid-base separation to purify alkaloids and these were subsequently weighed and the presence of alkaloid confirmed using Dragendorff's reagent. The total weight of alkaloid was divided by the total number of beetles extracted from to give the values above. *A. bipunctata* n = 628*, A. decempunctata* n = 133*, C.quatuordecimguttata* n = 88, *C. septempunctata* n = 355, *E. quadripustulatus* n = 198*, H. sedecimguttata* n = 29, *H. axyridis* n = 314 and *P. quatuordecimpunctata* n = 52. (Below) Plot showing the relationship between alkaloid content (% of mean body mass) and mean body mass. These figures show that beetles of different species contain very similar total quantities of alkaloid, irrespective of body mass (with the exception of *C. quatuordecimguttata*). To account for this, species with a lower body mass generally possess a higher proportion of alkaloid.

4.2 nAChR Activity of Ladybird Alkaloids

4.2.1 Inhibition of ACh induced nAChR currents

Both TE671 cells and locust neurons were subjected to whole-cell patch-clamp and increasing concentrations of alkaloid extract were co-applied with ACh over cells at V_H = -75 mV. The current response was measured and normalised against a control response (Figures 4.2.1-4.2.7). Both 2-spot and 10-spot ladybirds produce the same alkaloids (Table 4.1.1) so only the alkaloid extract of the 10-spot ladybird was used in patch-clamp experiments. All seven alkaloid extracts caused concentration dependent inhibition of ACh induced currents in both TE671 cells and locust neurons. The IC_{50} values calculated for each extract are shown in Tables 4.2.4-4.2.6. In most comparisons (Figure 4.2.8), alkaloid extracts showed significantly higher potency towards locust neuron nAChRs with the exception of *H. sedecimguttata* peak and AUG, and *H. axyridis* 1 s currents (Figure 4.2.5 and 4.2.6). The greatest inhibition was on the late current (1 s current) with IC_{50}s ranging from 0.29–4.9 μg/mL against locust nAChRs and 1.3–47.8 μg/mL against TE671 cell nAChRs. The smallest effect was on the peak current, with IC_{50} values ranging from 3.6 – 65 μg/mL on locust nAChRs and 12.5 μg/mL–*24.9mg/mL (*extrapolated from curve) against TE671 cell nAChRs. As would be expected from a biphasic response such as that of nAChR towards ACh, the IC_{50}s calculated for the net-charge fell approximately in between the peak and late current values, ranging from 0.44–16.2 μg/mL against locust nAChRs and 0.87–108.9 μg/mL against TE671 cell nAChRs. In all comparisons (Figure 4.2.8), alkaloid extract from the 10-spot ladybird (Figure 4.2.1) was found to be most potent to locust nAChRs (IC_{50}s 0.29-3.66 μg/mL) and one-way ANOVA (Tables 4.2.1-4.2.3) revealed that it was significantly more potent

than all other alkaloid extracts with the exception of 7-spot. 7-spot alkaloid extract was the second most potent tested (0.32-3.9 μg/mL), and significantly more potent than the other alkaloid extracts tested. Harlequin and pine ladybird alkaloids showed intermediate potency (0.76-4.4 and 0.68-4.34 μg/mL respectively) with no significant differences between IC_{50} values, but significantly more potent than 14-spot (1.6-21 μg/mL), cream spot (1.8-24.6 μg/mL) and orange ladybird alkaloids. Orange ladybird alkaloid extract showed the lowest nAChR antagonism (4.9-65 μg/mL) and displayed significantly lower potency than all other extracts tested on locust neurons.

The IC_{50} results calculated for TE671 cell nAChRs revealed a different trend with harlequin ladybird alkaloids showing the highest potency in all comparisons (0.87-12.25 μg/mL) and the IC_{50} values for AUG and 1 s current were significantly lower than all other alkaloid extracts tested with the exception of 10-spot ladybird 1 s current. Pine ladybird alkaloid was found to be the second most potent against human muscle-type nAChR (5.82-21.9 μg/mL) and significantly more potent than 7-spot (18.7-133 μg/mL), cream-spot (31.8-940 μg/mL) and 14-spot (47.8-*24,920 μg/mL [*extrapolated from curve]) ladybird alkaloids. Orange ladybird alkaloid displayed intermediate antagonism of human muscle-type nAChRs, significantly lower than that of harlequin ladybird. However, no other comparisons were statistically significant.

4.2.2 Selectivity

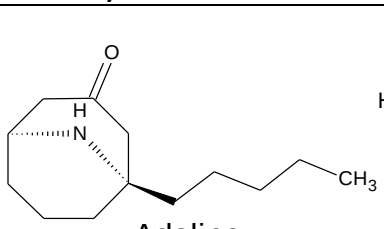
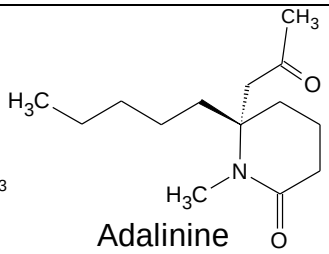
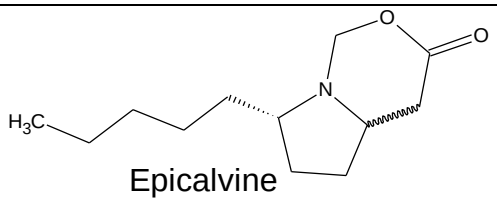
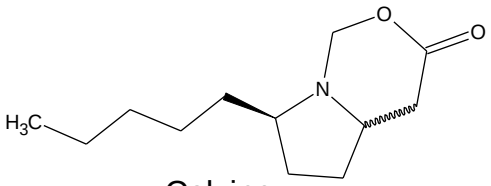
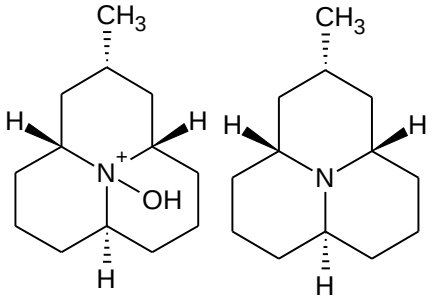
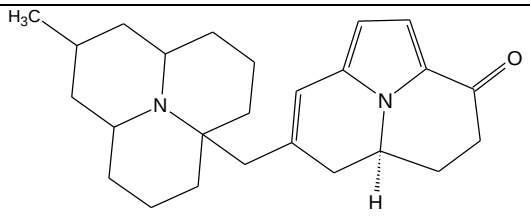
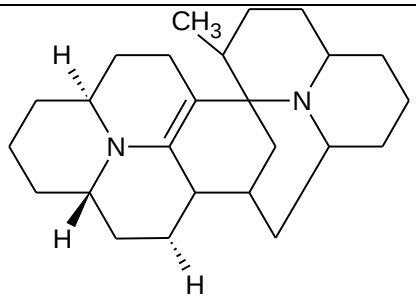
In most comparisons the IC_{50} values calculated for locust neurons were significantly lower than those calculated for TE671 (Figure 4.2.8). The exceptions to this were the peak and AUG IC_{50}s calculated for the orange ladybird (Figure 4.2.5),

the 1 s current calculated for harlequin ladybird alkaloids (Figure 4.2.6) and the peak IC_{50} calculated for the cream spot ladybird alkaloid (4.2.2). Four of the ladybird extracts showed moderate to high levels of selectivity for locust over human nAChRs in all three current measurements (Figure 4.2.8). 7-spot ladybird alkaloid showed the highest level of insect selectivity (38, 124 and 58 fold [peak, AUG and 1 s respectively]). 14-spot ladybird alkaloids displayed considerably greater inhibition of locust peak current over that of human muscle-type nAChRs (119 fold), but the TE671 cell value was extrapolated from a curve owing to the low potency of this extract. The AUG and 1 s selectivity values were 34 and 29 fold respectively. The cream spot ladybird showed a consistently high level of selectivity across all comparisons, 38, 35 and 18 fold and 10-spot ladybird alkaloid showed slightly lower selectivity of 13, 19 and 35 fold. Alkaloid from the pine ladybird displayed a lower level of selectivity for insect over vertebrate nAChRs, with selectivity values of 5, 3 and 10 fold. Both harlequin and orange ladybirds showed marginally higher potency for locust peak and 1s current (2.8, 2 fold and 2.2 and 1 fold for harlequin and orange, respectively) but slightly lower potency for locust over TE671 AUG current (1 and 2 fold respectively). On balance, neither of these alkaloid extracts appears to show selectivity for either locust of TE671 nAChR.

4.2.3 Activation of Inward Currents in ACh Responsive Cells

None of the alkaloid extracts tested consistently induced inward currents when applied to locust neurons or TE671 cells which had been voltage-clamped at V_H = -75 mV (Figures 4.2.1-4.2.7). Some locust neurons did display small inward currents in response to application of extracts (Figures 4.2.1, 4.2.3, 4.2.5 and 4.2.7), but in all cases, these cells also displayed similar responses to the application of

Ringer solutions. Interestingly, application of high concentrations (25 µg/mL) of 10-spot, 7-spot and 14-spot alkaloid appeared to block this inward current (Figures 4.2.1, 4.2.3 and 4.2.7) When high concentrations of harlequin ladybird alkaloid (25 µg/mL) were applied to locust cells, a small outward current was detectable (Figure 4.2.6).

Ladybird	Previously Identified Alkaloids	Reference/s
2-spot – *A. bipunctata* **10-spot –** *A. decempunctata*	 Adaline  Adalinine	Tursch *et al*, 1973. Lognay *et al*, 1996.
Cream spot – *C. quatuordecimguttata*	 Epicalvine  Calvine	Braekman *et al*, 1999.
7-spot – *C. septempunctata*	 Coccinelline Precoccinelline	Tursch *et al*, 1971.
Pine – *E. quadripustulatus*	 Exochomine	Timmermans *et al*, 1992.
Orange – *H. sedecimguttata*	 Isopsylloborine	Schröder & Tolasch, 1998. Lebrun *et al*, 1999.

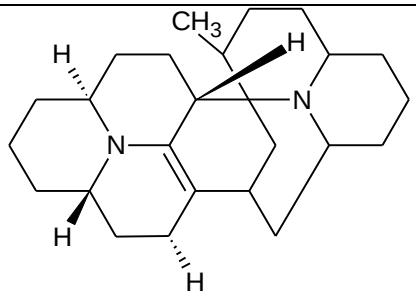
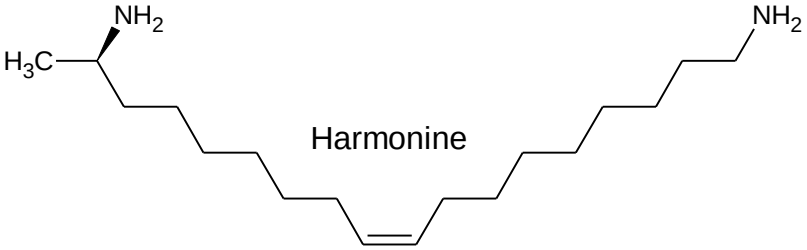
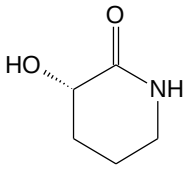
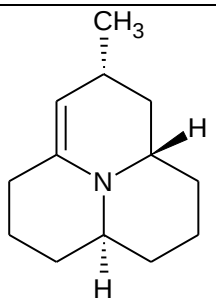
	 Psylloborine	
Harlequin – *H. axyridis*	 Harmonine  (*S*)-3-Hydroxypiperidin-2-one	Braconnier *et al*, 1985. Alam *et al*, 2002.
14-spot – *P. quatuordecimpunctata*	 Propyleine	Tursch *et al*, 1972.

Table 4.1.1 The alkaloids that have been identified in each of the ladybird species used in this study.

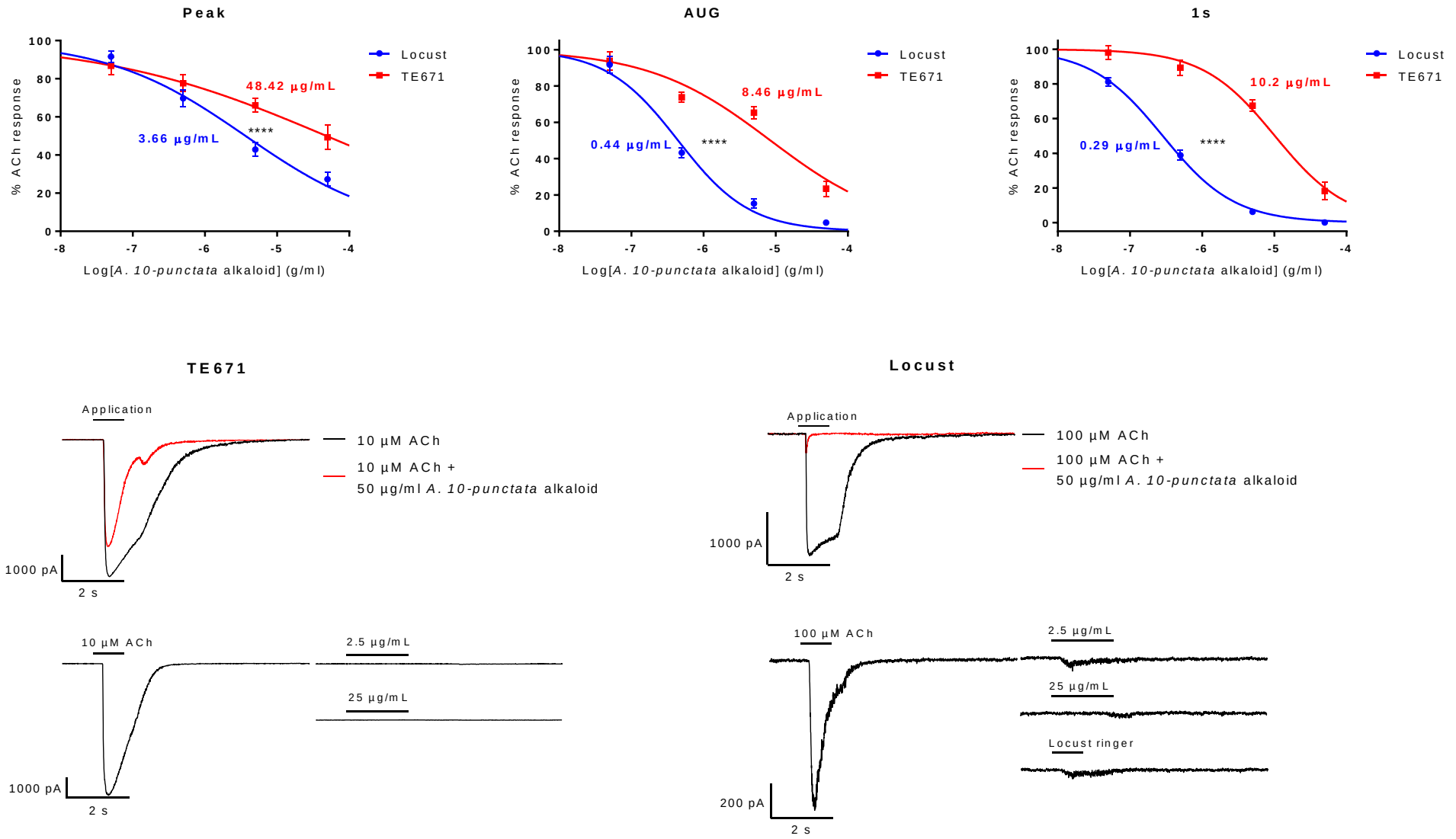


Figure 4.2.1 (Above) Comparative IC_{50} curves showing the inhibition of ACh induced currents in human muscle-type (red, N = 10) and locust neuronal nAChRs (blue, N = 15) by *A. decempunctata* alkaloid extract. IC_{50}s are shown and asterisks represent the p-value calculated by a sum-of-squares F-test. (Centre) Representative traces showing the inhibitory action of 50 µg/mL *A. decempunctata* alkaloid extract towards human muscle type (left) and locust neuronal nAChRs (right). (Below) Traces showing the effect of applying 2.5 and 25 µg/mL *A. decempunctata* alkaloid extract in the absence of ACh. No effect was seen in either model. V_H = -75mV for all experiments shown and error bars show ±SEM.

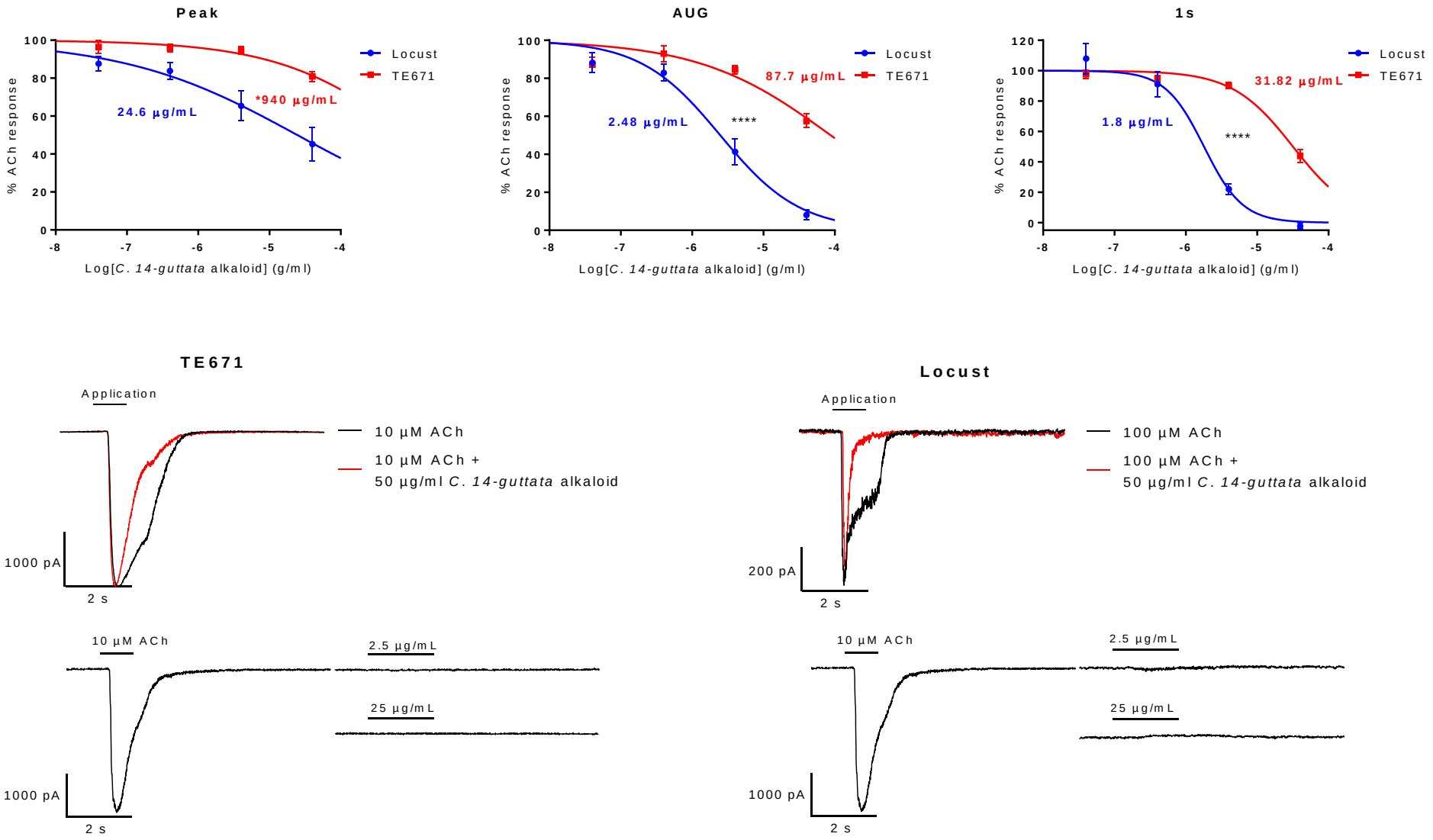


Figure 4.2.2 (Above) Comparative IC_{50} curves showing the inhibition of ACh induced currents in human muscle-type (red, N = 12) and locust neuronal nAChRs (blue, N = 8) by *C. quatuordecimguttata* alkaloid extract. IC_{50}s are shown and asterisks represent the p-value calculated by a sum-of-squares F-test. (Centre) Representative traces showing the inhibitory action of 50 µg/mL *C. quatuordecimguttata* alkaloid extract towards human muscle type (left) and locust neuronal nAChRs (right). (Below) Traces showing the effect of applying 2.5 and 25 µg/mL *C. quatuordecimguttata* alkaloid extract in the absence of ACh. No effect was seen in either model. V_H = -75mV for all experiments shown and error bars show ±SEM. * = IC_{50} value extrapolated from curve.

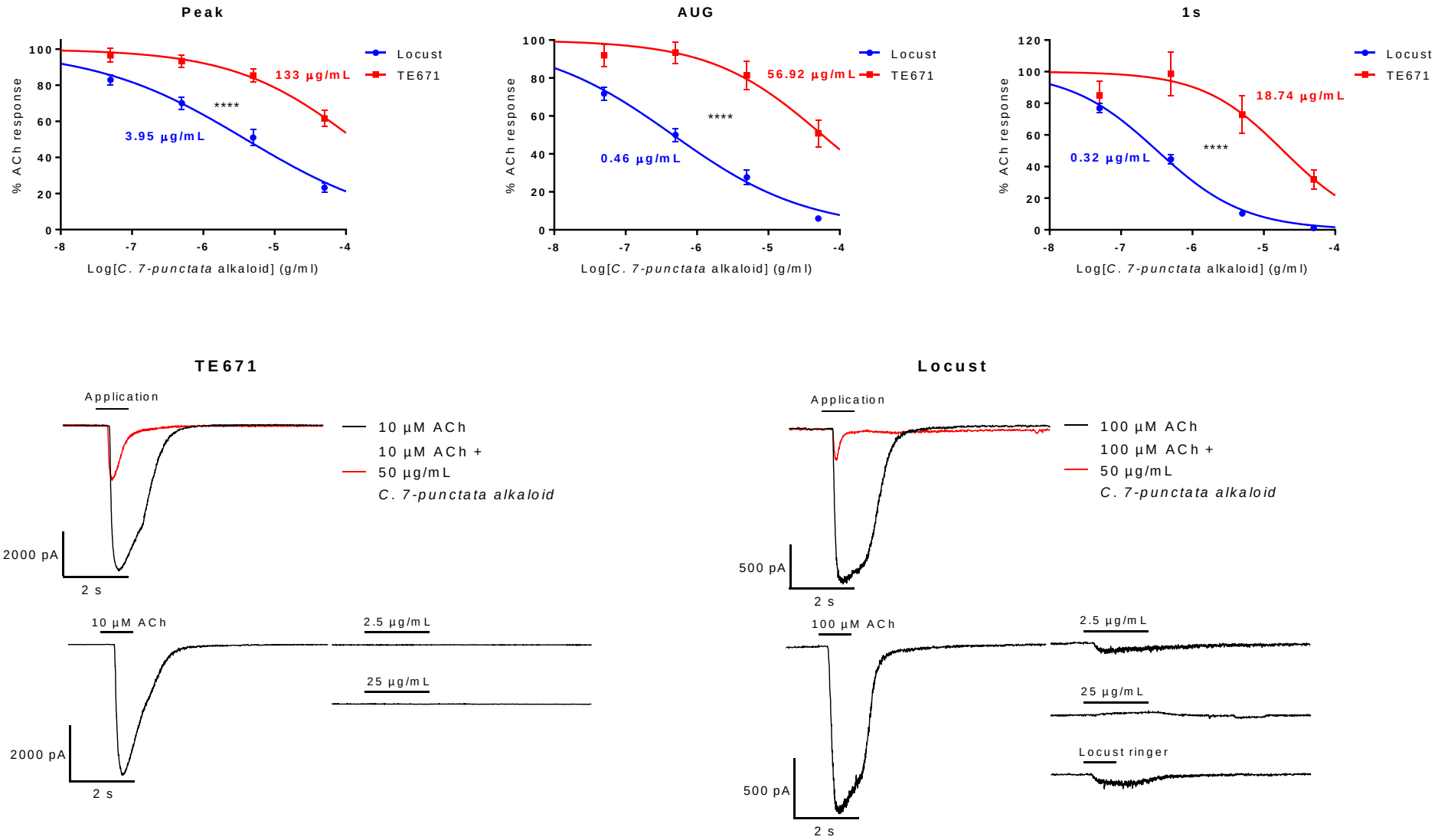


Figure 4.2.3 (Above) Comparative IC_{50} curves showing the inhibition of ACh induced currents in human muscle-type (red, N = 12) and locust neuronal nAChRs (blue, N = 16) by *C. septempunctata* alkaloid extract. IC_{50}s are shown and asterisks represent the p-value calculated by a sum-of-squares F-test. (Centre) Representative traces showing the inhibitory action of 50 µg/mL *C. septempunctata* alkaloid extract towards human muscle type (left) and locust neuronal nAChRs (right). (Below) Traces showing the effect of applying 2.5 and 25 µg/mL *C. septempunctata* alkaloid extract in the absence of ACh. No effect was seen in either model. V_H = -75mV for all experiments shown and error bars show ±SEM.

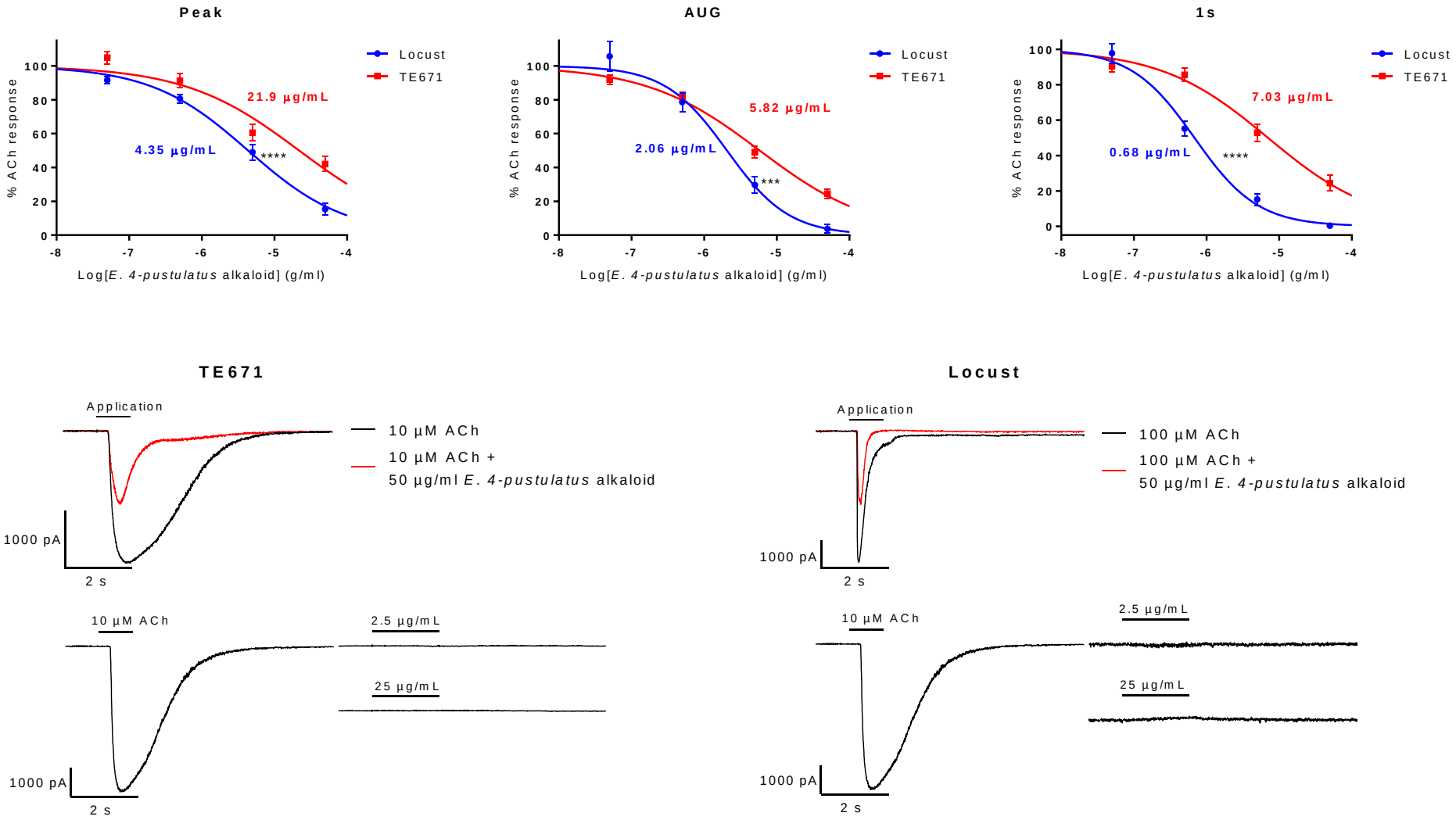


Figure 4.2.4 (Above) Comparative IC_{50} curves showing the inhibition of ACh induced currents in human muscle-type (red, N = 11) and locust neuronal nAChRs (blue, N = 15) by *E. quadripustulatus* alkaloid extract. IC_{50}s are shown and asterisks represent the p-value calculated by a sum-of-squares F-test. (Centre) Representative traces showing the inhibitory action of 50 µg/mL *E. quadripustulatus* alkaloid extract towards human muscle type (left) and locust neuronal nAChRs (right). (Below) Traces showing the effect of applying 2.5 and 25 µg/mL *E. quadripustulatus* alkaloid extract in the absence of ACh. No effect was seen in either model. V_H = -75mV for all experiments shown and error bars show ±SEM.

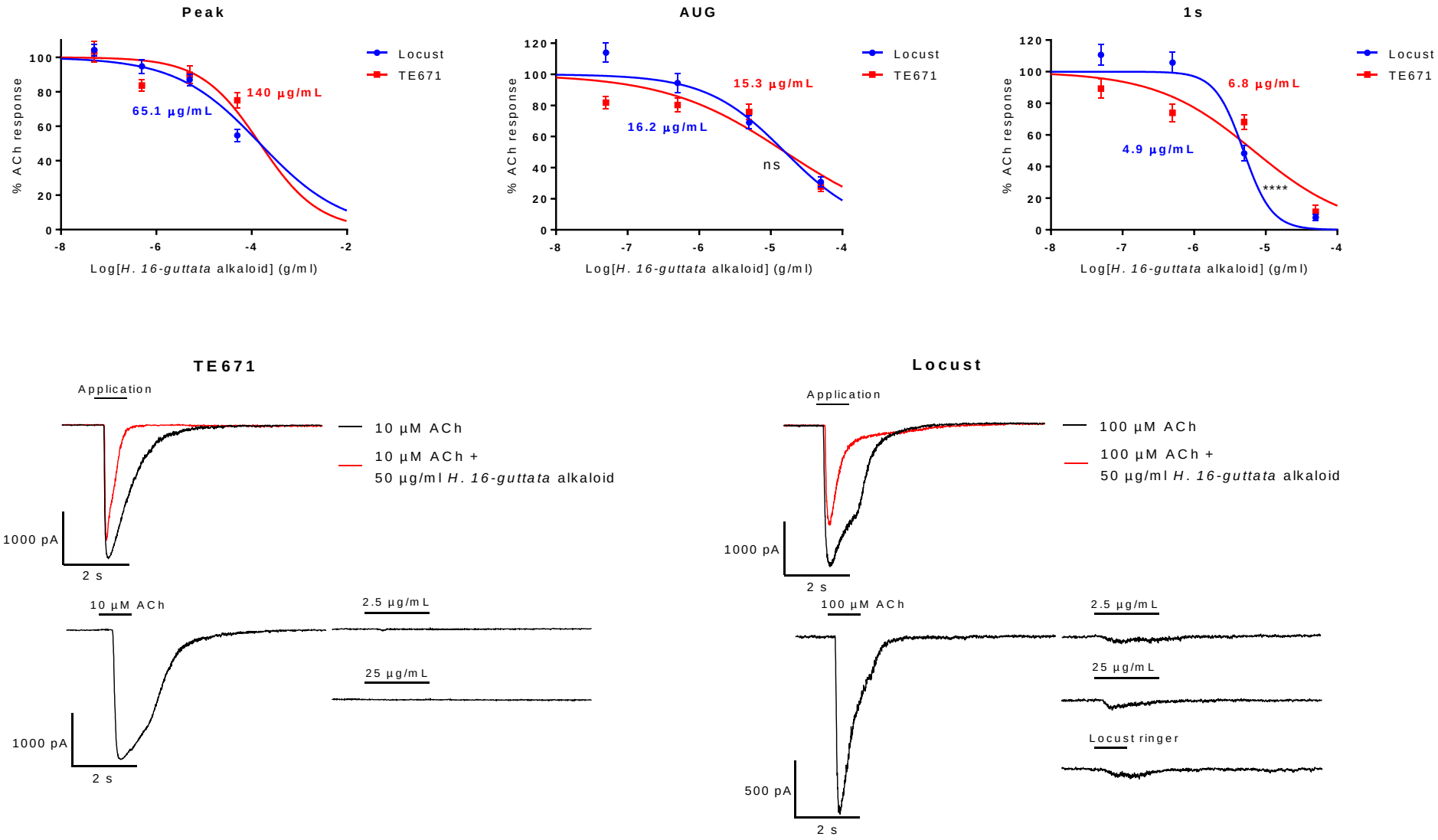


Figure 4.2.5 (Above) Comparative IC_{50} curves showing the inhibition of ACh induced currents in human muscle-type (red, N = 11) and locust neuronal nAChRs (blue, N = 16) by *H. sedecimguttata* alkaloid extract. IC_{50}s are shown and asterisks represent the p-value calculated by a sum-of-squares F-test. (Centre) Representative traces showing the inhibitory action of 50 µg/mL *H. sedecimguttata* alkaloid extract towards human muscle type (left) and locust neuronal nAChRs (right). (Below) Traces showing the effect of applying 2.5 and 25 µg/mL *H. sedecimguttata* alkaloid extract in the absence of ACh. No effect was seen in either model. V_H = -75mV for all experiments shown and error bars show ±SEM.

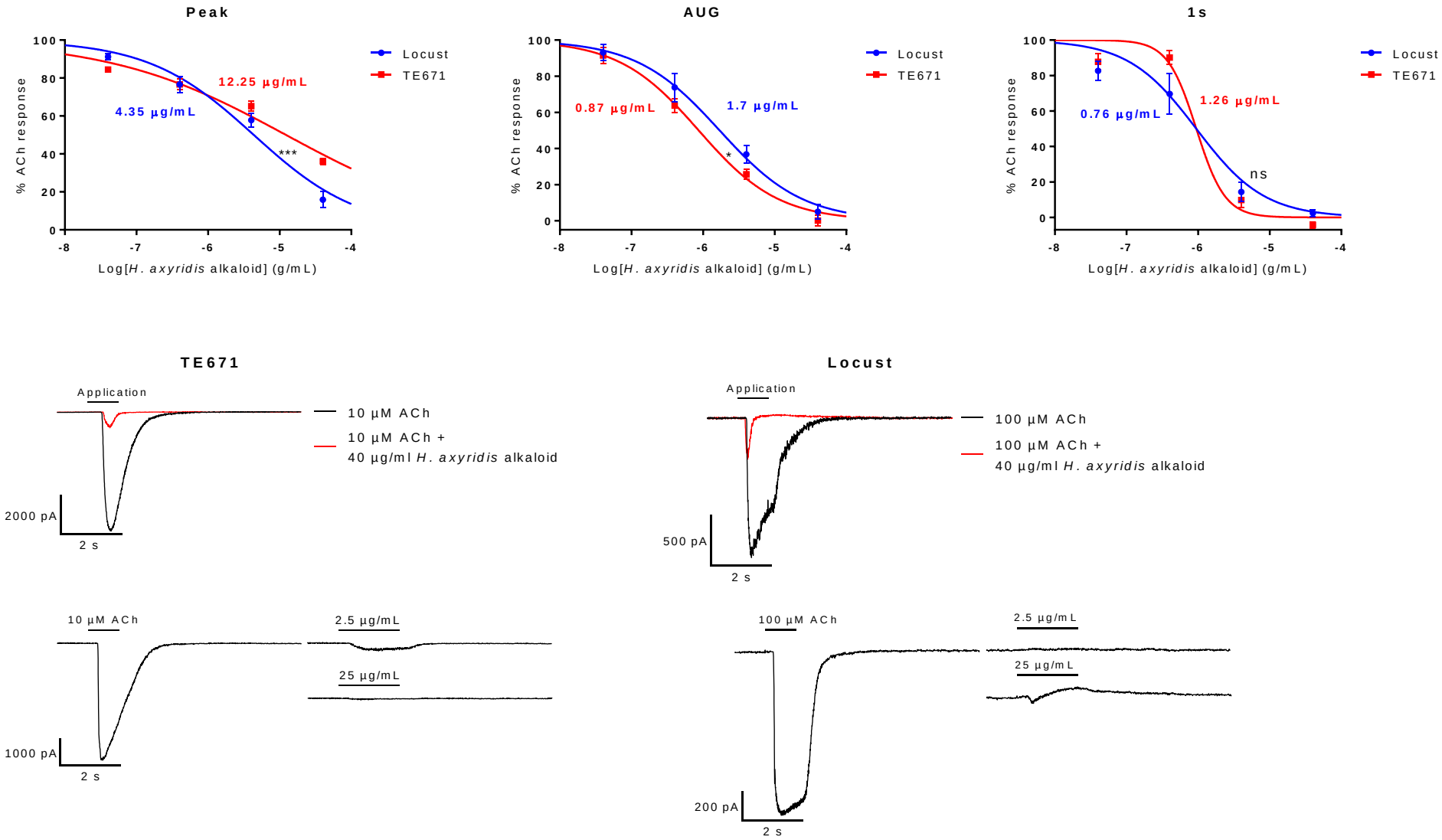


Figure 4.2.6 (Above) Comparative IC_{50} curves showing the inhibition of ACh induced currents in human muscle-type (red, N = 10) and locust neuronal nAChRs (blue, N = 10) by *H. axyridis* alkaloid extract. IC_{50}s are shown and asterisks represent the p-value calculated by a sum-of-squares F-test. (Centre) Representative traces showing the inhibitory action of 40 µg/mL *H. axyridis* alkaloid extract towards human muscle type (left) and locust neuronal nAChRs (right). (Below) Traces showing the effect of applying 2.5 and 25 µg/mL *H. axyridis* alkaloid extract in the absence of ACh. A small inward current is seen in TE671 cells at low concentration, and a small inward followed by an outward current is seen in the locust neuron at high concentration. V_H = -75mV for all experiments shown and error bars show ±SEM.

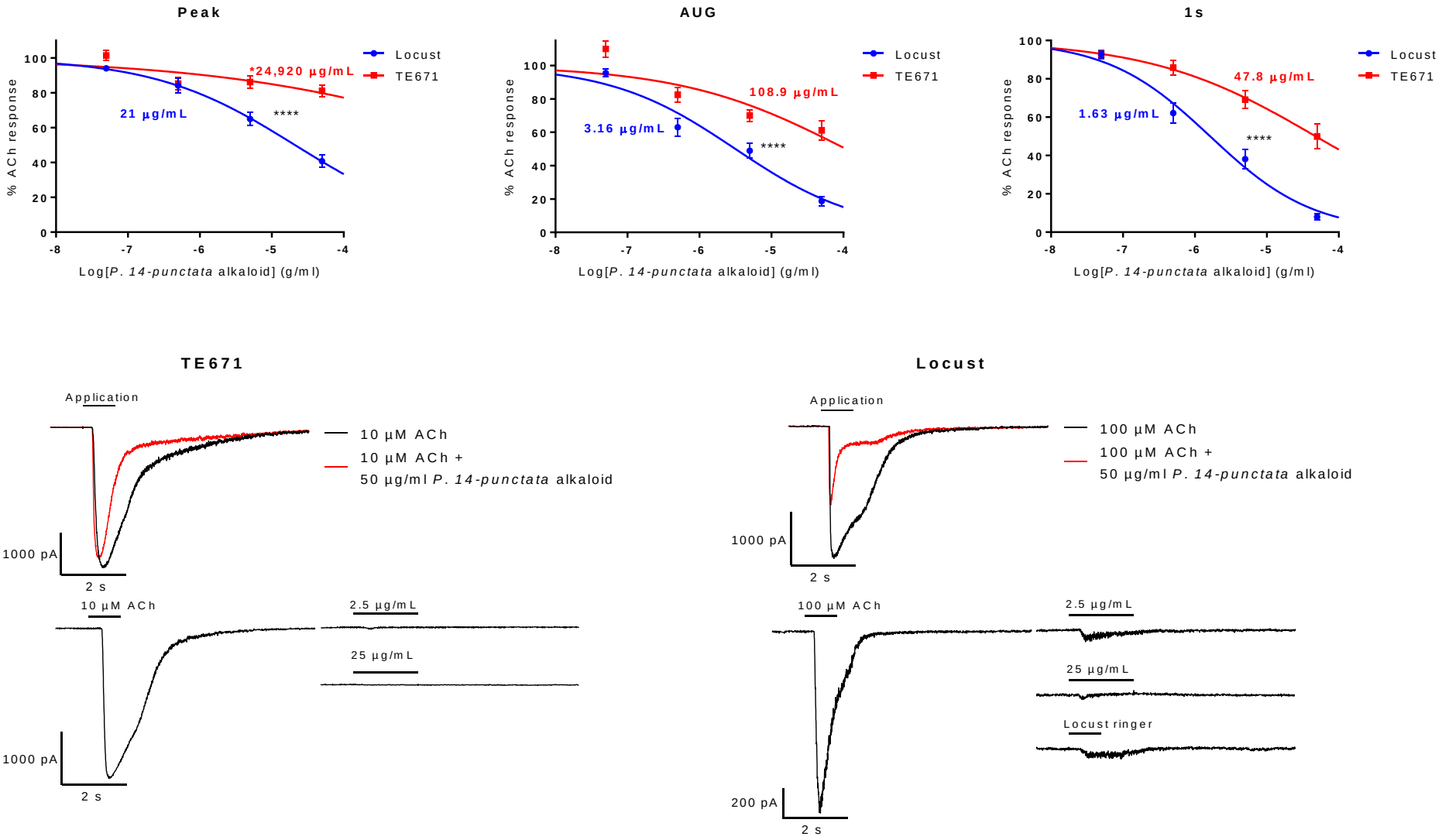


Figure 4.2.7 (Above) Comparative IC_{50} curves showing the inhibition of ACh induced currents in human muscle-type (red, N = 16) and locust neuronal nAChRs (blue, N = 12) by *P. quatuordecimpunctata* alkaloid extract. IC_{50}s are shown and asterisks represent the p-value calculated by a sum-of-squares F-test. (Centre) Representative traces showing the inhibitory action of 50 µg/mL *P. quatuordecimpunctata* alkaloid extract towards human muscle type (left) and locust neuronal nAChRs (right). (Below) Traces showing the effect of applying 2.5 and 25 µg/mL *P. quatuordecimpunctata* alkaloid extract in the absence of ACh. No effect was seen in either model. V_H = -75mV for all experiments shown and error bars show ±SEM. * = IC_{50} value extrapolated from curve

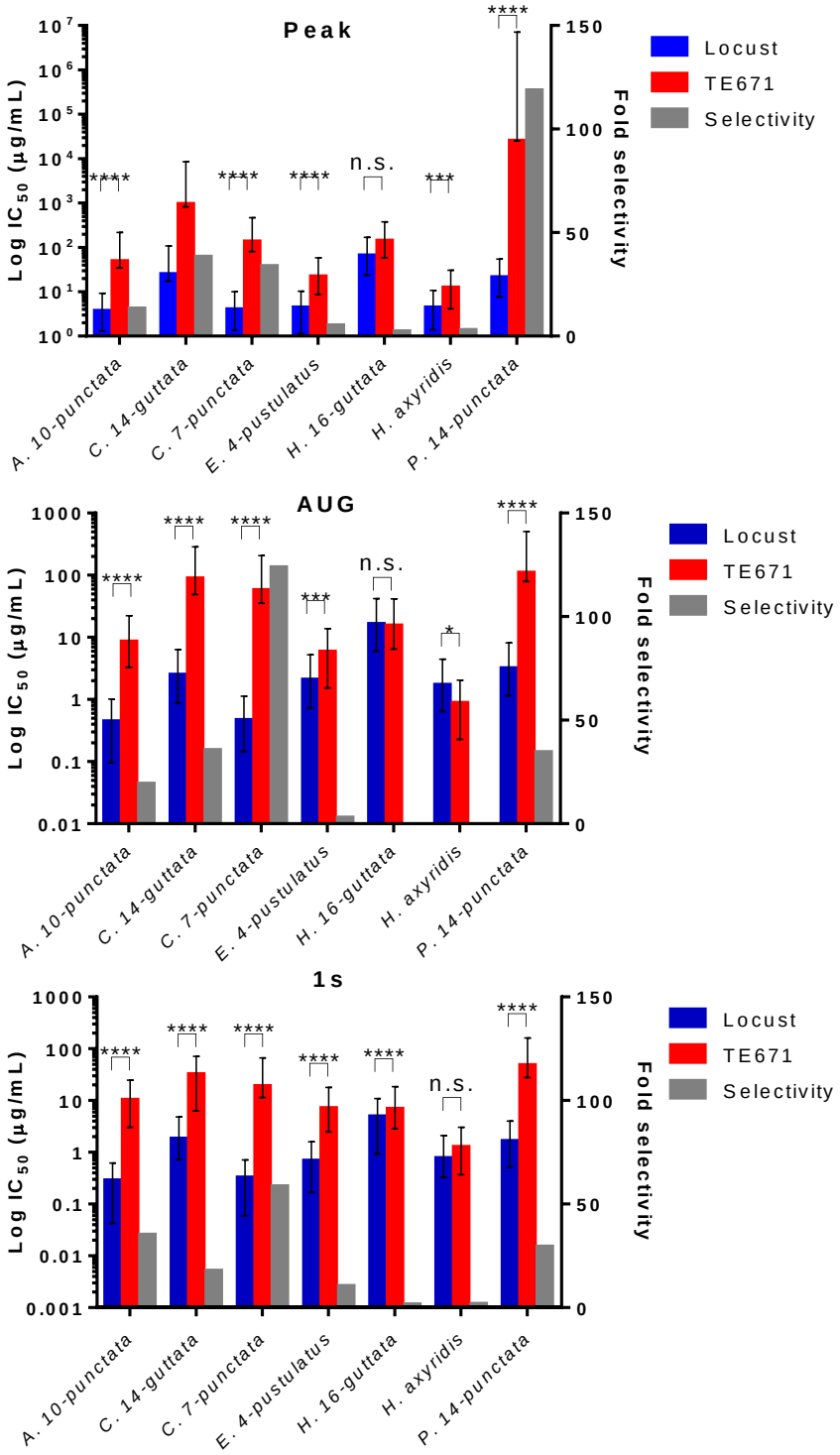


Figure 4.2.8 Column charts showing the IC_{50} ±95% CL calculated for alkaloid extracts from seven ladybird species on human muscle-type (red) and locust neuronal nAChRs (blue). The selectivity of each extract (the difference in IC_{50} values) is shown in grey. Asterisks indicate whether the IC_{50}s are significantly different for locust over TE671 nAChRs, calculated by sum-of-squares F-test. * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001 and **** = P ≤ 0.0001. IC_{50}s could not be compared for the peak inhibition caused by *C. 14-guttata* because the TE671 IC_{50} was calculated from an extrapolated curve and the 95% confidence intervals were too wide.

	C. 7-punctata	*C. 14-guttata*	*E. 4-pustulatus*	*H. 16-guttata*	*H. axyridis*	*P. 14-guttata*
A. 10-punctata		****	****	****	****	****
C. 7-punctata		****		****		****
C. 14-guttata			***		**	
E. 4-pustulatus				****		**
H. 16-guttata					****	*
H. axyridis						*** *

Table 4.2.1. Showing the results of one-way ANOVA pair-wise comparisons the peak IC_{50}s calculated against locust nAChRs (blue) and TE671 cells expressing human muscle-type nAChRs (red). Asterisks indicate whether the IC_{50}s calculated for two species were significantly different. If there was no significant difference between IC_{50} values, the box was left blank * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001 and **** = P ≤ 0.0001.

	C. 7-punctata	*C. 14-guttata*	*E. 4-pustulatus*	*H. 16-guttata*	*H. axyridis*	*P. 14-guttata*
A. 10-punctata		**** **	****	****	*** *	**** ***
C. 7-punctata		****	**** **	****	*** ****	****
C. 14-guttata			****	**** *	****	
E. 4-pustulatus				****	*	****
H. 16-guttata					**** ***	**** **
H. axyridis						****

Table 4.2.2. Showing the results of one-way ANOVA pair-wise comparisons of the AUG IC_{50}s calculated against locust nAChRs (blue) and TE671 cells expressing human muscle-type nAChRs (red). Asterisks indicate whether the IC_{50}s calculated for two species were significantly different. If there was no significant difference between IC_{50} values, the box was left blank * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001 and **** = P ≤ 0.0001.

	C. 7-punctata	*C. 14-guttata*	*E. 4-pustulatus*	*H. 16-guttata*	*H. axyridis*	*P. 14-guttata*
A. 10-punctata		****	***	****	*** ***	**** **
C. 7-punctata		****	**	****	** ****	****
C. 14-guttata			**	*** **	* ****	
E. 4-pustulatus				****	**	** ****
H. 16-guttata					**** **	**** ****
H. axyridis						* ****

Table 4.2.3. Showing the results of one-way ANOVA pairwise comparisons of the steady-state IC_{50}s calculated against locust nAChRs (blue) and TE671 cells expressing human muscle-type nAChRs (red). Asterisks indicate whether the IC_{50}s calculated for two species were significantly different. If there was no significant difference between IC_{50} values, the box was left blank * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001 and **** = P ≤ 0.0001.

Extract	Locust IC_{50} µg/mL (95% CL) [N]	TE671 IC_{50} µg/mL (95% CL) [N]
A. 10-punctata	3.66 (2.38-5.63) [15]	48.42 (13.85-169.3) [10]
C. 7-punctata	3.95 (2.58-6.04) [16]	133.0 (53.24-332.4) [12]
C. 14-guttata	24.64 (7.35-82.56) [8]	*940.1 (117.6-7515) [12]
E. 4-pustulatus	4.35 (3.21-5.89) [15]	21.9 (13.1-36.59) [11]
H. 16-guttata	65.1 (41.58-101.9) [16]	140.0 (82.15-238) [11]
H. axyridis	4.35 (2.97-6.38) [10]	12.25 (8.15-18.42) [10]
P. 14-guttata	21.01 (13.35-33.1) [12]	*24,920 (88.18-7042,000) [16]

Table 4.2.4 Peak IC_{50} values calculated for ladybird alkaloid extracts at V_H = - 75 mV against the ACh response of locust neurons and TE671 cells expressing M-nAChRs. Increasing concentrations of alkaloid extracts were co-applied with 100 µM (locust) and 10 µM (TE671) ACh. The responses to alkaloid were normalised against the control response and an IC_{50} curve constructedl and fitted with the 4PL equation. 95 % CL are shown in brackets and the number of cells used to create each graph shown in square brackets. * IC_{50} derived from extrapolated curve.

Extract	Locust IC_{50} µg/mL (95% CL) [N]	TE671 IC_{50} µg/mL (95% CL) [N]
A. 10-punctata	0.44 (0.35-0.57) [15]	8.46 (5.16-13.86) [10]
C. 7-punctata	0.46 (0.32-0.67) [16]	56.92 (21.76-148.9) [12]
C. 14-guttata	2.48 (1.61-3.83) [8]	87.73 (38.82-198.2) [12]
E. 4-pustulatus	2.1 (1.33-3.19) [15]	5.82 (4.29-7.91) [11]
H. 16-guttata	16.18 (10.22-25.63) [16]	15.29 (8.85-26.39) [11]
H. axyridis	1.7 (1.06-2.73) [10]	0.87 (0.64-1.18) [10]
P. 14-guttata	3.16 (2.01-4.98) [12]	108.9 (30.2-392.5) [16]

Table 4.2.5 AUG IC_{50} values calculated for ladybird alkaloid extracts at V_H = - 75 mV against the ACh response of locust neurons and TE671 cells expressing M-nAChRs. Increasing concentrations of alkaloid extracts were co-applied with 100 µM (locust) and 10 µM (TE671) ACh. The responses to alkaloid were normalised against the control response and an IC_{50} curve constructedl and fitted with the 4PL equation. 95 % CL are shown in brackets and the number of cells used to create each graph shown in square brackets.

Extract	Locust IC_{50} µg/mL (95% CL) [N]	TE671 IC_{50} µg/mL (95% CL) [N]
A. 10-punctata	0.29 (0.24-0.33) [15]	10.19 (7.15-14.51) [10]
C. 7-punctata	0.32 (0.26-0.39) [16]	18.74 (7.35-47.76) [12]
C. 14-guttata	1.8 (1.07-3.03) [8]	31.82 (25.52-39.66) [12]
E. 4-pustulatus	0.68 (0.52-0.91) [15]	7.03 (4.57-10.82) [11]
H. 16-guttata	4.89 (3.96-6.05) [16]	6.80 (3.95-11.71) [11]
H. axyridis	0.76 (0.43-1.35) [10]	1.26 (0.89-1.78) [10]
P. 14-guttata	1.63 (1.11-2.39) [12]	47.81 (20.0-114.3) [16]

Table 4.2.6 1 s IC_{50} values calculated for ladybird alkaloid extracts at V_H = - 75 mV against the ACh response of locust neurons and TE671 cells expressing M-nAChRs. Increasing concentrations of alkaloid extracts were co-applied with 100 µM (locust) and 10 µM (TE671) ACh. The responses to alkaloid were normalised against the control response and an IC_{50} curve constructedl and fitted with the 4PL equation. 95 % CL are shown in brackets and the number of cells used to create each graph shown in square brackets.

4.3 Discussion

4.3.1 Comparing the Alkaloid Content of Ladybird Species

Eight species of ladybird were collected in sufficient numbers to allow alkaloid extraction. Thin-layer chromatography and subsequent staining with Dragendorff's reagent (Figure 4.1.1) indicated that each extract contained alkaloid, but the quantity and composition varied between species (Figure 4.1.2), with extremes of 9.1 and 90.5 μg/beetle (cream spot and harlequin ladybird respectively) but most species averaged 35-60 μg/beetle. When body mass was factored in, it was clear that smaller ladybird species tended to have higher alkaloid content relative to body size than larger species, and this accounts for the consistent average alkaloid content. For example the 7-spot ladybirds used in this study had a mean body mass 5.6 times greater than the 2-spot ladybirds, but the mean alkaloid per beetle values were very similar (53.69 and 58.44 μg/beetle respectively). This may represent a critical quantity of alkaloid that is required to achieve toxicity to predators, or possibly the maximum that each beetle can tolerate without suffering self-toxicity. The exception to this was the cream spot ladybird which has both low body mass and alkaloid content. This species is notably different to the other species included in this study in that it does not appear to display aposematic elytra colouration. Larch ladybirds are similarly non-aposematic and extracts from this species were found to be the least toxic to *Daphnia* (Arenas *et al*, 2015). It has been suggested that ladybirds may use elytra colouration as an honest signal of chemical defence (Bezzerides *et al*, 2007; Blount *et al*, 2012; Winters *et al*, 2014; Wheeler *et al*, 2015). It has been shown that alkaloid quantity correlates with the proportion of red colouration displayed by non-melanic harlequin ladybirds (Bezzerides *et al*, 2007), precoccinelline levels are positively correlated with elytra

carotenoid concentration in the 7-spot ladybird (Blount *et al*, 2012) and that red convergent ladybirds (*Hippodamia convergens*) contain significantly higher quantities of alkaloid than their orange counterparts (Wheeler *et al*, 2015). The three species with the highest alkaloid content in this study all display similar red or orange and black colouration whereas those with the lowest mean alkaloid content, the cream spot ladybird lacks aposematic colouration, indicating that the use of aposematic elytra colouration as an honest signal of chemical defence may be an interspecific phenomenon. Unfortunately it was not possible to investigate individual differences in alkaloid content in this study, but comparing the quantity of alkaloid present in different individuals of polymorphic species, such as the harlequin, 2-spot and 10-spot ladybird would reveal whether elytra colouration is truly acting as an indicator of alkaloid content and possibly chemical defence status. The 10-spot ladybirds used in this study consisted of individuals possessing aposematic and non-aposematic colouration. Determining the alkaloid content of each colour form would reveal whether colouration is used as signal of alkaloid status, an attempt at mimicry of more alkaloid rich species or unrelated to alkaloid content. These data must be treated with caution because they are the result of single extractions and it is not known what proportion of alkaloid was retained during methanol extraction and acid-base separation or how variable alkaloid content may be between individuals, sexes, age classes and between seasons. Furthermore, some species were more inclined to reflex-bleed after capture and may have exhausted stores of defensive fluid, causing alkaloid to be lost prior to freezing and extraction. The 7-spot ladybird is known to lose up to 23.7 % of fresh body weight in defensive fluid (Holloway *et al*, 1991). A more

accurate way to address this question would be to capture ladybirds and freeze them instantly. Gas-chromatography coupled with mass spectrometry could then be used to quantify the alkaloid content of individual beetles. Unfortunately the alkaloid would not be recoverable using this procedure, so it was not possible to use it in this instance.

4.3.2 Screening Ladybird Alkaloid Extracts for Nicotinic Acetylcholine Receptor Activity

Alkaloid extracts from seven of the eight species were used for patch-clamp experiments. Both 2-spot and 10-spot ladybirds produce the same alkaloids (Lognay *et al*, 1996) and it was not deemed necessary to test both for nAChR activity. All seven of the alkaloid extracts tested caused concentration dependent inhibition of ACh induced currents in both TE671 cells and locust neurons (Figures 4.2.1– 4.2.7) adding weight to the theory that ladybirds are toxic and alkaloids play some role in chemical defence (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). The potency and selectivity of alkaloid extracts varied considerably between species (Figure 4.2.8). Five of the seven species showed significantly greater antagonism of locust nAChR than human muscle-type in all three current parameters (Figures 4.2.8), these were: 10-spot, 7-spot, 14-spot, cream spot and pine ladybirds, and species that showed high potency towards locust nAChRs didn't necessarily correspond to those that showed high potency towards human M-nAChRs. The alkaloid extracts of the 10-spot, 7-spot, harlequin and pine ladybird all showed high inhibitory activity towards locust nAChRs, with IC_{50}s ranging between 0.29–0.76 µg/mL for late current and 3.7–4.4 µg/mL for peak (net-charge IC_{50}s were

intermediate). Alkaloids isolated from the 14-spot and cream spot ladybird had considerably higher IC_{50}s (1.63 and 1.8 µg/mL for 1 s and 21 and 24.6 µg/mL for peak) with the orange ladybird the least potent, with 1 s and peak IC_{50}s of 4.9 and 65.1 µg/mL. This is in contrast to the results collected with TE671 cells where IC_{50} values were generally about one order of magnitude higher than those calculated for locust neurons. The exceptions to this were the alkaloid extract of the harlequin and orange ladybird (0.87–12.25 µg/mL and 6.8–140 µg/mL), which showed similar levels of activity in both locust and human nAChRs. The harlequin ladybird was found to be the most potent to human M-nAChRs, followed by the pine, 10-spot and orange ladybird. Alkaloids isolated from the cream-spot, 7-spot and 14-spot ladybird all showed very low activity against TE671 nAChRs and peak IC_{50}s had to be extrapolated from the curve (133-24,920 µg/mL and 18.75–47.8 µg/mL for 1 s). There does not appear to be an apparent correlation between body mass and potency to either insects or vertebrates. The differential potency toward the insect and vertebrate nAChRs used in this study may reflect the selective pressure imposed on ladybird species by invertebrate predators. Further experiments on nAChRs from other insects and vertebrates, as well as other possible molecular targets will need to be conducted to establish if this is the case. All but two of the extracts show consistent selectivity for locust over human nAChR but the most striking contrast in potency is shown by the alkaloid extract of the 7-spot ladybird, which shows 33.7–123.7 fold selectivity. It is known that the aposematic colouration and odour of this species is an effective deterrent to bird predation (Marples *et al*, 1994). It is also known that the eggs of this species are regularly predated upon by other ladybirds (Hemptinne *et al*, 2000; Sato &

Dixon, 2004; Ware & Majerus, 2008; Sloggett, 2009a; Sloggett, 2009b; Kajita *et al*, 2010), so it is possible that the comparatively high potency for locust nAChRs reflects selective pressure imposed by intraguild predation. Unfortunately, little is known about how well defended other ladybird species are from vertebrate predators but those that show higher insect potency may be under strong predation pressure from other insects, notably other ladybirds. The species that show the highest insect potency, the 10-spot, 7-spot, harlequin and pine ladybird are often found in close association, forming a 'guild' (Roy *et al*, 2011). Intraguild predation is likely to be a significant driver of defensive chemistry in these species, especially when other prey sources are scarce. The 14-spot ladybird is also a member of this guild of habitat generalists (Roy *et al*, 2011) but the alkaloid extract only showed intermediate insect potency in the patch-clamp assay. It could be that this species employs an alternative defensive mechanism, or the alkaloids have a molecular target other than the nAChR. This species is notable in the fact that the aposematic colouration displayed is yellow, unlike the other aposematic species included in this study, which are red or orange. It may be the case that this species has evolved to repel different predators and differences in aposematism are also reflected in defensive chemistry.

The species that show low insect potency, the cream spot and orange ladybird are arboreal specialists (Roy *et al*, 2011) and are less likely to encounter heterospecific predatory ladybirds. The appearance of the harlequin ladybird to the UK in 2004 (Majerus *et al*, 2006), a habitat generalist and ferocious predator (Cottrell & Yeargan, 1998; Ware & Majerus, 2008) may be a significant threat to these specialist species and possibly the reason that so few individuals were caught in this study. It may be the

case that predation by harlequin ladybirds will cause a change in the composition or quantity of toxins produced by these species, as is often the case in evolutionary 'arms races' where toxic prey is under strong predatory pressure. The fact that the harlequin ladybird is a habitat generalist (Ware & Majerus, 2008) and therefore may be likely to come into contact with a more diverse range of potential predators could account for the high potency that this species displayed towards both insect and human nAChRs. This calls into question the toxicity assay conducted by Vilcinskas *et al* (2013) whereby harmonine, the major alkaloid found in *H. axyridis* haemolymph was injected into 7-spot ladybirds and found to lack toxic activity. They concluded that harmonine was not toxic, whereas the results presented in Figure 4.2.6 clearly demonstrate high nAChR inhibitory activity, *in vitro*. It could be that 7-spot ladybirds are a poor model as regular intraguild predators that occur in sympatry with *H. axyridis* in Asia (Hironori & Katsuhiro, 1997), as they may have some level of resistance to heterospecific ladybird alkaloids or inherent resistance to their own alkaloids may confer cross resistance to harmonine.

The alkaloid extract of the orange ladybird showed comparatively low activity towards locust nAChRs and similar potency towards human nAChRs. Considering the apparent lower sensitivity of human nAChRs to the alkaloid extracts, the potency of the orange ladybird alkaloid extract was comparatively quite high to TE671 cells compared to those of other species. This relatively high vertebrate toxicity may be because the orange ladybird is often found in tree canopies (Roy *et al*, 2011), away from other ladybird species but presumably in close association with predatory birds, which also possess M-nAChR. As with harlequin ladybirds, this may impose a selective

pressure for higher vertebrate toxicity. Arenas *et al* (2015) used *Daphnia pulex* to test the toxicity of several ladybird extracts. They found the whole-beetle extract of the orange ladybird to be marginally more potent than 2-spot, pine or 14-spot ladybirds. It is possible that this species possesses another form of chemical defence other than alkaloids, or *Daphnia* may be a particularly susceptible model.

The degree to which alkaloid extracts affected different current parameters provides a basic insight into possible mode of action. The activity of alkaloid extracts against peak currents was variable; those of the 7-spot, pine and harlequin caused considerable inhibition of this current parameter whereas that caused by cream spot and orange ladybird alkaloid was minimal. The alkaloids of the 10-spot and 14-spot caused inhibition of locust nAChR peak responses but minimal inhibition of that of human M-nAChRs. This alternate action could suggest that different alkaloids are exploiting multiple binding sites, those that cause significant inhibition of peak responses may utilise an extracellular or shallow pore binding site. Those that cause minimal peak inhibition may rely on a deeper binding site that is only accessible in the activated channel. For all alkaloid extracts tested, the inhibition of net-charge and 1 s current was greater than that of peak current. The traces reveal that the action of alkaloids causes the ACh induced currents to return rapidly to baseline level. This is in contrast with the actions of both d-tbc (Figure 3.2.2) which diminished peak current response and other current parameters roughly in proportion. This would be expected of competitive antagonism, whereby the toxin is able to act on the closed conformation of the channel, competing with ACh for binding sites and causing fewer channels to activate. The inhibition caused by the ladybird alkaloid extracts tested

appears to act on the activated channel, suggestive of non-competitive action. The results suggest that either the alkaloids are binding within the open pore of the channel, preventing the flow of current or alternatively are causing a rapid desensitisation of the channel subsequent to activation by ACh. Leong *et al* (2015) concluded that the action of coccinelline, an alkaloid produced by the 7-spot ladybird was competitive with [3H]-TCP (which binds luminally) but not [3H]-cytisine (which binds orthosterically), indicating a non-competitive mode of action. The wasp polyamine toxin philanthotoxin-343 displays both open-channel block and enhancement of desensitisation by acting at two separate binding sites (Brier *et al*, 2003). Unfortunately it was not possible to elucidate the mode of action of all seven extracts because of very limited quantities available. Further work to determine the voltage-dependence of antagonism, whether binding is competitive with ACh and effects on single-channel conductance would clarify the mode of action and shed further light on the location of binding site/s. The sensitivity of other nAChR subtypes would also provide clues as to possible binding sites.

When applying alkaloids in the absence of ACh, to screen for agonist activity (Figures 4.2.1–4.2.7) only the harlequin ladybird alkaloid caused small inward currents in TE671 cells at a concentration of 2.5 µg/mL but this activity was not seen at the higher concentration of 25 µg/mL (Figure 4.2.6). This extract also generated small inward currents followed by an outward current in locust neurons (Figure 4.2.6), but only at a higher concentration of 25 µg/ mL. This activity was not seen with any other alkaloid extract. These small inward currents at high alkaloid concentrations could be caused by the cytotoxic activity that has been attributed to harmonine, the

predominant alkaloid produced by this species resulting in membrane damage (Alam *et al*, 2002; Röhrich *et al*, 2012). They may also be the result of activation of ion channels. The outward current is unlikely to be the result of nAChR modulation because of the negative holding potential and the fact that N-nAChRs display inward rectification (Haghigi & Cooper, 2000). A similar outward current can also be seen when high concentration *H. axyridis* alkaloid is co-applied with ACh (Figure 4.2.6). The inward current rapidly returns to baseline level but briefly an outward current is detectable. This could be caused by the action of potassium channels and this could be confirmed by using specific blockers or single-channel studies in potassium-free saline and electrode solutions. The locust neurons occasionally displayed inward currents simply as a response to perfusion with saline (Figures 4.2.1, 4.2.3, 4.2.5 & 4.2.7). These currents were considerably smaller than those generated in response to ACh exposure and are likely to be flow artefacts. These may have been caused by residual ACh in the perfusion manifold, the presence of mechanosensitive ion channels or disruption of the electrode seal. None of the alkaloid extracts generated currents larger than those caused by Ringer alone so were not deemed to cause receptor activation. These currents were not generated at higher concentration (25 μg/mL) of 10-spot, 7-spot and 14-spot extracts, this may have been because these were applied after the lower alkaloid concentration, and residual ACh may have been washed away. Alternatively, if these responses were the result of ion channel activation, a higher concentration may have caused desensitisation or channel block. The alkaloid extract of the orange ladybird did not block these responses at 25 μg/mL.

Chapter 5: Characterising the Nicotinic Acetylcholine Receptor Antagonism of Harlequin Ladybird Alkaloids

Introduction

Having identified nAChR inhibitory activity in a range of ladybird alkaloid extracts in Chapter 4, it was necessary to carry out further experiments to examine the mode of action. The work presented in the previous chapter identified the alkaloid extract of the harlequin ladybird as a potent inhibitor of both human muscle-type and locust neuronal nAChR. This species was abundant throughout summer months (unlike many of the other species shown in Chapter 4), invasive and potentially damaging to other ladybirds (Roy & Brown, 2015). It was therefore decided to focus upon the alkaloid extract of this species to further characterise the nAChR activity. To establish whether alkaloids were the main cause of nAChR antagonism, the inhibitory activity of the alkaloid extract was compared with a crude methanol extract. To establish mode of action, the voltage-dependence of inhibition was compared at five holding potentials ranging from +50 mV to -120 mV. An ACh concentration-response curve in the presence and absence of alkaloid was then used to determine whether the inhibition was competitive or non-competitive with ACh. Finally, the alkaloid extract was separated into fractions using HPLC, liquid chromatography and thin-layer chromatography and the inhibitory activity of fractions compared.

5.1 Comparing the nAChR Inhibitory Activity of Ladybird Methanolic and Alkaloid Extracts

To ensure that the alkaloids are indeed the major basis of nAChR inhibition, the activity of methanol extract was compared with that of purified alkaloid extract. The alkaloid was prepared as described previously, using acid-base separation. 2.4 g of

methanol extract resulted in 135 mg of alkaloid extract. Assuming that acid-base separation was 100 % effective in separating alkaloid from non-alkaloid constituents, the alkaloid represented 5.6 % of the total methanol extract, by mass. Both methanol extract and alkaloid extract were co-applied with 10 µM ACh, to TE671 cells at 10 and 100 µg/mL (Figures 5.1.1 and 5.1.2) at V_H = -75 mV. At a concentration of 10 µg/mL the alkaloid extract caused 53 % inhibition of the 1 s current whereas the methanol extract, (which would be expected to contain 0.56 µg/mL of alkaloid) did not cause any detectable inhibition. Co-application of 100 µg/mL alkaloid caused 79 % reduction in the 1 s current whereas the methanol extract (containing 5.6 µg/mL alkaloid) caused just 15 % reduction. Application of 10 and 100 µg/mL of both extracts without ACh to ACh responsive cells at V_H = -75 mV failed to elicit any inward or outward currents (Figure 5.1.3).

5.1.2 Insights Into the Mode of Action of *H. axyridis* Alkaloid Extract: Non-competitive Inhibition of ACh Induced Currents

To determine whether the action of *H. axyridis* alkaloid was competitive or non-competitive with ACh, a concentration-response curve in the absence and presence of alkaloid was constructed (Figure 5.2.1) as described for imidacloprid in Chapter 3, with an alkaloid concentration of 30 µg/mL. The results for all current measurements clearly indicate that the action of this alkaloid extract was non-competitive. The alkaloid EC_{50} curves plateau at 80 %, 30 % and 41 % of the equivalent peak, AUG and 1 s control, rather than shifting to the right, as would be expected of a competitive antagonist. The EC_{50} values were (control and in the presence of alkaloid respectively) 3.3 (95% CL = 2.61-4.29) and 6.31 µM (95% CL = 3.61-11) for peak, 5.48

(95% CL = 2.69-11.2) and 11.24 µM (95% CL = 9.34-13.5) for AUG and 1.48 (95% CL = 0.07-31.2) and 2.26 µM (95% CL = 1.6-3.19) for 1 s currents.

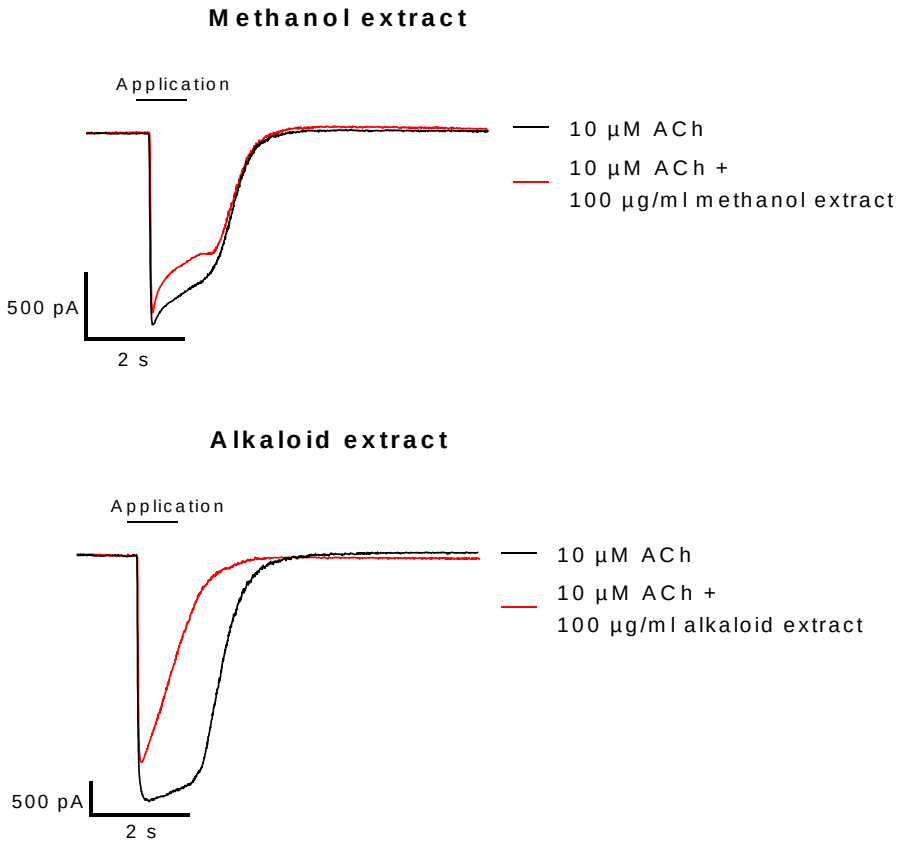


Figure 5.1.1 Representative traces showing inhibition of ACh induced currents by (left) 100 µg/mL *H. axyridis* methanol extract and (right) concentrated alkaloid extract in TE671 cells at V_H = -75 mV. The alkaloid extract causes considerably greater inhibition than the methanol extract.

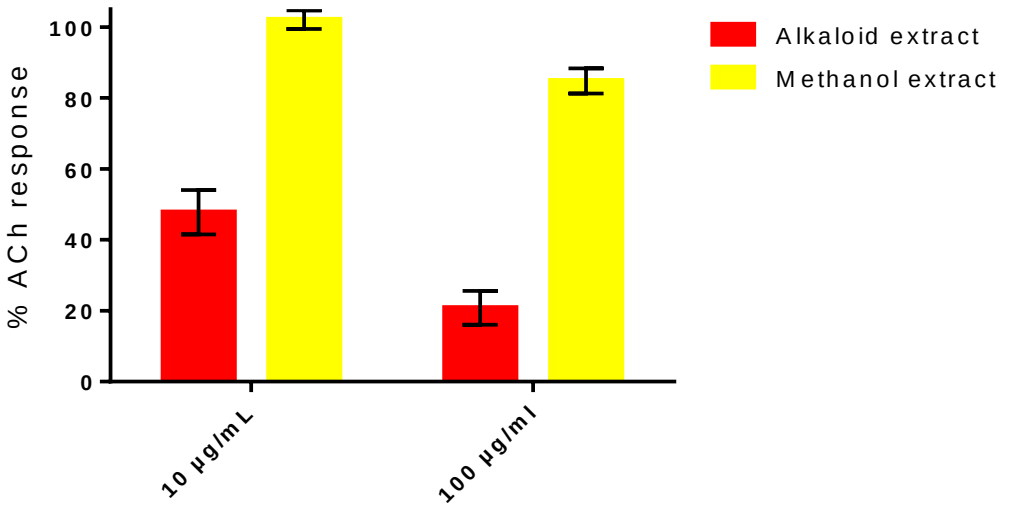


Figure 5.1.2 Column chart showing the inhibition of 1 s current by co-application of 10 µM ACh with 10 and 100 µg/mL of *H. axyridis* alkaloid (red) and methanol extracts at V_H = -75 mV. The alkaloid extract caused 53 and 79 % inhibition at 10 and 100 µg/mL respectively whereas the methanol extract caused 0 and 15 % inhibition, respectively. These data suggest that the alkaloids are the major cause of nAChR inhibition. Error bars indicate ±SEM. N = 11 and 6 for alkaloid and methanol extracts, respectively.

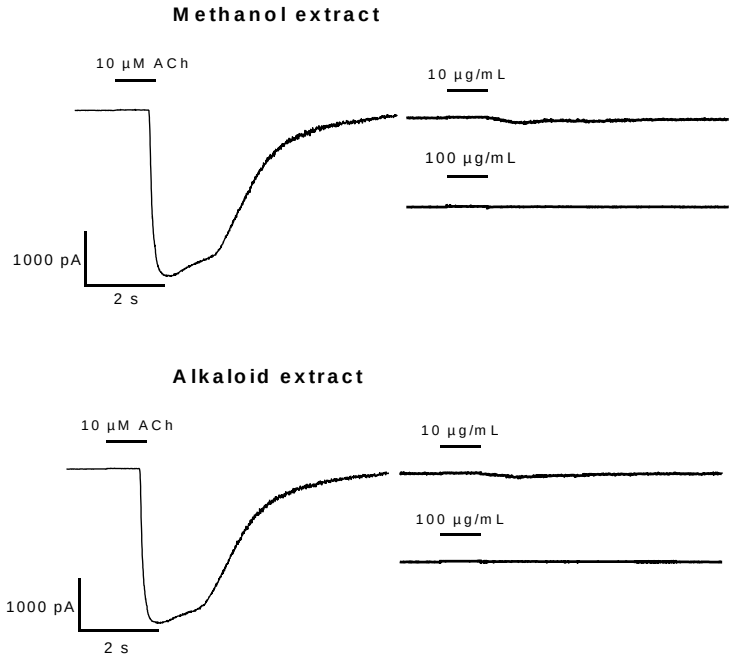


Figure 5.1.3. TE671 cells were whole-cell patch-clamped at V_H = -75 mV and perfused with 10 µM ACh to ascertain a nicotinic response. After a 60 s wash-off period, *H. axyridis* whole and alkaloid extracts were perfused over the cell for one second at concentrations of 10 and 100 µg/mL. No cells (N = 10) showed any response to either extract and either concentration.

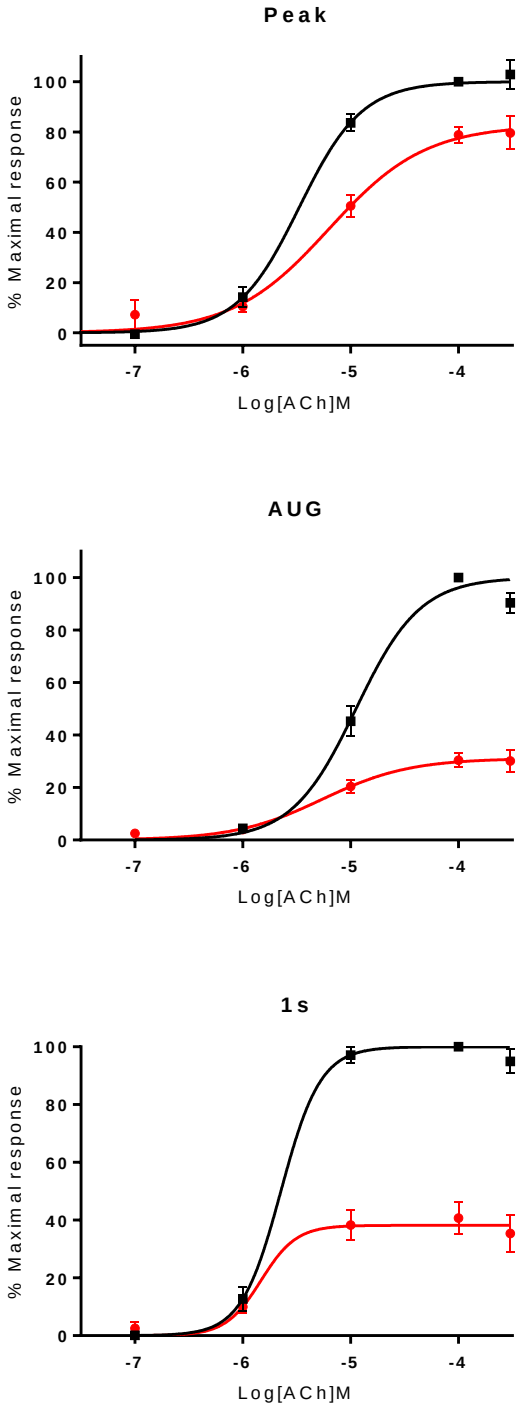


Figure 5.2.1. Concentration-response curves showing the current response of TE671 cells to increasing concentrations of ACh (black). The same ACh concentration range was co-applied with 30 µg/ml *H. axyridis* alkaloid extract and the results normalised against the control response which gave the largest response (red). The results show that the action of co-application of *H. axyridis* alkaloid with ACh causes the response to plateau at 80 %, 30 % and 41 % compared to ACh alone, rather than shift to the right, as would be expected if ACh was able to displace alkaloid binding. The EC_{50} values were (control and in the presence of alkaloid respectively) 3.3 and 6.31 µM for peak, 5.48 and 11.24 µM for AUG and 1.48 and 2.26 µM for 1 s currents. This is indicative of non-competitive antagonism. Error bars show ±SEM, V_H = -75mV and N = 12.

5.1.3 *H. axyridis* Alkaloid nAChR Inhibition is Voltage-Dependent

To determine whether the inhibitory activity of *H. axyridis* alkaloid was voltage-dependent, concentration-inhibition plots at a range of holding potentials were generated. The holding potentials were +50, -50, -75, -100 and -120 mV. TE671 cells were subjected to whole-cell patch-clamp and perfused with 10 µM ACh to generate a control response. Following a 60 s wash-off period, a single concentration of *H.* axyridis alkaloid was co-applied with ACh and current response measured. This procedure was repeated for the five holding potentials with alkaloid concentrations ranging from 0.1 – 100 µg/mL. Peak, AUG and 1 s currents were then normalised against control values and mean ±SEM were plotted and fitted with 4PL curves (Figure 5.2.2). The inhibition of peak current was very minor (15-20 % at 100 µg/mL) and accurate curves could not be fitted or IC_{50} values calculated. The inhibitory activity increased at greater membrane voltages and the IC_{50} values decreased concomitantly with holding potential (Table 5.2.1). IC_{50} values calculated for AUG and 1 s current were plotted against holding potential and a regression fitted with the Woodhull equation (Figure 5.2.3). The mixed composition of the alkaloid extract meant that an accurate $_z$ value could not be assigned, so $_z$ and δ were combined. The slope of the Woodhull-fitted regression differed significantly from zero (P = 0.0007 and 0.012 for AUG and 1 s respectively) indicating strong voltage-dependence of inhibition.

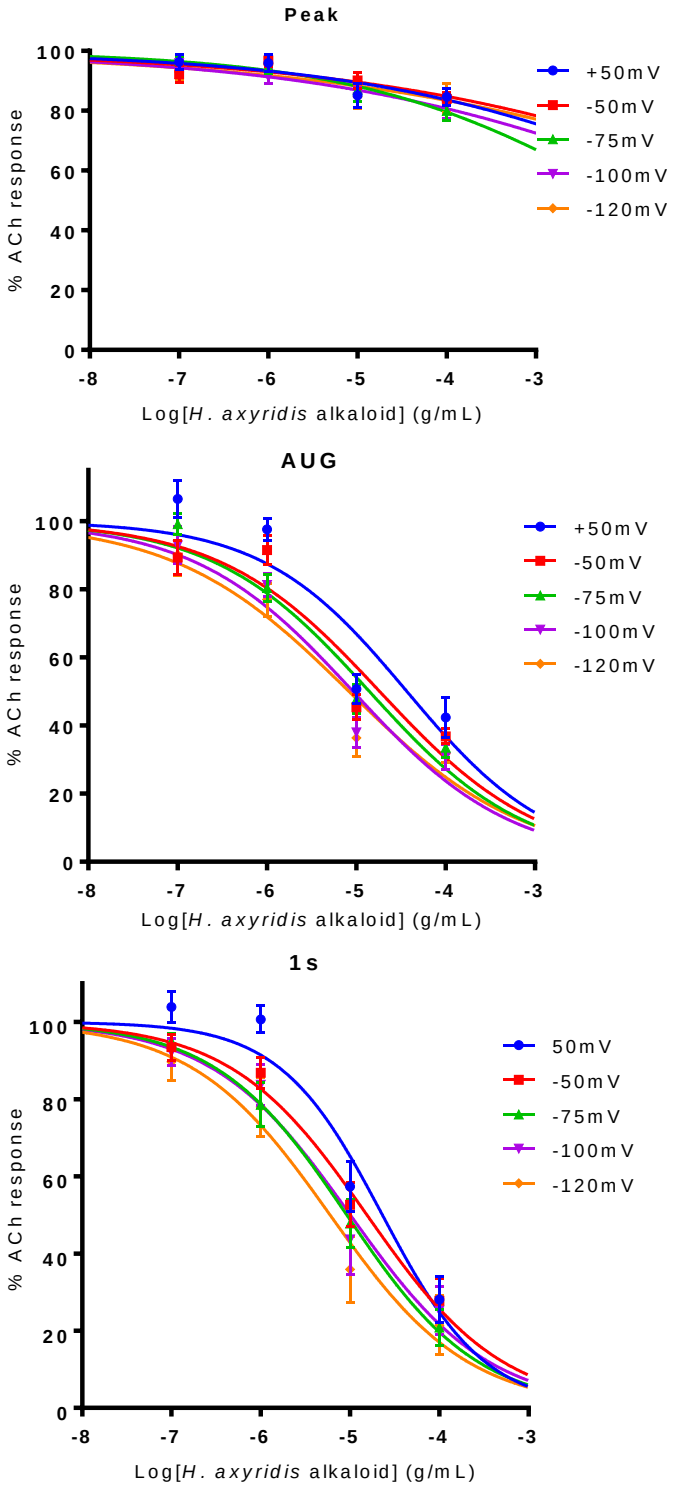


Figure 5.2.2 Concentration-inhibition plots showing the voltage-dependence of *H. axyridis* alkaloid inhibition of human muscle-type nAChRs. TE671 cells were clamped at holding potentials ranging from +50 to -120 mV and perfused with 10 µM ACh followed by co-application of 10 µM ACh and 0.1 – 100 µg/mL *H. axyridis* alkaloid. Responses were normalised against an ACh control and mean ±SEM plotted and fitted with 4PL regression curves. The inhibition of peak current was minimal, and it was not possible to accurately estimate IC_{50} values from these data. The results for AUG and 1 s clearly show greater inhibition at more negative holding potentials, indicative of voltage-dependent inhibition.

V_H (mV)	AUG IC_{50} µg/mL (95% CL) [N]	1s IC_{50} µg/mL (95% CL) [N]
+50	**36.9** (18.6-73.4) [7–11]	**23.1** (13.9-38.1) [7-11]
-50	**18.3** (10.5-31.7) [9-10]	**15.5** (9.4-25.4) [9-12]
-75	**14** (8.9-22) [8-12]	**9.2** (5.7-14.7) [8-12]
-100	**9.2** (5.4-15.7) [9-13]	**10.1** (5.3-19.3) [11-14]
-120	**8.3** (4.3-16.2) [7-11]	**6** (2.8-12.6) [8-11]

Table 5.2.1 IC_{50} values calculated for *H. axyridis* alkaloid extract at holding potentials ranging from +50 - -120 mV on TE671 cells expressing M-nAChRs. A single concentration of alkaloid extract in the range of 0.1 – 100 µg/mL was co-applied with ACh to a TE671 cell following a control response at five holding potentials. The response to alkaloid was normalised against the control response and an IC_{50} curve constructed for each holding potential and fitted with the 4PL equation (Figure 5.2.2). N shows the range of repeats used to generate each point used to fit the curve.

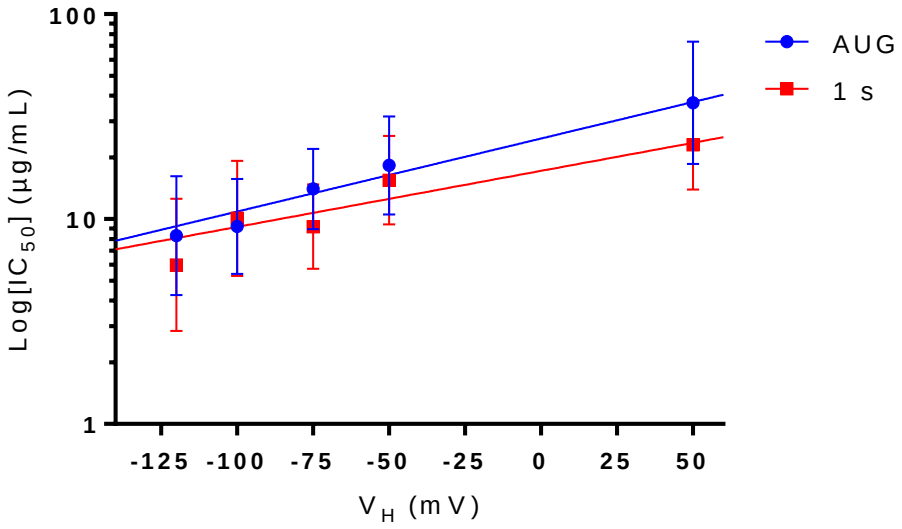


Figure 5.2.3 The IC_{50} values calculated for the AUG (blue) and 1s (red) current of *H. axyridis* alkaloid inhibition of human muscle-type nAChRs at holding potentials ranging from -120 - +50 mV. When fitted with the Woodhull equation ($_z$ and δ combined because of mixed composition of alkaloid extract), it is clear that the IC_{50} values decrease as holding potential becomes increasingly negative. The slope of both regression lines differ significantly from zero, P = 0.0007 and 0.012 for AUG and 1s respectively. This is indicative of voltage-dependent inhibition.

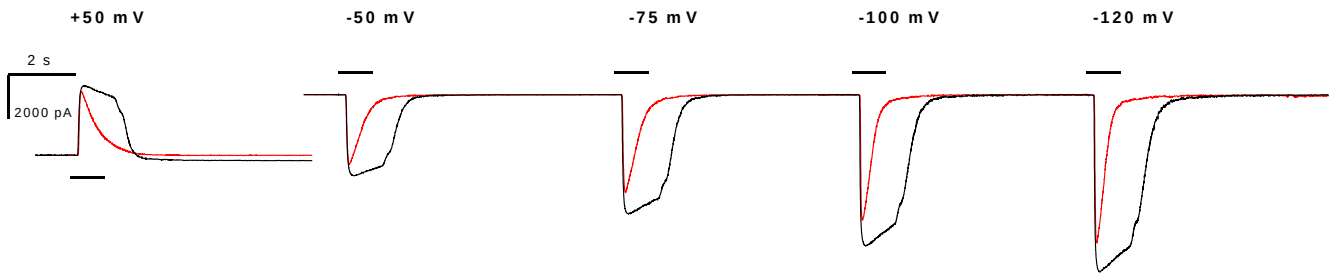


Figure 5.2.4 Example trace of *H. axyridis* alkaloid extract 100 µg/mL on TE671 at holding potentials ranging from +50 - -120 mV. Horizontal bars show the 1 s perfusion period. It can be seen that as holding potential becomes increasingly negative, the inhibition caused by alkaloid is greater.

5.2 Fractionating *H. axyridis* Alkaloid Extract

Mass spectrometry and thin-layer chromatography revealed that the *H. axyridis* alkaloid extract consisted of multiple compounds. The major alkaloid produced by the harlequin ladybird is harmonine (Braconnier *et al*, 1985) which has been shown to display antibiotic and antifungal activity (Röhrich *et al*, 2012, Vilcinskas *et al*, 2013) but no mechanism of action has been put forward. In order to isolate harmonine and other alkaloids, it was necessary to separate the alkaloid constituents. Various separation techniques were attempted, including: HPLC, liquid chromatography and thin-layer chromatography. The nAChR inhibitory activity and insect selectivity of fractions was then calculated by producing concentration-inhibition plots for nAChRs in both TE671 cells and locust neurons.

5.2.1 HPLC

Dried alkaloid extract was dissolved in 1:1 acetonitrile:water, added to a 250 x 4.6 mm column (ACE HPLC Columns) packed with C18 (octadecylsilane) at a concentration of 1 mg/mL and then subjected to reverse phased HPLC (Shimadzu SCL-10AVP, Shimadzu Instruments, Japan). The organic solvents used were either acetonitrile (CH_3CN) or methanol (CH_3OH) and the aqueous solvent was HPLC grade water with 0.1 % formic acid. The concentration of organic solvent increased from 5 to 100 % over fifty minutes, remained at 100 % for ten minutes before returning to 5 % for a further five minutes. The UV absorbance was measured as compounds eluted off the column at 215, 230, 254, 270, 280 and 360 nM. Unfortunately harmonine displayed very poor UV absorbance and increasing extract concentration to 33 mg/mL failed to overcome this problem. Therefore fractions containing this alkaloid could not be detected or collected using HPLC without employing secondary mass spectrometry.

This made HPLC an impractical method for separation of the alkaloids we wished to isolate.

5.2.2 Liquid chromatography

5 mg of alkaloid was dissolved in 1 mL of DCM and added to a 30 cm glass column that had been packed with 2 cm neutral aluminium oxide, activity grade I (Woelm Pharmaceuticals) and washed with DCM. Ten 1 mL fractions were collected in clean, glass vials and dried under nitrogen. The running solvent was then replaced with 4:1 DCM:methanol for the following ten fractions before being replaced again with methanol with 1 % ammonium hydroxide. When the resulting fractions were subjected to mass spectrometry (MS), thin-layer chromatography (TLC) and the nAChR inhibitory activity tested using patch-clamp, these fractions were found to be very similar in composition and subsequently rejected (data not shown). The procedure was repeated with 60 Å silica gel (Fluka) as the stationary phase and methanol with 1 % ammonium hydroxide as the running solvent, but this also failed to separate *H. axyridis* alkaloids to generate useful fractions.

5.2.3 Thin-Layer Chromatography

H. axyridis alkaloid extract was dissolved in methanol to a concentration of 5 mg/mL and 1 mL was loaded along a 20 cm^2 silica gel glass plate (Fluka) in a straight line, 2 cm from one edge, using a glass capillary tube. The plate was transferred to a 3000 mL Pyrex beaker with a small volume of methanol with 1 % ammonium hydroxide (below the loading level of the plate), covered and allowed to run for 4 cm. Two fractions were clearly visible (Figure 5.3.1) without staining, and Dragendorff's reagent confirmed that these fractions were discrete, one mobile and one that

remained at the loading level. The silica containing the two fractions was separately scraped off the plate using a flat measuring spatula. The silica was collected in a glass vial and extracted in methanol. The supernatant was removed to a clean glass vial and dried under nitrogen. The total quantity of each fraction recovered amounted to 560 µg of the mobile fraction (A) and 1.27 mg of the stationary fraction (B). Mass spectrometry revealed that the low solubility fraction (Fraction B) was harmonine whereas the mobile fraction (Fraction A) was a mixture of other, unidentified alkaloids with a low harmonine concentration (Figure 5.3.2). The remaining 3.17 mg of material was unaccounted for and may have been lost in the re-extraction procedure.

5.2.4 Comparing the nAChR Activity of *H. axyridis* Alkaloid Fractions

To assess whether harmonine was responsible for the inhibition of nAChR currents displayed by whole alkaloid extract, increasing concentrations of both TLC fractions A and B (harmonine) were co-applied with ACh to TE671 cells at V_H = -75 mV. Both fractions were also applied to locust neurons to determine whether either fraction shows selective potency for either human muscle-type or locust neuronal nAChRs. Responses were normalised against a control response and plotted in Figure 5.3.3. Both fractions showed inhibitory activity towards both TE671 cell and locust N-nAChRs. The inhibition of peak current by Fraction A was minimal, with locust and TE671 cell IC_{50}s respectively 90.2 (95% CL = 25.1-324.6) and 745 µg/mL (95% CL = 57.4-9669) (Figures 5.3.3 and 5.3.4). Fraction B showed greater activity with IC_{50}s of 5.3 (95% CL = 2.87-9.88) and 69.6 µg/mL (95% CL = 24.9-194.2) for locust and TE671 respectively. Fraction A showed very similar activity towards both human muscle-type and locust neuronal nAChRs against net-charge and late currents (Figure 5.3.5). The

locust and TE671 cell IC_{50}s were 15.94 (95% CL = 10.5-24.1) and 12.21 µg/mL (95% CL = 6.0-24.8) for net-charge and 3.23 (95% CL = 1.69-6.18) and 4.77 µg/mL (95% CL = 2.12-10.7) for 1 s current. Fraction B showed considerably greater inhibition of locust nAChR with net-charge IC_{50}s of 0.074 (95% CL = 0.04-0.13) and 4.16 µg/mL and 1s IC_{50}s of 0.072 (95% CL = 0.038-0.14) and 1.68 µg/mL (95% CL = 1.16-2.45) (locust and TE671 cells respectively). One-way ANOVA revealed that in all comparisons, Fraction B was significantly more potent to locust than human muscle-type nAChRs (Peak P = ≤0.05, AUG P = ≤0.0001 & 1 s P = ≤0.0001). It was also significantly more potent than Fraction A to either locust or human nAChRs (Peak P = ≤0.05, AUG P = ≤0.0001 & 1 s P = ≤0.0001). No other comparisons were statistically significant (Figure 5.3.4).

Neither fraction elicited a response when applied to TE671 cells patch-clamped at V_H = -75 mV but application of a high concentration (25 µg/mL) of Fraction B generated a small outward current in five of nine locust neurons (Figure 5.3.6).

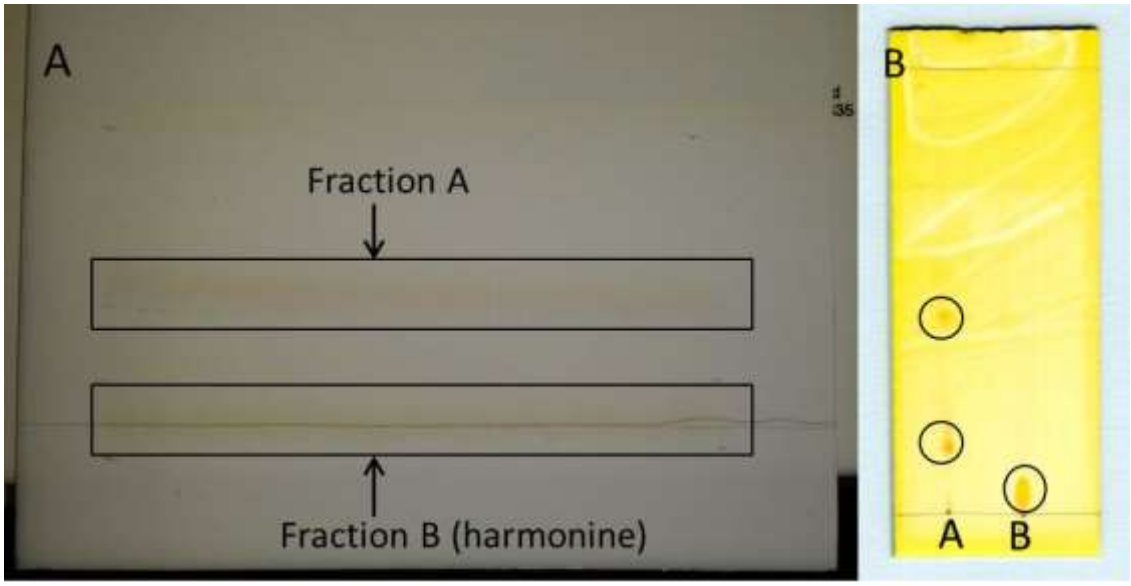


Figure 5.3.1 (A) Two fractions can be seen on the preparative scale silica plate after running a 5 mg/mL solution of *H. axyridis* alkaloid extract in methanol with 1 % ammonium hydroxide, Fraction A is mobile whereas fraction B, subsequently confirmed as harmonine using MS, remains at the loading line. **(B)** Shows a qualitative silica TLC plate spotted with fractions A and B, allowed to run in methanol with 1 % ammonium hydroxide and then stained with Dragendorff's reagent.

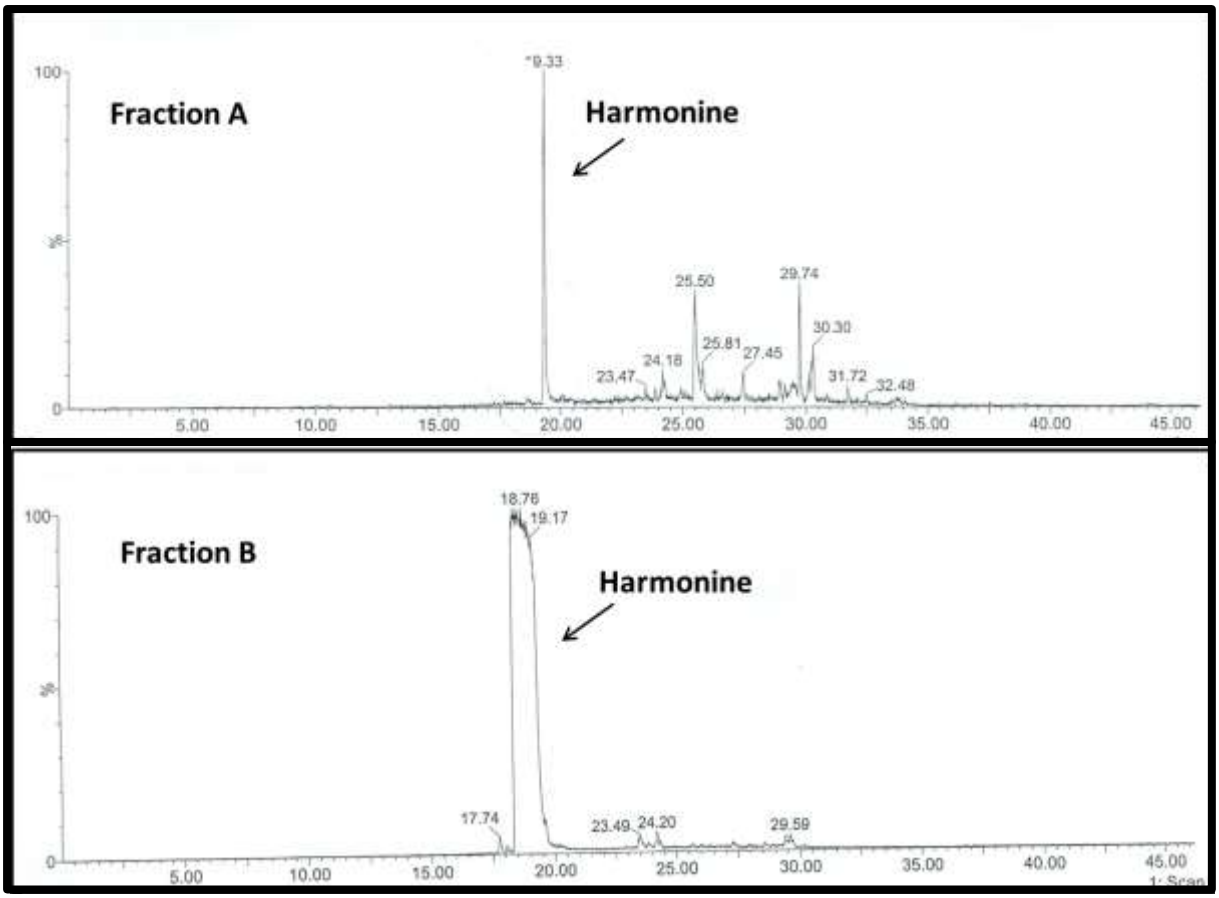


Figure 5.3.2 Mass spectrum for fractions generated by TLC. Harmonine is present in both fractions, but at much higher concentration in Fraction B. Mass spectrometry conducted by John Caulfield and Mike Birkett (Rothamsted Research).

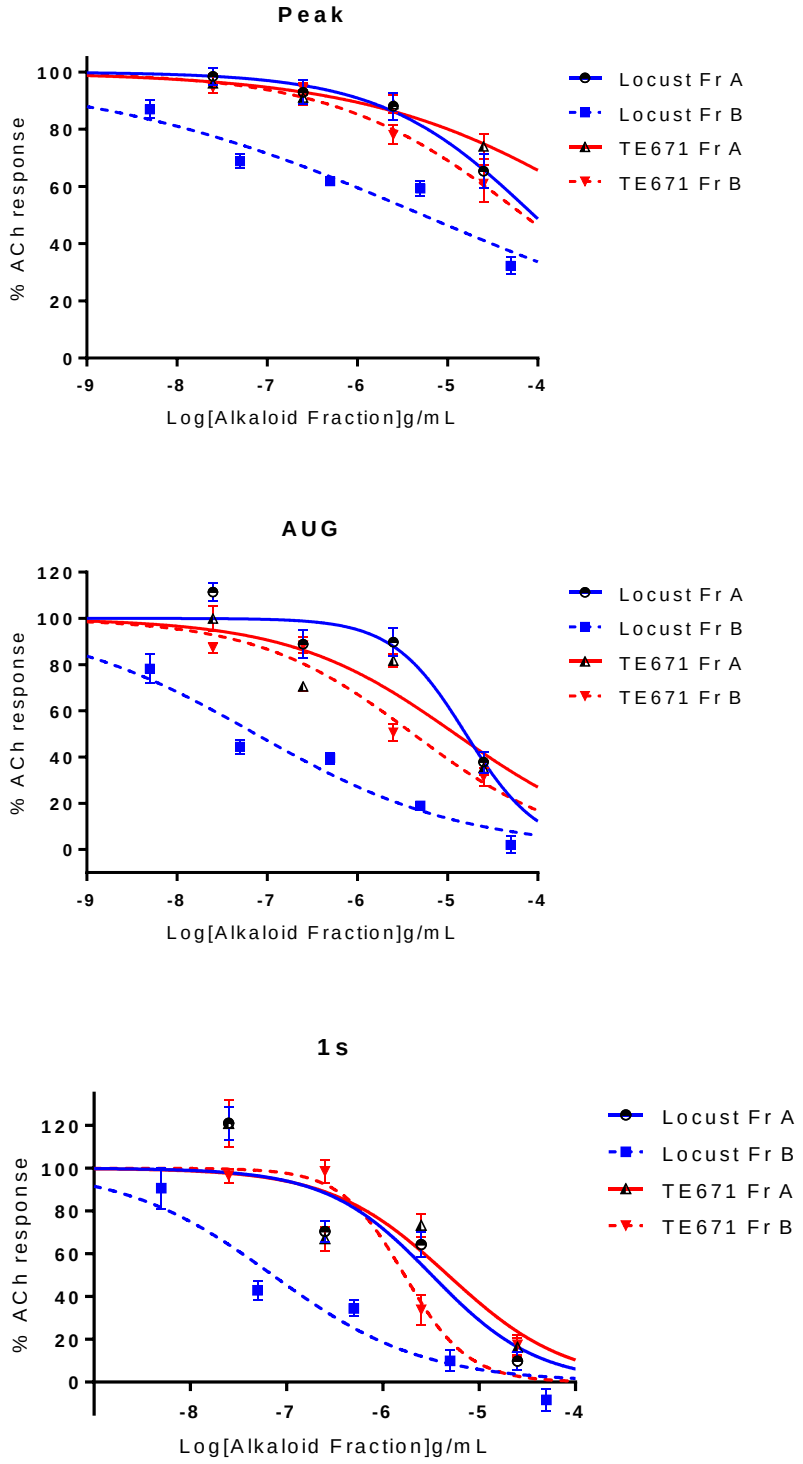


Figure 5.3.3 Increasing concentrations of Fractions A and B were individually co-applied with ACh to both TE671 cells (red) and locust neurons (blue) which had been clamped at V_H = -75 mV. The peak, net-charge and 1 s currents were measured and normalised against a control response. A 4PL curve was fitted to the points, from which IC_{50} values were derived. N = 10 (Locust Fr A), 9 (Locust Fr B), 9 (TE671 Fr A) and 12 (TE671 Fr B).

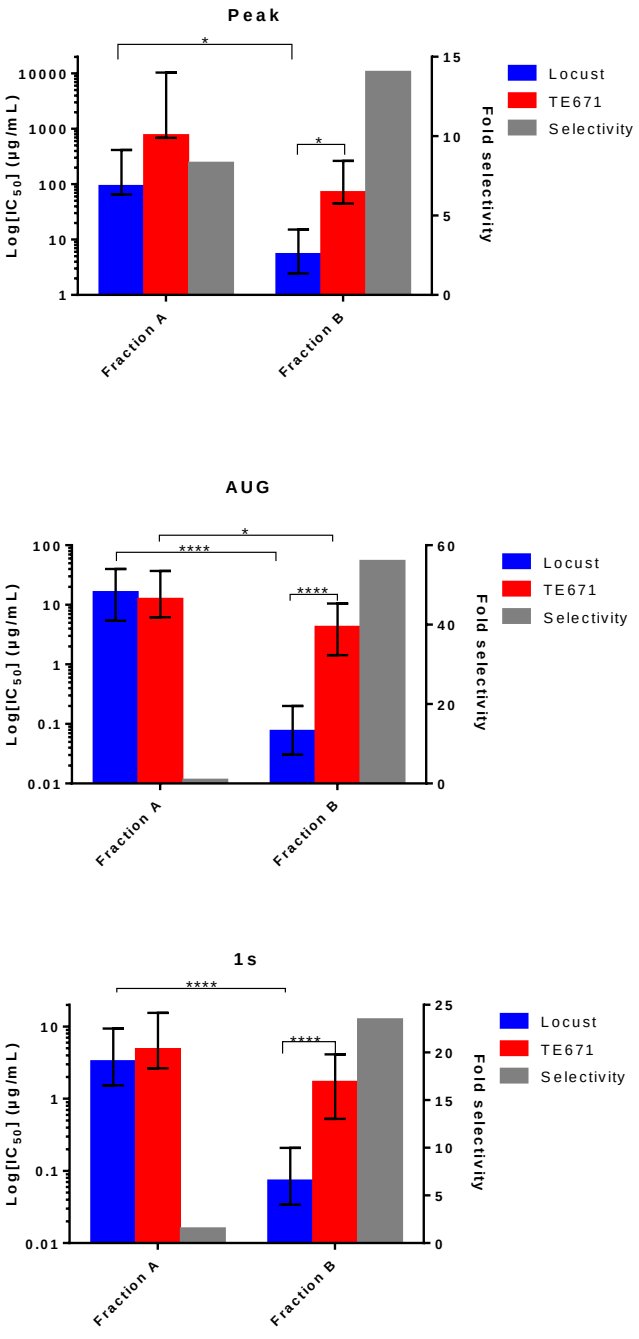


Figure 5.3.4 Column charts comparing the IC_{50} and selectivity values for two TLC derived fractions of *H. axyridis* alkaloid extract against human muscle-type (red) and locust neuronal (blue) nAChRs. Asterisks represent the result of one-way ANOVA comparison of IC_{50} values. Fraction B (harmonine) is significantly more potent to locust nAChRs than it is to human muscle-type, or than Fraction A is to either locust or TE671 nAChR in all comparisons. ANOVA comparison of net-charge IC_{50}s reveals that Fraction B is significantly more potent to TE671 nAChR than Fraction A is to either locust or TE671. V_H = -75 mV and N = 10 (Locust Fr A), 9 (Locust Fr B), 9 (TE671 Fr A) and 12 (TE671 Fr B). Error bars show 95 % CL.

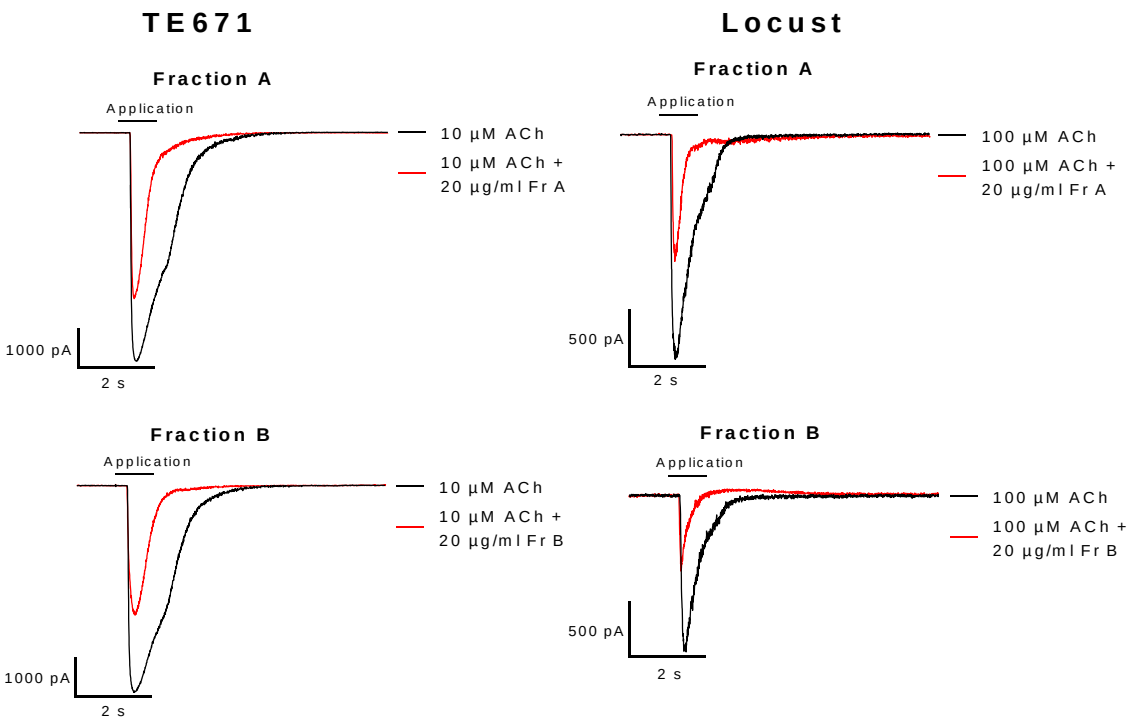


Figure 5.3.5 Representative traces showing the inhibition of ACh induced currents in both TE671 cells (left) and locust neurons (right) by *H. axyridis* thin-layer chromatography alkaloid fractions. Both fractions antagonised nAChR currents in both cells types, but fraction B (harmonine) was significantly more active against locust receptors than fraction A and showed selectivity for locust over human nAChRs. V_H = -75 mV.

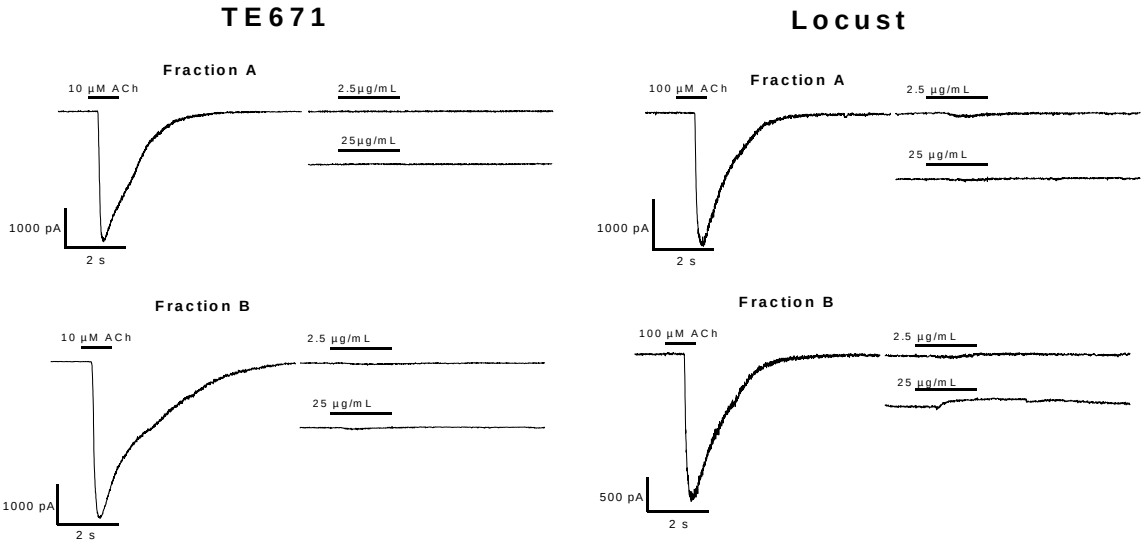


Figure 5.3.6 Representative traces showing the effect of perfusing two harlequin ladybird alkaloid fractions generated by thin-layer chromatography over ACh responsive TE671 cells and locust neurons. Cells were subjected to whole-cell patch-clamp at V_H = -75 mV and alkaloid was applied at 2.5 and 25 µg/mL concentrations. Neither fraction displayed activity against TE671 cells but fraction B (harmonine) caused small outward currents in five of nine locust neurons tested.

5.3 Discussion

5.3.1 Non-Competitive Inhibition of Human Muscle-Type Nicotinic Acetylcholine Receptor Expressed in TE671 Cells by Harlequin Ladybird Alkaloid Extract

The alkaloid extract of the harlequin ladybird was shown to be a potent antagonist of both locust neuronal and human muscle-type nAChR. To better understand the action of this alkaloid extract, it was decided to focus upon TE671 cells. The inhibition of ACh induced currents was roughly similar between locust neurons and TE671 cells, and the latter had the advantage of being easily cultured and marginally better able to tolerate repeated perfusion of test compounds whilst maintaining high-resistance seals with patch-clamp electrode.

To ensure that alkaloids were the major cause of nAChR inhibition produced by ladybirds, the activity of a crude methanol extract was compared with an alkaloid extract prepared by acid-base separation. As can be seen in Figures 5.1.1 and 5.1.2, the methanol extract failed to cause any detectable inhibition of nAChR current at a concentration of 10 μg/mL and just 15 % reduction of 1s current at 100 μg/mL. As expected, the alkaloid extract caused significantly greater inhibition, 53 % at 10 μg/mL and 79 % at 100 μg/mL. From these data it is possible to confirm that alkaloids are responsible for activity observed with methanol extract.

To elucidate whether the active component/s of this extract act competitively or non-competitively with ACh, concentration-response plots in the presence and absence of alkaloid extract were constructed (Figure 5.2.1). The concentration-response curves show that increasing concentrations of ACh are not able to overcome the inhibitory action of alkaloid. From these data we can conclude that the inhibition

caused by alkaloid extract is non-competitive. The response size plateaus at 80, 30 and 41 % of control response when co-applied with 30 µg/mL of alkaloid. This would suggest that binding of the active component/s is not competitive with ACh and therefore antagonism is caused by binding to an allosteric site.

To examine whether the alkaloid/s act to enhance desensitisation or bind within the pore region to block ion conductance, the voltage-dependence of inhibition was measured (Figure 5.2.2). Concentration-inhibition plots were prepared at five holding potentials, from +50 mV to -120 mV (Figure 5.2.2) to identify the degree to which nAChR antagonism of this extract showed voltage-dependence. The plots and IC_{50} values derived from them are shown in Table 5.2.1 and clearly show voltage-dependent action, with IC_{50} values decreasing as holding potential becomes increasingly negative. The alkaloid extract was 2.2–2.6 fold more potent when cells were clamped at -120 mV as opposed to -50 mV. When the IC_{50} values were plotted against holding potential and fitted with the Woodhull equation (Figure 5.2.3) (Woodhull, 1973) the slope of the line differed significantly from zero, confirming the voltage-dependent nature of antagonism. This finding indicates that the alkaloid extract of the harlequin ladybird is likely to act as an open-channel blocker rather than enhancing desensitisation of the channel. Unfortunately, it was not possible to generate accurate δ values from these data because the alkaloid extract is composed of a mixture of compounds (Figure 5.3.1) and assigning a definitive z value was therefore not possible.

This alkaloid extract showed inhibitory activity at positive holding potential of +50 mV (Figure 5.2.2), with IC_{50} values of 36.9 and 23.1 µg/mL for net-charge and 1 s

respectively. These IC_{50} values were lower than those calculated at negative holding potentials, as would be expected for a voltage-dependent compound, but the fact that activity remains suggests that a binding site may be quite shallow.

The activity observed was considerably lower than that measured in Chapter 4 with a different extract prepared from the same species. The most likely explanation for this is that the different protocols used to construct plots affected the level of inhibition. In Chapter 4, increasing concentrations of alkaloid were applied to the same cell and responses were normalised against a control response applied prior to alkaloid co-application. The plots shown in Figure 5.2.2 were compiled by applying a single alkaloid concentration at multiple holding potentials and the data for multiple concentrations assembled to create a concentration-inhibition plot. Another possible explanation is that that the quantity of stored alkaloid changes throughout the year (de Jong *et al*, 1991a: de Jong *et al*, 1991b) and it is possible that composition does as well. It may be that the extracts were compositionally different from one another because the beetles used to prepare extracts were collected 12 months apart, and this could account for the disparity in activity observed. Collecting beetles throughout the year and comparing alkaloid content using GCMS would reveal how important seasonal effects may be on alkaloid reserves in the harlequin ladybird.

5.3.2 Separating and Comparing the Activity of Harlequin Ladybird Alkaloid Fractions

Separating the components of harlequin ladybird alkaloid extract proved challenging for a number of reasons. Although it would have been desirable to isolate,

identify and quantify all of the individual alkaloid compounds from the mixture, this was not possible. When subjected to reverse phase HPLC, it was possible to separate compounds, but harmonine, the major alkaloid produced by this species could not be visualised with UV absorbance. It would have been possible to use HPLC coupled with secondary mass spectrometry, but the low-throughput nature of the technique made this an impractical solution. Liquid chromatography was attempted to increase the quantity of alkaloid extract that could be separated and fractions collected. Multiple attempts with different stationary phases, solvents and solvent gradients failed to separate alkaloids effectively and generate discrete fractions. Preparative scale TLC was then used to separate harmonine (Fraction B) from the other alkaloid components (Fraction A) within the alkaloid extract (Figure 5.2.1); this was confirmed with mass spectrometry (Figure 5.3.2). From 5 mg of whole alkaloid extract, 1.27 mg of harmonine and 560 µg of unidentified alkaloids with a very low concentration of harmonine were recovered. This confirms that harmonine is the predominant alkaloid within *H. axyridis*.

When tested with the patch-clamp assay, both TLC fractions antagonised the nAChR responses of both TE671 cells and locust neurons (Figure 5.3.3 & 5.3.5) in a concentration dependent manner. The activity of Fraction A was roughly similar towards both human muscle-type and locust neuronal nAChRs, with IC_{50}s of 15.94 and 12.21 µg/mL for net-charge and 3.23 and 4.77 µg/mL for 1 s current in locust and TE671 cells respectively. These values are considerably higher than those calculated for the whole alkaloid extract in Figure 4.2.6 which were, for locust and human nAChR respectively, 1.7 and 0.87 µg/mL for AUG and 0.76 and 1.26 µg/mL for 1 s current.

Fraction B displayed roughly similar activity to Fraction A against TE671 but was significantly more active against locust nAChRs with net-charge IC_{50}s of 0.074 and 4.16 µg/mL and 1 s IC_{50}s of 0.072 and 1.68 µg/mL (locust and TE671 cells respectively). From these data we can conclude that harmonine is not the sole active component of *H. axyridis* alkaloid extract but it does seem to show higher activity towards locust N-nAChRs than human M-nAChRs. It may be that the harlequin ladybird produces several alkaloids for chemical defence, targeted to different potential predators. The higher potency that harmonine displays towards locust nAChRs may indicate that this alkaloid is a chemical defence compound to combat predation from insect predators. This could be further tested by determining the potency of harmonine on other vertebrate and insect nAChRs. The very small quantity of both fractions available meant that it was not possible to repeat this experiment multiple times or conduct mode of action studies. It is therefore unfortunately not possible to infer mode of action or predict possible binding sites for these fractions.

When alkaloid fractions were applied to cells in the absence of ACh, Fraction A had no effect on either cell type but Fraction B caused small outward currents when applied at the highest concentration (25 µg/mL) to locust neurons (Figure 5.3.6) as with the whole alkaloid extract from this species (Figure 4.2.6). From this it is possible to conclude that harmonine is responsible for the activation activity observed for the whole alkaloid extract. The cause of this outward current is not known, but attempts to block this current with selective ligands may help to uncover the source. The fact that this current was generated in locust neurons, but not TE671 cells suggests that

this effect may be insect selective and worthy of further investigation as a possible insecticide target.

Chapter 6: (-)-Adaline an Alkaloid Produced by the 10-spot Ladybird Acts as a Selective Antagonist of Locust Nicotinic Acetylcholine Receptors

Introduction

In Chapter 4, the alkaloid extract of the 10-spot ladybird was identified as a potent antagonist of nAChRs which also displayed significant selectivity for locust over human nAChRs. *A. decempunctata* is known to produce two alkaloids, adaline and adalinine (Tursch *et al*, 1973 & Lognay *et al*, 1996). A limited quantity of the naturally occurring (-)- isomer of adaline was synthesised and donated by Dr Mike Birkett (Rothamsted Research). To elucidate mode of action, concentration-inhibition curves at holding potentials ranging from +50 to -120 mV and -50 to -120 mV were constructed for TE671 and locust neurons respectively. The resulting IC_{50} values were plotted against holding potential and a linear regression fitted using the Woodhull equation (Woodhull, 1973). This generated a δ value which was used to estimate the proportion of the membrane electric field penetrated by adaline, providing an indication of the nAChR binding site for this compound. The IC_{50} values calculated were compared using one-way ANOVA and F-tests, to ensure that insect selectivity is maintained irrespective of membrane potential. A concentration-response curve in the absence and presence of adaline was then constructed to verify if adaline action was competitive or non-competitive with ACh.

6.1 The Antagonistic Activity of (-)-adaline is Voltage-Dependent in Both Human and Locust nAChRs

Preliminary experiments revealed that adaline, like the whole alkaloid extract of *A. decempunctata* caused inhibition of ACh induced currents in both TE671 cells and locust neurons. In order to elucidate the mode of action by which adaline antagonises nAChR currents, it was necessary to determine to what degree the activity was voltage-dependent. We also wished to determine whether selectivity for locust over human nAChRs was maintained at a range of holding potentials. Concentration-inhibition plots were constructed at five holding potentials ranging from +50 to -120 mV in TE671 cells and four membrane potentials ranging from -50 to -120 mV in locust neurons (which display inward rectification and therefore cannot conduct current at positive holding potentials). Cells were subjected to whole-cell patch-clamp. A control response to ACh was established and a single concentration of synthetic (-)-adaline was co-applied with ACh. The holding potential was then changed and the process repeated. Current measurements were taken and normalised against an ACh control. This was then repeated with adaline concentrations ranging from 100 nM to 100 µM on TE671 and 10 nM to 30 µM on locust neurons. Adaline antagonised the ACh response in both cell types (Figure 6.1.1). Normalised response size was then plotted against adaline concentration and fitted with a 4PL curve (Figure 6.1.2 and 6.1.3). The inhibition of peak currents generated in TE671 cells was minimal (maximum 25 % inhibition at 100 µM) and accurate IC_{50} values could not be derived from the curves. Adaline caused concentration dependent inhibition of both human muscle-type and locust neuronal nAChRs. In both models, the potency increased with more negative holding potentials (Figures 6.1.4 and 6.1.5) as reflected by the IC_{50} values (Tables 6.1.1

& 6.1.2) which ranged from 28.02 to 87.66 µM and 10.26 to 45.83 µM (-120 to -50 mV) for AUG and 1 s current respectively in TE671 cells. The IC_{50} values calculated for inhibition of locust nAChRs were considerably lower, ranging from; 1.63 to 7.01 µM and 0.55 to 2.97 µM for AUG and 1 s respectively. Comparison of IC_{50} values with ANOVA revealed that adaline was significantly more potent to locust nAChRs at all holding potentials (P = <0.0001) for both the AUG and 1 s current. This was also the case when curves were compared using F-test in a pair-wise fashion (P = <0.0001). The inhibitory activity of adaline at positive membrane potentials in TE671 cells was modest, causing just 7 %, 17 % and 22 % reduction in ACh response for peak, AUG and 1 s respectively. When the IC_{50} values were plotted against holding potential and fitted using the Woodhull equation, the δ values were 0.51 and 0.59 (AUG) for TE671 cell and locust nAChRs, respectively and 0.62 and 0.74 (1 s). The slopes of all regressions differed significantly from zero (TE671 AUG P = 0.016, 1 s P = 0.003; Locust AUG P = 0.0038, 1 s P = 0.007) indicating strong voltage-dependence of inhibition.

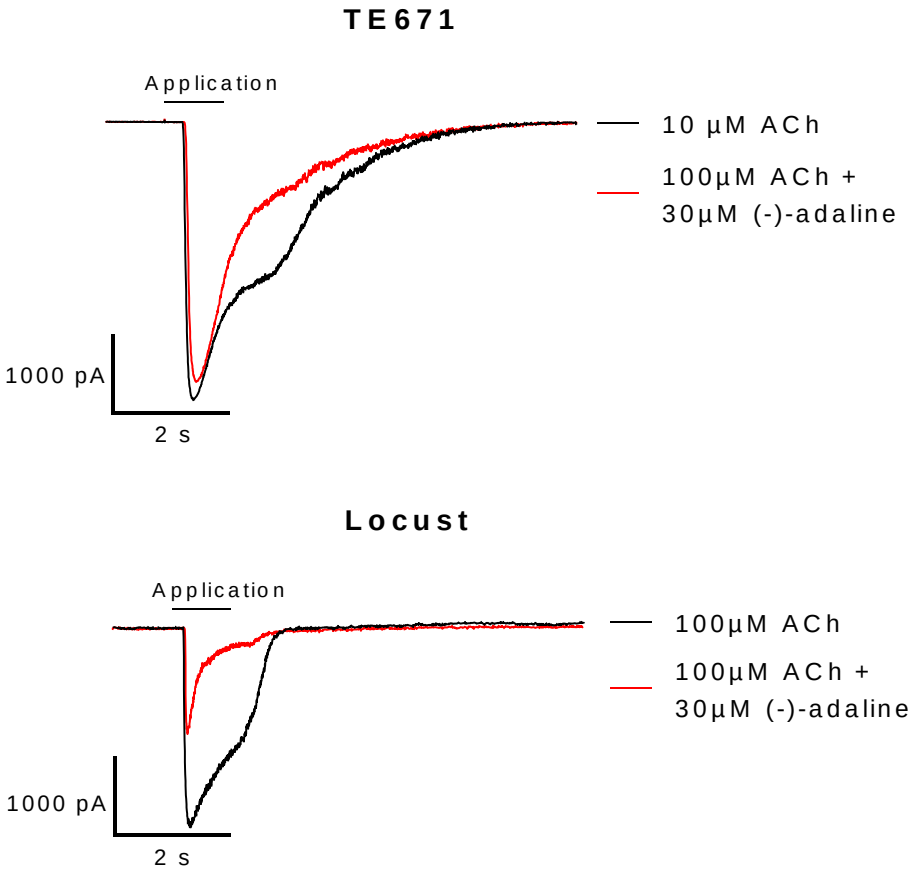


Figure 6.1.1 Representative traces showing the inhibition of ACh induced nAChR currents in both TE671 cells and locust neurons by 30 µM (-)-adaline. Cells were subjected to whole-cell patch-clamp at V_H = -75 mV.

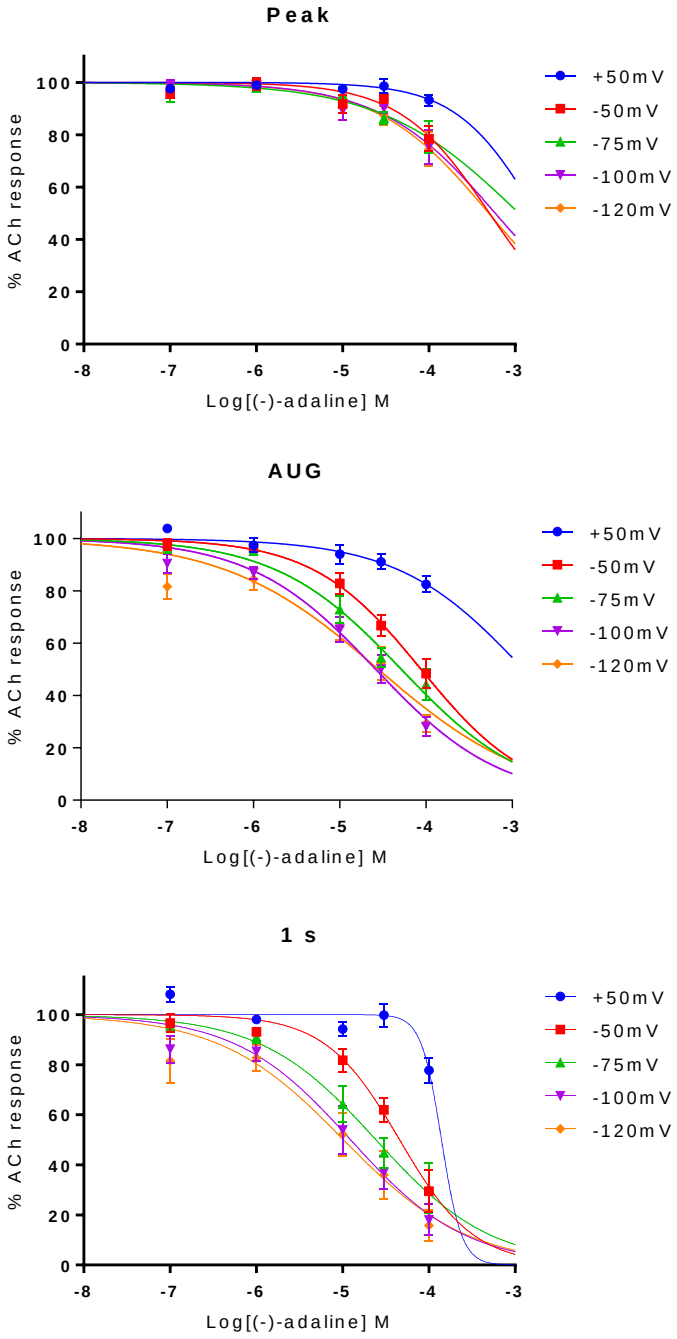


Figure 6.1.2 Concentration-inhibition plots showing the inhibition of human muscle-type nAChRs by increasing concentrations of (-)-adaline. TE671 cells were subjected to whole-cell patch-clamp and concentrations of synthetic (-)-adaline co-applied with 10 µM ACh at holding potentials ranging from +50 to -120 mV. The peak, AUG and late current values were then normalised against an ACh control and means plotted with ±SEM. 4PL non-linear regressions were fitted to AUG and late current plots from which IC_{50} values were derived (regressions could not be fitted for peak or +50 mV data to accurately predict IC_{50} values because of the low inhibitory activity exhibited by adaline). The inhibitory activity increased with holding potential and IC_{50} values ranged from 28.02 to 87.66 µM for AUG and 10.26 to 45.83 µM for 1 s indicative of voltage-dependent action. N = 7-14.

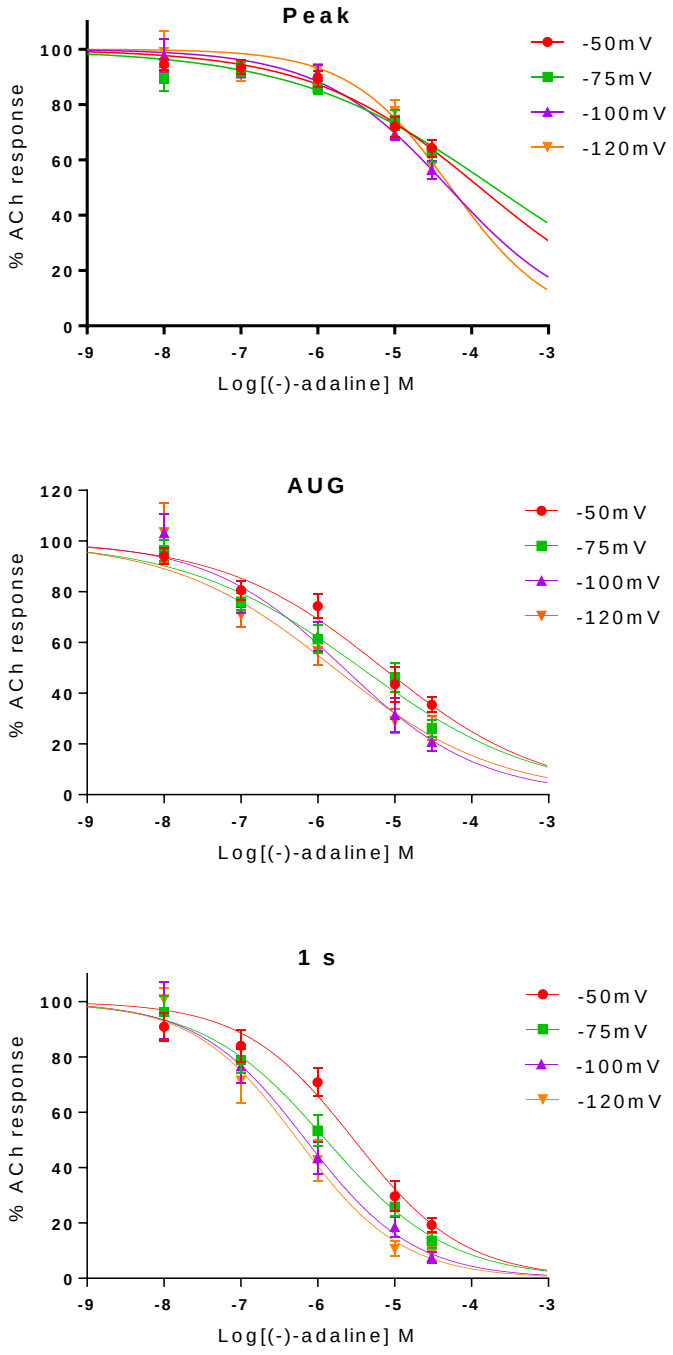


Figure 6.1.3 Concentration-inhibition plots showing the inhibition of locust neuronal nAChRs by increasing concentrations of (-)-adaline. Locust neurons were subjected to whole-cell patch-clamp and concentrations of synthetic (-)-adaline co-applied with 100 µM ACh at holding potentials ranging from -50 to -120 mV. The peak, AUG and late current values were then normalised against an ACh control and means plotted with ±SEM. 4PL non-linear regressions were fitted from which IC_{50} values were derived. The inhibition of peak current was minimal, and IC_{50} values were extrapolated from the fitted curve. These varied from 50.3 to 200 µM. The AUG and 1 s IC_{50} values varied respectively from 1.63 to 7.01 µM and 0.55 to 2.97 µM and decreased with increasingly negative holding potentials, indicative of voltage-dependent action. N = 6-18 (*N = 3 for 10 nM at V_H = -120 mV).

V_H (mV)	AUG IC_{50} µg/mL (95% CL) [N]	1s IC_{50} µg/mL (95% CL) [N]
-50	87.66 (59.72-128.7) [9-15]	45.83 (34.64-60.62) [9-15]
-75	51.75 (35.94-74.5) [8-14]	24.37 (15.6-38.09) [8-14]
-100	26.18 (18.97-36.13) [7-13]	12.39 (7.63-20.12) [7-13]
-120	28.02 (17.11-45.9) [7-11]	10.26 (5.42-19.41) [6-11]

Table 6.1.1 IC_{50} values calculated for (-)-adaline at holding potentials ranging from -50 - -120 mV against TE671 cells expressing human M-nAChR.

V_H (mV)	AUG IC_{50} µg/mL (95% CL) [N]	1s IC_{50} µg/mL (95% CL) [N]
-50	7.01 (4.16-11.8) [7-15]	2.97 (1.94-4.54) [7-15]
-75	3.64 (2.01-6.59) [7-18]	1.28 (0.83-1.97) [7-18]
-100	2.16 (1.26-3.71) [6-12]	0.7 (0.44-1.11) [6-12]
-120	1.63 (0.89-2.96) [3-13]	0.55 (0.33-0.93) [3-13]

Table 6.1.2 IC_{50} values calculated for (-)-adaline at holding potentials ranging from -50 - -120 mV against locust neuron nAChR.

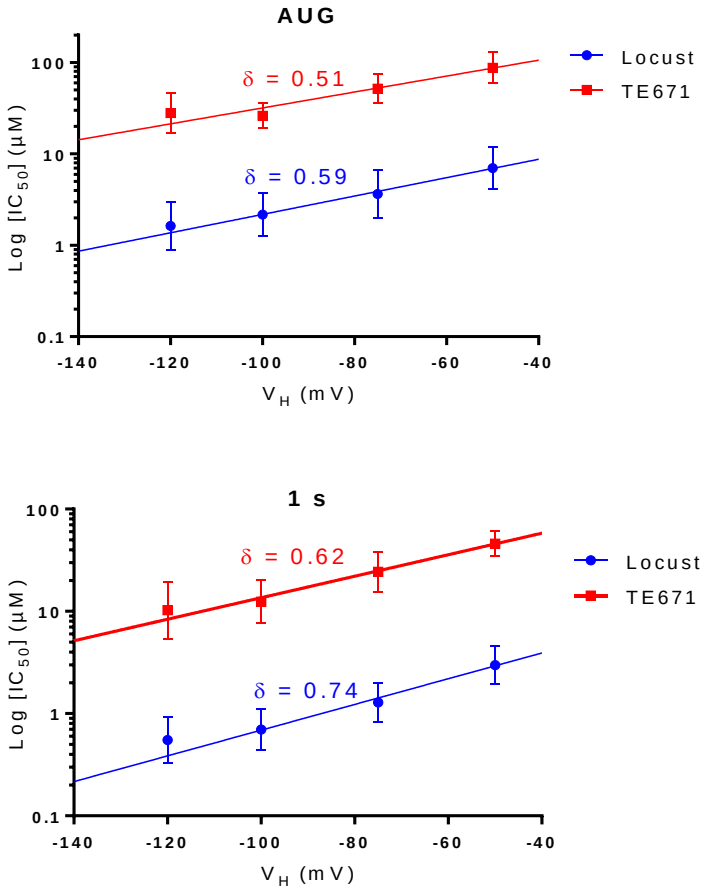


Figure 6.1.4 The IC_{50} values calculated in Figures 6.1.1 and 6.1.2 were plotted against holding potential and fitted with a non-linear regression using the Woodhull equation. The slope of each regression differs significantly from zero indicating strong voltage-dependence of (-)-adaline (TE671 AUG P = 0.016, 1 s P = 0.003; Locust AUG P = 0.0038, 1 s P = 0.007).The vertical shift between the TE671 cell and locust IC_{50} values illustrates the selectivity for insect nAChR. δ values indicate the Woodhull estimate of the fraction of the membrane's electric field that adaline has entered. Error bars indicate 95 % CL.

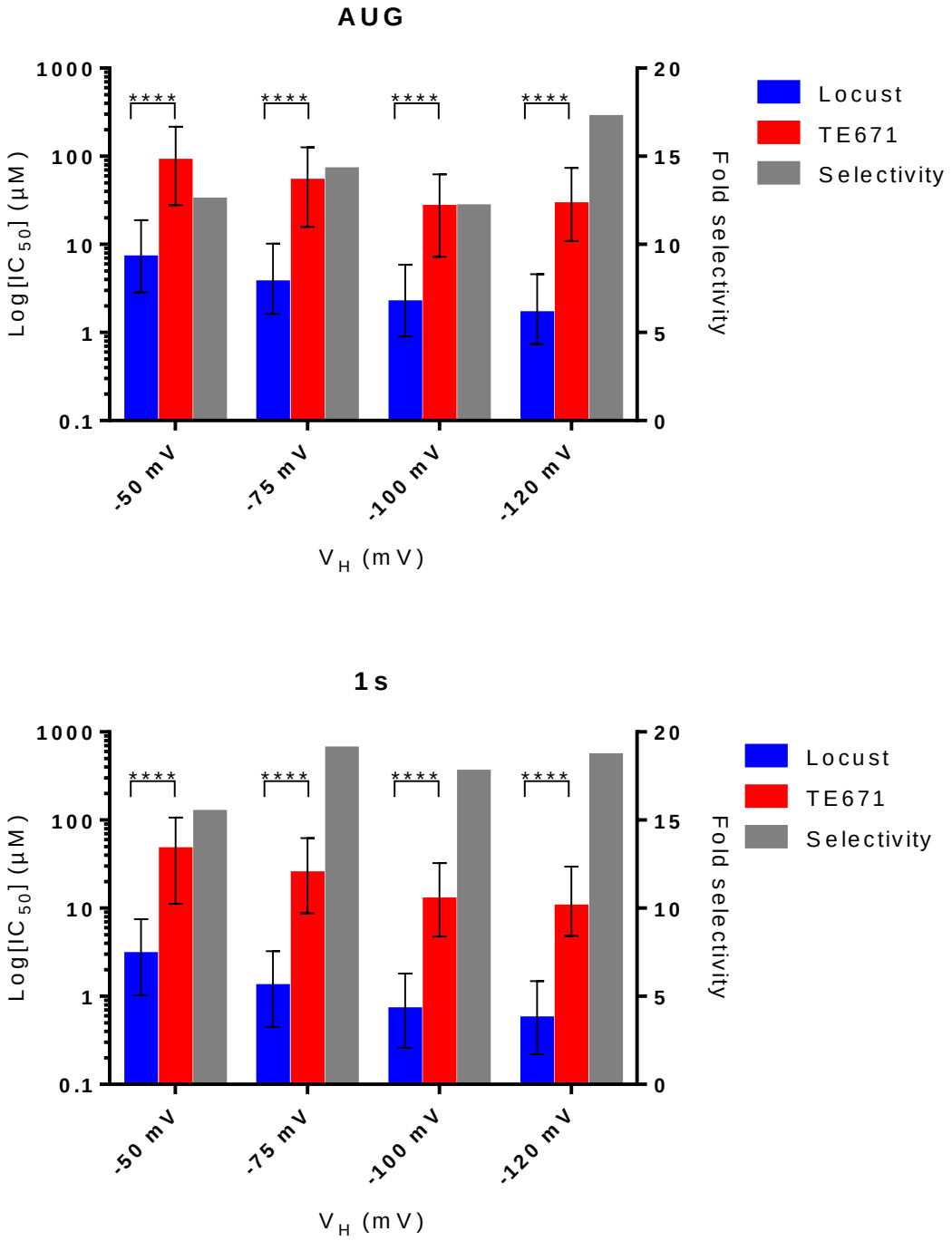


Figure 6.1.5 Column charts showing the IC_{50} values calculated for (-)-adaline against both human muscle-type (red) and locust neuronal (blue) nAChRs. Grey bars indicate selectivity for locust over human receptors and error bars show 95 % CL. Asterisks show the result of one-way ANOVA and F-test comparing IC_{50} value. (-)-adaline was significantly more potent towards locust nAChR (P = <0.0001) at all holding potentials tested.

6.2 The Antagonistic Action of (-)-adaline is Non-Competitive with ACh

To determine whether the inhibitory action of (-)-adaline was competitive with ACh, a concentration-response curve in the presence and absence of alkaloid was constructed as described previously with 20 μM (-)-adaline. This was co-applied with increasing concentrations of ACh to TE671 cells clamped at V_H = -75 mV. Current responses were measured and normalised against control responses of ACh applied to the same cell. The means ±SEM of 18 cells were plotted in Figure 6.2.1. The inhibition of currents was minimal at a 20 μM concentration of adaline but it can be seen that the current response is not significantly right shifted and plateau below the level of ACh alone. The EC_{50} values calculated in the absence and presence of adaline, respectively as 1.72 (95 % CL = 1.72-2.1) and 2.3 μM (95 % CL = 1.63-3.25) for peak, 11.59 (95 % CL = 9.13-14.7) and 9.05 μM (95 % CL = 6.71-12.21) for AUG and 1.17 μM for both 1 s curves. This is consistent with non-competitive inhibition. Unfortunately the limited quantity of adaline available, its apparent instability and the relatively large quantity required to conduct this type of experiment meant that it was not possible to repeat at a higher concentration.

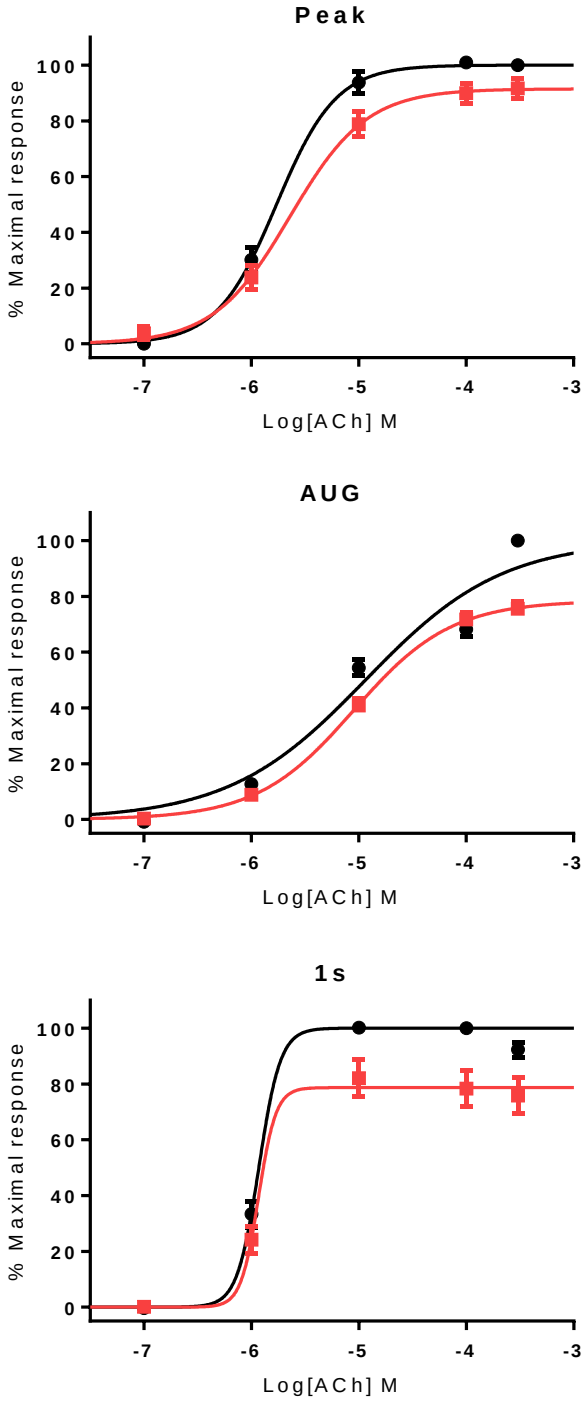


Figure 6.2.1 Concentration-response plots showing the current response of TE671 cells to increasing concentrations of ACh (black). The same ACh concentration range was co-applied with 20 µM (-)-adaline extract and the results normalised against the control response which gave the largest response (red). The results show that the action of co-application of (-)-adaline with ACh causes the response to plateau at 92 %, 76 % and 82 %, which is significantly lower than ACh alone (Unpaired T-test P = 0.027 for peak, P = <0.0001 for AUG and P = 0.016 for 1 s), rather than shift to the right, as would be expected if (-)-adaline was able to displace ACh binding. This is indicative of non-competitive antagonism. Error bars show ±SEM, V_H = -75mV and N = 18.

6.3 Activation Activity of Adaline

Adaline alone was applied to ACh responsive TE671 cells and locust neurons at concentrations of 2.5 and 25 µg/mL, equivalent to 11.9 and 119.4 µM respectively. TE671 cells showed no response to adaline in the absence of ACh, but 50 % of locust neurons (N = 10) displayed small inward currents (mean 6.6 % of 100 µM ACh peak response) after the application of 2.5 µg/mL. This response did not occur in response to the application of locust Ringer or at the higher adaline concentration of 25 µg/mL (Figure 6.3.1).

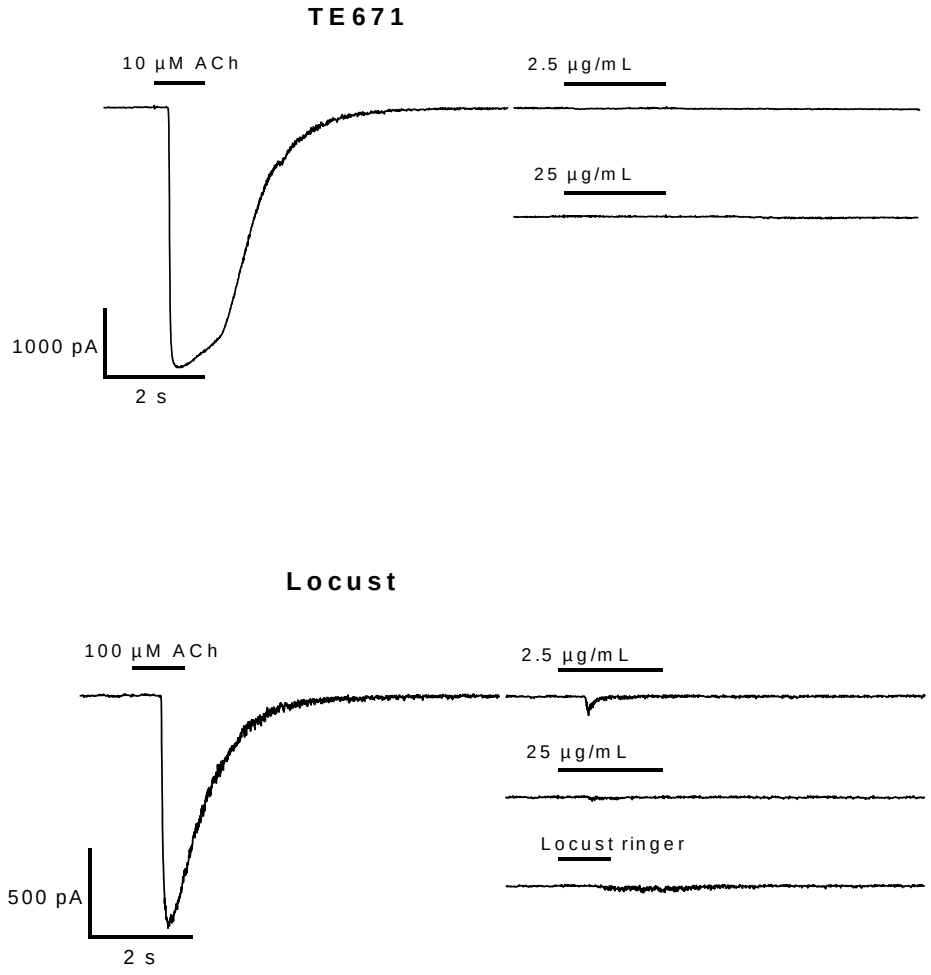


Figure 6.3.1 Representative traces showing the action of (-)-adaline in the absence of ACh on both TE671 and locust neurons subjected to whole-cell patch-clamp at V_H = -75 mV. The adaline concentrations used are equivalent to 12 and 120 µM. Neither concentration had any noticeable effect on TE671 cells but the lowest concentration generated a small inward current in locust neurons which was not seen in response to an application of locust ringer. This current was blocked at the higher adaline concentration.

6.4 Discussion

6.4.1 (-)-adaline Acts as a Selective, Non-Competitive Antagonist of Locust Nicotinic Acetylcholine Receptors

Adaline is one of two known alkaloid compounds produced by both 2-spot and 10-spot ladybirds. In Chapter 4, the alkaloid extract of the 10-spot ladybird was shown to display significant selectivity for locust over human muscle-type nAChR (Figure 4.2.1). Co-application of synthetic (-)-adaline with ACh caused concentration-dependent inhibition of inward currents, in exactly the same manner as alkaloid extracts prepared from beetles, in both TE671 cells and locust neurons (Figure 6.1.1). This further confirms that alkaloids are responsible for the antagonism displayed by alkaloid extracts prepared from beetles. Concentration-response plots in the presence and absence of 20 μM adaline (Figure 6.2.1) show that adaline inhibition does not right-shift the ACh EC_{50}, instead causing a reduction in the maximum response. As with harlequin ladybird alkaloid extract and the azaphenalene ladybird alkaloids tested by Leong *et al* (2015), this is suggestive of non-competitive antagonism. The effect is subtle but significant, causing 8, 24 and 18 % inhibition of peak, AUG and 1 s current respectively. This is because of the relatively low adaline concentration and comparative insensitivity of human M-nAChRs to this alkaloid. Unfortunately it wasn't possible to repeat this experiment with a higher adaline concentration because of the very limited quantity available.

To investigate nAChR antagonism by adaline further, the voltage-dependence of inhibition displayed by synthetic (-)-adaline was examined in both TE671 cells and locust neurons (Figures 6.1.2 & 6.1.3). As with *H. axyridis* alkaloid, nAChR antagonism is strongly voltage-dependent (Figure 6.1.4). The fact that adaline displays weak

activity at a positive holding potential of +50 mV in TE671 cells whereas harlequin ladybird alkaloids display comparatively strong activity (Figure 6.4.1) suggests that they are likely to be exploiting different binding sites with adaline's located deeper within the pore. Fitting the IC_{50} values with the Woodhull equation produces AUG and 1 s δ values of 0.51–0.62 in TE671 cells and 0.59–0.74 for locust. The strong voltage-dependence of inhibition suggests that adaline binds to the open pore, rather than enhancing desensitisation. Assuming that adaline exploits the same binding site in both locust and human nAChRs, these δ values suggest that adaline binds within the electric field of the membrane, in or below the central pore. These δ values must be treated with some caution because they cannot take into account the membrane surface charge, but this region of the M2 domain corresponds with the luminal high-affinity binding site for non-competitive blockers of nAChR such as chlorpromazine, phencyclidine, TCP, QX-222 and philanthotoxin-343 (Giraudat *et al*, 1986; Charnet *et al*, 1990; Arias, 1998; Brier *et al*, 2003). Five azaphenalene alkaloids (coccinelline, precoccinelline, convergine, hippodamine and exochomine) are known to displace [3H]-TCP from *Torpedo* membranes, suggesting that they also bind in this region (Leong *et al*, 2015). This site is located on the α-helical section of M2 which form the hydrophobic and hydrophilic rings involved in channel gating and ion selectivity (Miyazawa *et al*, 2003). The most straightforward way to confirm whether this is the case would be to conduct a competition assay with radiolabelled chlorpromazine, or [3H]-TCP (Leong *et al*, 2015) to determine whether adaline is able to displace this high-affinity ligand. This could then be further supported with site-directed mutagenesis and electrophysiological assays (e.g. Charnet *et al*, 1990).

When comparing the IC_{50} values estimated for locust and human nAChRs (Figure 6.1.5), it is clear that adaline displays roughly similar selectivity to the alkaloid extract of the 10-spot ladybird, with locust IC_{50}s between 12 and 19 fold lower than those of human muscle-type nAChR. The IC_{50} values calculated for adaline at V_H = -75 mV were very similar to those calculated for 10-spot ladybird alkaloid extract (locust AUG = 0.76 and 0.44 µg/mL, 1 s = 0.27 and 0.29 µg/mL: TE671 AUG = 10.8 and 8.46 µg/mL, 5.1 and 10.2 for adaline and 10-spot alkaloid respectively). This suggests that adaline is likely to form the majority of the alkaloid extract of the 10-spot ladybird, or alternatively adalinine, the other known alkaloid produced by this species, displays very similar potency to adaline.

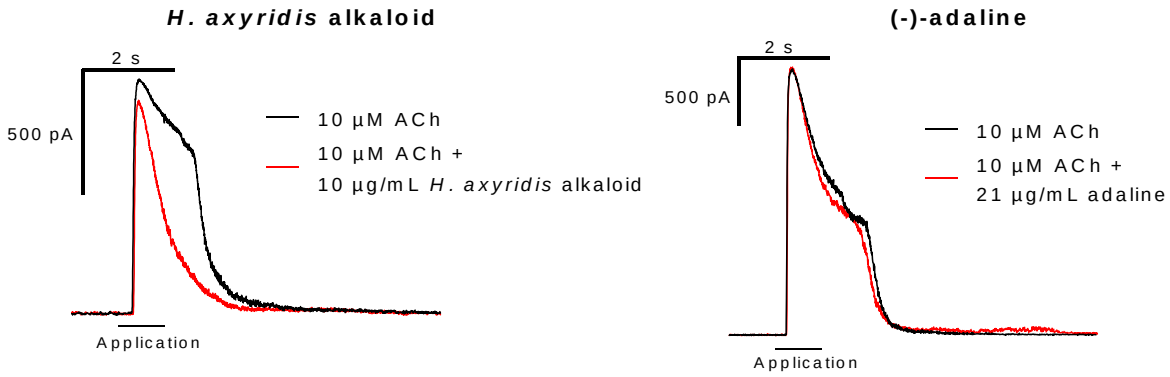


Figure 6.4.1 Representative traces showing the inhibition of outward nAChR currents in TE671 cells at V_H = +50 mV by harlequin ladybird alkaloid and adaline. The former causes inhibition of currents at a concentration of 10 µg/mL whereas the effect of adaline at 21 µg/mL (100 µM) is negligible. This concentration causes significant inhibition of currents at negative holding potentials. From this we infer that the active components of *H. axyridis* alkaloid extract are able to utilise a separate binding site to adaline. This site is likely to be comparatively shallow.

Aligning the M2 region of Sgα1, Sgβ1 and the human M-nAChR subunits expressed by TE671 reveal several key differences that may contribute to the higher potency that adaline appears to show for locust receptors. These are shown in Table 6.4.1, along with several vertebrate neuronal subunits that could be used to

determine which residues are of key importance, and whether the apparent selectivity represents insect/vertebrate or muscle/neuronal. It is not possible to accurately predict which residues are important for adaline binding or selective activity based on the data presented, not least because the nAChR subunits present in the locust neuron preparation have not been confirmed, but several key differences are noteworthy. At position 4, Sgα1 contributes a moderately hydrophobic alanine, this is replaced with a polar threonine in human α1, γ and Sgβ1. The human δ subunit possesses a polar serine residue at this site which could be involved in the apparent insensitivity of the human M-nAChR to adaline. If this is the case, human α7 would also be expected to show concomitant insensitivity because it also possesses a serine at this site, as do both *Drosophila* and *Anopheles* α1. Position 8 is a conserved serine residue in both locust subunits, but in the human β1, this is replaced with an aromatic phenylalanine. This amino acid change is not seen in other human β subunits, so the sensitivity of human N-nAChRs may provide clues as to whether this contributes to selectivity. The presence of hydrophobic alanine residues in both γ and δ subunits in contrast to the polar serine residues in both locust subunits at position 12 could be important, and the presence of alanine at this same position on human β2 and β4 could be used to test whether this is the case. Despite the voltage-dependence displayed by adaline indicating a deep binding site, it is possible that shallower pore-lining residues contribute to the probability of adaline entering the channel. Sgα1 possesses a methionine residue at position 15, as opposed to a well conserved aliphatic valine. At this same location, human β4 has an aromatic phenylalanine which could be used to test the importance of this site. Finally, human γ has an aromatic

phenylalanine at position 18, a site that is otherwise a completely conserved aliphatic leucine. Again, the sensitivity of vertebrate N-nAChRs will reveal whether this site is involved in adaline binding.

Comparing the sensitivity of locust N-nAChRs and human M-nAChRs with that of human α3β4, α4β2 and α7 N-nAChR will confirm whether adaline selectively binds to insect or vertebrate nAChR, or whether M-nAChRs happen to be relatively insensitive. Comparison with other insect nAChR will clarify whether *S. gregaria* nAChR are particularly sensitive to adaline, or whether this is a common feature of insect receptors. The results of these experiments will ultimately determine whether adaline could serve as an insecticide lead structure.

Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
***Sg*α1**	E	K	I	A	L	C	I	S	I	L	L	S	Q	T	M	F	F	L	L	I	S
Hα1	E	K	M	T	L	S	I	S	V	L	L	S	L	T	V	F	L	L	V	I	V
Dα1	E	K	I	S	L	C	I	S	I	L	L	S	L	T	V	F	F	L	L	L	A
Aα1	E	K	I	S	L	C	I	S	I	L	L	S	L	T	V	F	F	L	L	L	A
Sgβ	E	K	V	T	L	G	I	S	I	L	L	S	L	V	V	F	L	L	L	V	S
Hβ1	E	K	M	G	L	S	I	F	A	L	L	T	L	T	V	F	L	L	L	L	A
Hγ	Q	K	C	T	V	A	I	N	V	L	L	A	Q	T	V	F	L	F	L	V	A
Hδ	E	K	T	S	V	A	I	S	V	L	L	A	Q	S	V	F	L	L	L	I	S
Hα3	E	K	V	T	L	C	I	S	V	L	L	S	L	T	V	F	L	L	V	I	T
Hα4	E	K	I	T	L	C	I	S	V	L	L	S	L	T	V	F	L	L	L	I	T
Hα7	E	K	I	S	L	G	I	T	V	L	L	S	L	T	V	F	M	L	L	V	A
Hβ2	E	K	M	T	L	C	I	S	V	L	L	A	L	T	V	F	L	L	L	I	S
Hβ4	E	K	M	T	L	C	I	S	V	L	L	A	L	T	F	F	L	L	L	I	S

Table 6.4.1 Alignment of the M2 domain of nicotinic acetylcholine receptor subunits from *Schistocerca gregaria*, *Drosophila melanogaster*, *Anopheles gambiae*, human embryonic M-nAChR and human N-nAChR. Columns highlighted in blue are pore facing and underlined positions represent chlorpromazine binding sites. Amino acids highlighted in red represent novel changes that may promote adaline binding in locust subunits or reduce binding in human M-nAChR. Sequences from Genbank. Pore lining positions corresponding to: Position 1 = Hydrophilic Intermediate. 4 = Threonine, 8 = Serine, 11 & 12 = Equatorial, 15 = Valine, 18 & 19 = Outer Leucine.

Chapter 7: Discussion

The alkaloids produced by ladybirds have been the subject of research attention and speculation since their initial isolation in the early 1970s (Tursch *et al*, 1971). A definitive biological function has not yet been demonstrated beyond doubt, but it has long been assumed that chemical defence is the main purpose of these compounds (e.g. de Jong *et al* 1991a & 1991b; Daloze *et al*, 1995; Bezzerides *et al*, 1997), possibly to target intraguild predators such as other ladybirds and lacewing larvae (Agarwala & Dixon, 1992; Hemptinne *et al*, 2000; Sloggett *et al*, 2009; Kajita *et al*, 2010; Nedvěd et al, 2013). The fact that many ladybird species from which alkaloids have been isolated display aposematic colouration and that the alkaloids are found at high concentration in the haemolymph, which is expelled in response to perceived threat (Holloway *et al*, 1991), is compelling. Furthermore, toxicity of beetles (Marples *et al*, 1989) and beetle extracts (Nedvěd *et al*, 2010; Arenas *et al*, 2015), larvae (Yasuda & Ohnuma, 1999) and eggs (Hemptinne *et al*, 2000) has been demonstrated. Several azaphenalene ladybird alkaloids have been shown to act as negative allosteric modulators of vertebrate nAChRs (Leong *et al*, 2015) but other molecular targets have not yet been explored. Therefore the bioactivity and molecular mechanisms of action remain unknown for the majority of ladybird alkaloids.

The results of this study along with those of Leong *et al* (2015) indicate that a common characteristic of ladybird alkaloids is the ability to antagonise nAChRs. Each of the alkaloid extracts from seven ladybird species showed inhibitory activity against both locust N-nAChRs and human M-nAChRs (Figures 4.2.1-4.2.7) but the potency of these compounds varied significantly between ladybird species and also between

locust and human nAChRs (Figure 4.2.8). Comparison of concentration/inhibition curves with sum of squares F-test (Figure 4.2.8) demonstrated that extracts from five of the seven species studied were significantly more potent against nAChRs of locust neurons than of TE671 cells in all three current comparisons. These species were: 10-spot (13.2-35 fold), cream spot (17.7-38.2 fold), 7-spot (33.7-123.7 fold), pine (2.8-10.3 fold) and 14-spot (29.3-118.6 fold). The extracts of the cream spot, 14-spot and 7-spot ladybird showed such low inhibitory activity against the ACh response of TE671 cells that peak IC_{50} values had to be derived from extrapolated curves. The higher potency that the majority of ladybird alkaloid extracts showed for locust over human nAChRs may reflect evolved selectivity of chemical defence compounds to target the nAChRs of insect predators. Alternatively it may be that these differences in potency reflect differences in sensitivity between M-nAChR and N-nAChR rather than selectivity for insect over vertebrate receptors. For this reason, it is essential to verify the potency shown towards vertebrate N-nAChRs as well as a range of other insect nAChRs. Furthermore, conducting bioassays of both vertebrate and insect models will reveal whether the differences in potency observed in *in vitro* patch-clamp experiments are reflected in LD_{50} values. These further experiments will reveal whether ladybird alkaloids really do show selectivity for insects over vertebrates and will highlight which compounds, if any are worthy of further investigation as insecticide leads.

The alkaloid extracts of the harlequin and orange ladybird showed roughly similar potency towards both locust and TE671 nAChRs. That of the orange ladybird showed the lowest potency of all extracts tested towards locust neurons (4.9-65.1 µg/mL) but was of intermediate potency towards TE671 nAChRs (6.8-140 µg/mL)

when compared with the other extracts. This species is a habitat specialist often found in deciduous trees (Roy *et al*, 2011) and possibly less likely to encounter heterospecific intraguild predators, but more likely to encounter predatory birds. If the nAChR is the molecular target for ladybird chemical defence alkaloids, it could be that predation pressure on the orange ladybird favours vertebrate toxicity. The alkaloid extract of the harlequin ladybird was found to be the most potent antagonist of TE671 nAChRs (0.87-12.3 µg/mL) (Figure 4.2.8) and also displayed high potency towards locust nAChR. The harlequin ladybird is a habitat generalist (Cottrell & Yeargan, 1998; Ware & Majerus, 2008) that presumably comes into contact with a range of predators.

The inhibitory action of 30 µg/mL of the alkaloid extract of the harlequin ladybird was not overcome by increasing concentrations of ACh (Figure 5.2.1) indicating that the active component/s are non-competitive and likely to bind at an allosteric site. This is further supported by the fact that the antagonism caused by this alkaloid extract was strongly voltage-dependent (Figures 5.2.2. & 5.2.3, Table 5.2.1) with IC_{50} values at V_H = -50 mV 2.2-2.6 fold higher than those at V_H = -120 mV in TE671 cells. When comparing the effect of this alkaloid against different current parameters; the peak current is inhibited considerably less than the late current or net-charge movement (Figures 5.2.2 & 5.2.4). This use-dependent block suggests that the binding site for the active component(s) is not accessible when the channel is closed. On balance, it is likely that the active component(s) within harlequin ladybird alkaloid extract are acting as negative allosteric modulators, possibly acting as open-channel blockers. This extract showed considerable inhibitory activity in TE671 cells at V_H = +50 mV, suggesting that the active component(s) may be occupying a shallow binding site.

The alkaloid extract was separated into harmonine (Fraction B), the predominant alkaloid produced by the harlequin ladybird (Braconnier *et al*, 1985; Alam *et al*, 2002) and other alkaloid constituents (Fraction A) using thin-layer chromatography. Both fractions displayed inhibitory activity to ACh induced currents in both TE671 cells and locust neurons (Figure 5.3.3). Activity towards nAChRs of TE671 cells was similar for both fractions, but harmonine was significantly more potent to nAChRs of locust neurons than the other alkaloid components. The number of repeats used to construct these concentration inhibition plots was limited by the very small quantities, so further work to verify the apparent differences in activity against the nAChRs of TE671 and locust neurons is needed. Application of 25 µg/mL harmonine alone to locust neurons at V_H = -75 mV resulted in small outward currents in five of the nine cells tested (Figure 5.3.6). This activity was not observed in TE671 cells under the same conditions. Determining the cause of this outward current with the use of selective pharmacological blockers could reveal another molecular target of harmonine.

Adaline is one of two alkaloids produced by both 2-spot and 10-spot ladybirds (Tursch *et al*, 1973; Lognay *et al*, 1996). The alkaloid extract of the latter species was found to show significantly higher inhibition of locust nAChRs than TE671 (Figures 4.2.1 & 4.2.8). As with the harlequin ladybird alkaloid extract, synthetic (-)-adaline was found to show strong voltage-dependence of inhibition of ACh induced currents in both TE671 cells and locust neurons (Figures 6.1.2, 6.1.3 & 6.1.4, Tables 6.1.1 & 6.1.2) with IC_{50}s 3.1 and 4.5 (AUG and 1 s, respectively) fold lower at -120 mV than at -50 mV in TE671 cells and 4.3 and 5.4 fold lower in locust neurons. When the IC_{50} values were plotted against holding potential at V_H = -50, -75, -100 and -120 mV and fitted with the

Woodhull equation (Figure 6.1.4) δ values between 0.51-0.62 and 0.59-0.74 are predicted for TE671 and locust neuron nAChRs, respectively. These estimates agree that the binding site of adaline is within the electric field of the membrane. These values must be treated with caution because the Woodhull equation was developed to describe block by protons rather than comparatively complex organic cations (Brier *et al*, 2003), such as adaline. Application of increasing concentrations of ACh in the presence and absence of 20 μM adaline to TE671 cells resulted in EC_{50} curves that were not significantly right-shifted (Figure 6.2.1). The minimal activity of adaline towards TE671 cells meant that inhibition of ACh responses was limited at this concentration, and the limited quantity of adaline available meant it was not possible to repeat this experiment at a higher adaline concentration. The results of these experiments suggest that the mode of action of adaline is similar to that of the harlequin ladybird alkaloid extract and that it also acts as a negative allosteric modulator of nAChR, probably an open-channel blocker. Adaline showed very minimal activity at a positive holding potential of +50 mV. This is in contrast to the alkaloid extract of the harlequin ladybird, the activity of which was reduced but still present at this holding potential (Figure 6.4.1). This may be because adaline exploits a deeper binding site than one of the active components of harlequin ladybird alkaloid extract, which is not accessible from the extracellular surface when the nAChR is conducting outward current. This could be tested by examining the effect of intracellular application of adaline to the outward nAChR currents of TE671 cells, either by including adaline in the pipette solution, or by inside-out patch-clamp.

As with the alkaloid extract of the 10-spot ladybird, the inhibition of locust neuron nAChRs by adaline was significantly greater than those of TE671 cells (Figure 6.1.5). The net-charge IC_{50} at V_H = -75 mV was 14.2 fold lower in locust neurons than TE671 cells (51.75 and 3.64 μM, respectively) and the 1 s current IC_{50} was 19 fold lower against locust neuron nAChR (24.37 and 1.28 μM, respectively). Testing the activity of this compound against more vertebrate and insect nAChR as well as conducting bioassays will allow us to evaluate to whether this compound does show selective toxicity to insect nAChR. A radioligand binding assay to examine whether adaline is able to displace the binding of [3H]-TCP would reveal whether adaline occupies the same binding site as the azaphenalene alkaloids tested by Leong, *et al* (2015). As with the other alkaloids and alkaloid extracts tested in this study, further experiments to test for similar inhibitory activity at other Cys-loop receptors is required to find out whether the action seen at nAChR in this work and Leong *et al* (2015) is the result of a specific effect at nAChR, or caused by non-selective pore-block.

When the activity and selectivity of adaline is compared with d-tbc and imidacloprid (Figure 7.1.1) it can be seen that d-tbc is significantly more potent to human M-nAChRs than to locust in all three current parameters, but potency is very high for both, with IC_{50}s in the low μM range. Imidacloprid shows significantly higher potency for locust nAChRs than for human M-nAChRs when comparing peak and late current, as would be expected for an insecticidal compound but there is no significant difference for the inhibition of net-charge movement. This reflects the dual activity of imidacloprid as a partial agonist of locust nAChR (Nagata *et al*, 1997; Matsuda *et al*,

2005) and resulted in wide 95 % confidence limits for the AUG IC_{50} of imidacloprid on locust neurons (IC_{50} = 42 µm, 95 % CL =9.8-181). Adaline also shows significant selectivity for locust nAChRs across all three current parameters, but lower than that of imidacloprid. The inhibition of peak current is considerably higher for both d-tbc and imidacloprid than for adaline, this is likely to be because d-tbc acts as a competitive antagonist of the nAChR and can act on the closed conformation of the channel, therefore the effect on the peak current is high, roughly in proportion with the net-charge and 1 s current. Adaline acts as a non-competitive antagonist showing use-dependent block and appears to act on the activated channel, thus the inhibition of peak current is significantly lower than the action on net-charge and 1 s currents. Imidacloprid acts as a partial agonist and causes both activation in the absence of ACh and inhibition in the presence of agonist. Thus the effect on peak and 1 s currents is high, but the effect on net-charge is slightly lower. The IC_{50} calculated for the late current inhibition of locust neuron nAChR by adaline is similar to that of imidacloprid, with IC_{50}s of 1.28 (95 % CL = 0.83-1.97) and 1.60 µM (95 % CL = 0.80-3.12). If inhibition of this current parameter proves to be an effective insecticidal mechanism, adaline could show promise as an insecticide lead compound.

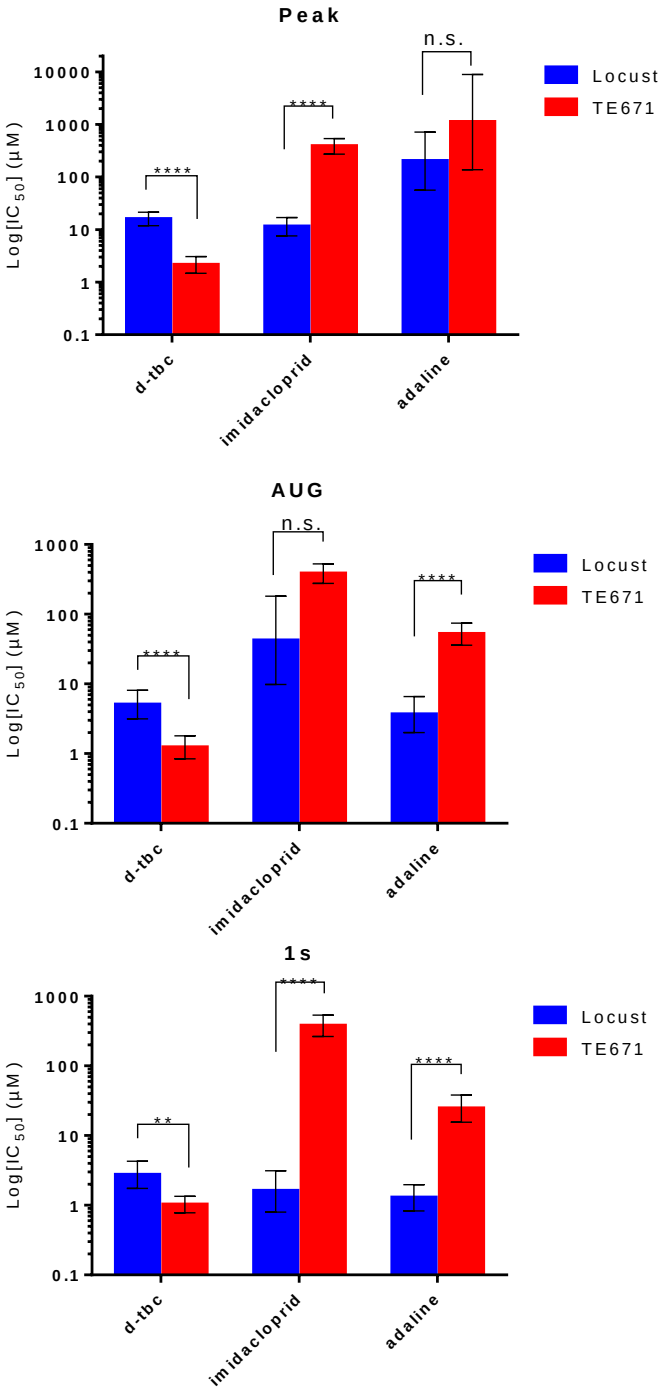


Figure 7.1.1 Column charts comparing the potency of d-tbc, imidacloprid and adaline towards both locust N-nAChRs and human M-nAChRs expressed by TE671 cells. Columns represent the µM IC_{50} value and error bars indicate 95% CL. It can be seen that d-tbc was significantly more potent to human M-nAChRs in all current parameters. Imidacloprid was significantly more potent to locust nAChRs by several orders of magnitude in both peak and late current, but not when comparing the IC_{50}s for net-charge movement. Adaline showed significant selectivity for locust over human nAChRs against both net-charge movement and late current, but not for peak current IC_{50}. IC_{50}s calculated at V_H = -75 mV. Asterisks indicate significance calculated by multiple T-test comparison * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001 and **** = P ≤ 0.0001

Further work

The results presented herein demonstrate that a range of ladybird alkaloid extracts and pure alkaloids act as antagonists of both human and insect nAChR. All ladybird alkaloids tested at this receptor show inhibitory activity, including several structural classes. This suggests that the nAChR represents a possible target for ladybird chemical defence and ladybird alkaloids may represent a library of novel nAChR modulators. To date, 36 structurally unique alkaloids have been isolated from 41 species of ladybird (Durieux *et al*, 2010). This means that >99 % of known ladybird species (roughly 5960 species) remain unstudied and this potentially presents an excellent source of novel ion channel toxins.

Of the compounds tested in this study, it is necessary to determine the potency shown towards vertebrate N-nAChRs as well as other insect nAChRs. Comparing the IC_{50}s for ladybird alkaloid extracts, harmonine and adaline with those calculated on other nAChRs, notably vertebrate α7, α3β4 and α4β2 will allow us to evaluate which subunits confer sensitivity to alkaloid action and gain further insights into the possible binding site(s) and mode of action. In order to establish whether the alkaloid extracts and compounds which show higher potency to *S. gregaria* nAChRs also show similar activity at other insect nAChRs. Heterologously expressing insect nAChRs of known stoichiometry, such as αL1 or Dα2/chicken β2 and testing sensitivity to ladybird alkaloids will reveal whether the differences seen by comparing activity at locust N-nAChR and human M-nAChR reflect genuine insect selectivity, or simply differences between the two models used in this study.

The activity that ladybird alkaloids display towards other molecular targets, particularly other Cys-loop receptors and other cation channels remains to be seen. The degree to which these compounds show selectivity for nAChRs over other receptors should be examined to determine whether this receptor represents a specific target for ladybird alkaloids, or whether these compounds are non-selective antagonists. Philanthotoxins show similar nAChR inhibitory action to ladybird alkaloids against the M-nAChRs of TE671 cells (Brier *et al*, 2003) and also show strong inhibition of NMDA and AMPA receptors (Mellor *et al*, 2003). It could be that ladybird alkaloids act as non-selective pore blocking compounds, making them undesirable as insecticide lead compounds.

The insecticidal efficacy of ladybird alkaloids also needs to be established. The majority of widely used ion channel targeting insecticides operate by activating, potentiating or blocking the inactivation of excitatory ion channels (e.g. neonicotinoids, pyrethroids), inhibiting enzymes that hydrolyse excitatory neurotransmitters (organophosphates and carbamates) or blocking inhibitory ion channels (fipronil, lindane and endosulfan). The net result of these insecticides is neuronal hyperexcitability which results in rapid and sustained immobilisation. Insect bioassays of ladybird alkaloids will reveal whether they show insecticidal efficacy, and studying the *in vivo* effects of alkaloid toxicity will also suggest whether the pathophysiology is consistent with antagonism of nAChRs.

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